Cytoplasmic transfer: the risks?

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This review focuses on cytoplasm transfer. Cytoplasmic control of preimplantation development is not a ''new'' concept, but ooplasm transfer have been amazingly rapidly applied in humans, with relative success, in the absence of extensive research to evaluate the efficacy and the potential risks of the method, resulting in some publications highlighting the potential dangers (Winston and Hardy 2002, De Rycke *et al*., 2002, Templeton, 2002,) and unpredictable outcomes (Cummins 2001, 2002).

Cytoplasmic transfer

The first experiment of cytoplasmic transfer was realized in mouse, in the early eighties (Muggleton-Harris, 1982). The authors transferred cytoplasm from non blocking strains to blocking stains in mouse, thus alleviating the 2-cell block. Since 1997, when Cohen and colleagues announced the first human pregnancy following cytoplasm transfer from donor oocytes into the eggs of a patient with a history of poor embryo development and recurrent implantation failure, over thirty children have been born after direct injection of ooplasm, from fresh, mature or immature, or even frozen-thawed donor oocytes, into recipient oocytes via modified ICSI (Cohen *et al*., 1997, Lanzendorf *et al*., 1999, Huang *et al*., 1999, Hwang *et al*., 2002). As live birth of healthy children was obtained, this method was subsequently proposed to restore some defect in the recipient oocyte using a small amount of injected cytoplasm.

Processes affected by cytoplasmic transfer

1/Positive aspects

Correction of a putative imbalance between anti and pro apoptotic factors
Following ovulation, zygote survival almost exclusively depends on maternal mRNAs and proteins that accumulate during oocyte growth and maturation (Braude *et al*. 1988). Maternal transcripts are thus responsible for the first few cleavage divisions and for transition of the maternally controlled zygote into an activated embryonic genome. Morphological and biochemical hallmarks of apoptosis have been described in mammalian pre-embryo (Levy *et al*., 1998). *In vitro* sub-optimal culture conditions *i.e.* composition of the culture medium, glucose concentrations, lack of free radical scavengers, excessive reactive oxygen species generation (ROS), excessive spermatozoa exposition, quality of gametes such as chromosomal and DNA anomalies (Levy, 2001) are the most known candidates. Apoptosis must be considered as a normal feature in preimplantation development, both *in vivo* and *in vitro*, playing an active and crucial role in the developing embryo through the removal of deficient cells. Contrary to these beneficial effects, apoptosis may have detrimental effects if either the number of apoptotic cells or ratio of these cells to the normal cells is over a critical threshold. Embryo development is regulated by a balance of pro- (i.e. Bax) and anti- apoptotic (i.e. Bcl-2) genes, since oogenesis, throughout the preimplantation period, with successive checkpoints. Thus, mature oocytes with appropriate anti-apoptotic mRNAs will succeed through maturation and fertilization whereas poor quality oocytes with high levels of cell death inducers or low levels of cell death antagonists will failed to fertilize, or fragment and/or arrest.

Would it be possible to rescue some apoptotic embryos? Ooplasm donation from healthy oocyte is supposed to provide appropriate anti apoptotic mRNAs to restore the balance of apoptosis-related mRNAs. Thus, when cytoplasm donation is ''justified'' by constant high level of fragmentation and poor embryo development after ICSI, it is of interest to note that the pregnancy reported after cytoplasmic transfer was associated with a strong reduction in embryo fragmentation.

Correction of defective mitochondrial membrane potential:
Healthy mitochondria are essential for accurate chromatid segregation at the time of fertilization and during subsequent mitotic divisions. Mitochondria, present in cytoplasm, are responsible for respiratory process and ATP production (OXPHOS): they generate reactive oxygen species (ROS) deleterious for biological material including DNA. Injection of a bolus of cytoplasm from healthy donor oocytes would restore global mitochondrial

activity, in case of damage. This could avoid, at least in part, the chaotic mosaïcism related to low mitochondrial potential (Wilding *et al* 2003). In addition to their role as the cell's powerhouse, mitochondria play a central role in the control of apoptosis (Ravagnan *et al*., 2002). Mitochondrial malfunction may contribute to cell cycle arrest, followed by cell death, triggered by mild oxidative stress (Liu *et al*., 2000 a, b). Thus, healthy mitochondria transfusion was experimented by Liu using reconstructed zygotes with nuclei and cytoplasm from H_2O_2 treated or untreated zygotes. Arrested, reconstituted zygotes displayed TUNEL staining at a similar rate to that of H_2O_2-treated controls, suggesting that apoptotic potential could be transferred cytoplasmically. On the other hand, rates of cleavage and development of the reconstituted zygotes, derived from stressed pronuclei and untreated cytoplasm, showed that healthy cytoplasm could partly rescue pronuclei from oxidative stress. Although oxidation stressed both nuclei and cytoplasm, cytoplasm was more sensitive than nuclei to oxidative stress. Mitochondria, plays a central role in mediating both development and apoptotic cell death induced by oxidative stress in mouse zygotes.

2/ Negative aspects

Mitochondrial heteroplasmy, mitochondrial disease and nuclear/mitochondrial interaction:

The presence of donor mitochondria was detected in the cells of two out of 15 children at 1 year of age (Brenner *et al*., 2000, Barritt *et al*., 2000, 2001a). This heteroplasmy has been described as a possible therapeutic effect (Malter H.E., 2002). It has also raised significant concerns regarding the safety of this technique (Brenner *et al.,* 2000). In fact, the risk of mitochondrial disease transmission (mtDNA mutation) associated with ooplasmic transfer seems extremely low due to the population frequency of such symptomatic disease (1:8000). It could even be argued that ooplasmic transplantation represents a methodology for the reduction of the random transmission of such mitochondrial diseases compared with standard oocytes donation. There is a high degree of homoplasmy throughout the body, which is thought to be crucial for the prevention of conflict between nuclear and mitochondrial DNA. Ooplasmic transfer into human oocytes may induce conflicts between nuclear DNA, recipient mtDNA, donor mtDNA and lead to unpredictable outcomes (Cummins, 2001). Mitochondria are maternally inherited, so if the child is female, the mixture of mitochondria is supposed pass to future generations. However, mitochondria are thought to pass through a bottleneck during oogenesis or embryogenesis with clonal expansion from one or a few of these organelles resulting in restoration of homoplasmy.

Epigenetic aspects

Cytoplasm transfer can have immediate adverse effects on early embryo development through blastomere fragmentation and/or apoptosis. Blastomere fragmentation at the two-cell stage in the mouse embryos is under the control of both parental genotypes, and possibly results of either genomic imprinting or differences in mitochondrial origin (Hawes *et al*., 2001, 2002).

Chromosomal abnormalities following ooplasmic transplantation in humans

A frank follow-up of ooplasmic transplantation pregnancies and infants reports that two out of seventeen fetuses had an abnormal 45, XO karyotype (Barritt *et al*., 2000, 2001b). The authors assume the hypothesis of a link between chromosomal anomalies and oocytes manipulation, and reveal that one of the babies has been diagnosed at 18 months with Pervasive Developmental Disorder, a spectrum of autism-related diagnoses which have an incidence of as high as 1 in 250 children.

The regulatory protein polarization

Oocyte polarity is crucial for subsequent embryo development (Edwards and Beard, 1997). Polarity has been demonstrated for the regulatory protein leptin, the transcription factor STAT 3 (Antczak and Van Blerkom, 1997), b-actin and IL-1 mRNA, vascular endothelial growth factor (VEGF), the growth factor receptors c-kit and epidermal growth factor receptor (EGF-R) but also the apoptosis-associated proteins Bcl-x and Bax (Antczak and Van Blerkom, 1999). The injection of foreign ooplasm into the recipient can be deleterious for subsequent embryo development as it could disturb the regulatory protein polarization.

3/ Impossible to analyze

The mRNA, Polyadenylation aspect:

The first divisions of the preimplantation embryos are maternally driven. Translational activation of the oocyte mRNA is regulated via poly (A) length. Cytoplasmic polyadenylation element binding protein (CPEB), Cleavage and polyadenylation specific factor (CPSF) and poly(A) polymerase (PAP) are implicated in mRNA polyadenylation (Dickson *et al.* 2001, Hodgman *et al.* 2001)

Specific changes in polyadenylation contribute to gene expression in embryos: they have a direct relation with developmental competence (El Mouatassim *et al*. 1999, Brevini-Gandolfi *et al*. 1999, Brevini *et al* 2002). It is obvious that poor cytoplasmic maturation and to poor regulation of mRNA polyadenylation process are linked and thus lead to altered developmental competence (El Mouatassim *et al.* 1999, Brevini *et al*. 2002). The mRNA coding for Oct-4 (involved in early differentiation and totipotency), connexin 32 and 34 (important for blastocyst formation), for protection against free radicals and

even for Polyadenylate polymerase, are submitted to this fine regulation process. Whether or not injection of good quality cytoplasm could help a low quality recipient oocyte cytoplasm is completely a matter of speculation. It is obvious that it could help in increasing the polyadenylation process (via injection of PAP and CPE) and elongating the polyA tail of some MRNA. In the contrary, some of the polyadenylated mRNAs are submitted to a gradual reduction of their polyA tail (Connexin-43 and Oct-4). In this case a complete disturbance in the developmental process can be expected. For this aspect, cytoplasmic transfer is a question mark.

Unknown genetic maternal factor: the mouse model

A maternal effect affecting blastocyst formation was reported by Renard *et al*. where the transfer of ooplasm from the inbred mouse DDK strain or oocytes RNA to non-DDK oocytes converts the oocytes to a DDK phenotype resulting in post zygotic lethality (1994). It is difficult to speculate on the presence of such a syndrome in human: it cannot be, however, totally excluded. In this case, injection of healthy mRNAs could not rescue the embryos.

Conclusion

The arguments for gamete and embryo rescue *via* cytoplasmic transfer lead to the question of whether or not every embryo is capable of being improved. Cons have severely evaluated the process as ''trying to improve a bottle of spoiled milk by adding a cup of fresh'' (Hawes *et al.* 2002): It is a little bit hard. Nevertheless, basic research is really needed on animal models, to determine *a minima* which processes it implies.

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Human umbilical cord blood stem cells: can they replace embryonic stem cells?

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Summary

Embryonic stem cells (ES cells) are capable of unlimited self-renewal and have the ability to give rise to all tissue types in the body. The use of human ES cells for tissue and cell therapeutics has been suggested but is limited by ethical concerns since these cells are derived from the inner cell mass of human embryos. In addition, the need for HLA matching of ES cell-derived tissues for allogeneic transplantation would require a bank of several thousand ES cell lines to make tissue therapeutics practical. Recently, adult stem cells, of which those in bone marrow are the best studied, have been shown to be capable of multilineage differentiation into cells of various non-blood tissues. Umbilical cord blood hematopoietic stem cells have been shown to be equivalent to bone marrow stem cells for reconstitution of the hematopoietic system. Preliminary work has also demonstrated that umbilical cord blood hematopoietic stem cells are multipotent and capable of differentiating into non-blood cell types. This observation raises the exciting possibility of replacing human ES cells for tissue and cell therapeutics with umbilical cord blood hematopoietic stem cells that are normally discarded with the placenta after delivery.

Introduction

Stem cells are defined as cells capable of unlimited self-renewal and with the ability to give rise to multiple tissue types (Thomson, Itskovitz-Eldor et al. 1998). Embryonic stem cells (ES cells), derived from the inner cell mass of mammalian embryos, fit this definition since they have unlimited self-renewal properties and give rise to all embryonic tissue types *in vitro*. It is interesting to note that unlimited self-renewal is not a property of cells of the inner cell mass *in situ*, where they differentiate into various tissues of the body and the ES phenotype is lost. On the other hand, adult stem cells, of which hematopoietic stem cells

(HSC) are the best studied, give rise to a wide range of progenitor and mature cells within the confines of the hematopoietic system, and have self-renewal properties for the life of the organism (Harder, Henschler et al. 2002). It is, therefore, believed that HSC have a capacity for self-renewal but a more limited differentiation range than ICM derived cells. This view is now changing as described in the present review.

Experimentally, embryonic stem cells can be considered a blank slate capable of responding to external signals from surrounding cells or from exogenously added growth factors. The self-renewal ability of ES cells and their capacity to differentiate into a wide range of cell types make them potential candidates for tissue therapy (Amit and Itskovitz-Eldor 2002). This novel use of human ES cells has, however, created a moral and ethical dilemma (Green 2001). Although it is likely that human ES cells can eventually realize their clinical potential, we believe that the need to derive these cells from a human embryo will always limit their widespread use in tissue and cell therapeutics. A secondary problem limiting the use of human ES cells is the required HLA matching of the derived cells or tissues to the recipient, requiring a bank of several thousand ES cell lines to make tissue therapeutics or gene therapy practical (Trounson 2001). Thirdly, murine or human ES cells injected directly into immunosuppressed mice result in the formation of germ-cell tumours called teratomas (ref), a problem that requires correction before considering these cells for tissue therapeutics.

The exciting question at present is whether tissue specific stem cells (also called adult stem cells) have a 'blank slate' phenotype as well. UCB stem cells have the expected, and already demonstrated, potential to replace bone marrow for reconstitution of the hematopoietic system (Gluckman, Rocha et al. 2001) but may also have the exciting possibility of bypassing the need for ES cells for tissue therapeutics and gene transfer in the future. In this regard, UCB stem cells have an advantage over other types of stem cells as they can be collected non-invasively at the time of birth from normally discarded tissue Their use obviates the ethical problems associated with the creation of multiple human ES cell lines. In addition, we have found that they never form tumours when injected into immunosuppressed mice. The present review will, therefore, focus on the potential of adult derived stem cells, especially umbilical cord blood hematopoietic stem cells, as an alternative to embryonic stem cells.

Embryonic stem cells

Embryonic stem cells were originally derived from the inner cell mass of the murine blastocyst (Evans and Kaufman 1981; Martin 1981; Bradley, Evans et al. 1984). These cells represent the ultimate in stem cells because of their abilities

to be both self-renewing and multipotent. Chimeric analysis using murine ES cells has demonstrated their capacity to contribute to all tissues (Nagy, Gocza et al. 1990). Murine ES cells can be maintained in vitro in a multipotent state (Wiles and Johansson 1999) and induced to form embryoid bodies, which can be differentiated into multiple adult cell types. Murine ES cells have been used as a tool for the study of embryo development, cell-cell interactions, the unscrambling of biological pathways and to derive models of human disease (Roach, Stock et al. 1995). Furthermore, ES cells in which gene alterations have been made, can be manipulated *in vitro* and provide a constant source of cells for study.

Some cell types will form spontaneously (cardiac myocytes and hepatocytes) but other cell types require an induction step using growth factors (Jones, Tosh et al. 2002). For example during embryoid body growth, vascularization will occur spontaneously but the addition of both FGF and VEGF or endothelial growth supplement to the ES cell cultures enhances the production of endothelial cells (Vittet, Prandini et al. 1996; Balconi, Spagnuolo et al. 2000). ES cells can be differentiated into blood, endothelial and smooth muscle cells thus recapitulating the normal embryonic development of the vascular system that occurs in the blood islands of the yolk sac (Yamashita, Itoh et al. 2000). ES cells grown in similar conditions used to generate osteoblast cells from bone marrow aspirates will generate osteoblast cells that will form bone nodules containing calcium deposits. Spontaneous nodules form from embryoid bodies without bone enhancing factors, but the frequency is greatly enhanced with the addition of phosphate, dexamethasone and ascorbic acid (Buttery, Bourne et al. 2001).

Human embryonic stem cells (huES) have now been found to exhibit properties similar to those of murine stem cells (Odorico, Kaufman et al. 2001) with some minor differences. Both require LIF and feeder layers in order to maintain an undifferentiated state but huES cells require the addition of bFGF. HuES cells have been tested for gene expression by PCR and they have been grown in specific cultures in order to determine their ability and range of tissue formation. Removal of LIF from huES cells causes them to form embryoid bodies, which will cavitate to form a blastocyst-like mass and acquire characteristics of the endoderm, mesoderm and ectoderm. These huES can form beating myocytes, neuron-like cells and hematopoietic cells (Itskovitz-Eldor, Schuldiner et al. 2000; Schuldiner, Yanuka et al. 2000; Kehat, Kenyagin-Karsenti et al. 2001). Although the clinical therapeutic properties of embryonic stem cells are recognized to be immense, their origin leads to ethical controversy. In addition, the requirement for HLA matching with a recipient will result in the necessity of ES cell banks or therapeutic cloning.

Therapeutic Cloning

Recently the utility of therapeutic cloning combined with gene therapy was demonstrated in the mouse. An ES cell line was derived from Rag2-/- immunodeficient mice and the genetic defect was corrected in the ES cells with a gene insertion. The corrected ES cells were then used to engraft adult Rag2-/- mouse bone marrow resulting in a reestablishment of an immune system (Rideout, Hochedlinger et al. 2002). Differentiation of these genetically modified ES cells into fully function hematopoietic stem/progenitor cells for bone marrow transplantation has proved difficult, however. Part of the problem encountered is a result of the Rag2-/- mouse having active NK cells that results in the death of the donor cells. In addition, the bone marrow engraftment was of the myeloid lineage, not the lymphoid lineage. The low levels of immunohistocompatibility complexes on the ES cells resulted in depletion of these cells by NK cells and this effect was only prevented by the removal of NK cell function. This paper proves that therapeutic cloning combined with gene therapy is capable of restoring normal immune function but also illustrates the absolute requirement for proper levels and matching of the major histocompatibility proteins for clinically useful tissue therapeutics.

Adult Stem Cells

An alternative source of stem cells can be found in adult derived tissue specific stem cells. For example, having a reliable source of blood stem cells from bone marrow, peripheral or umbilical cord blood abrogates the need for deriving blood from an ES cell source. This is not the case, however, for neural and some other tissue specific stem cells, which are not readily available for collection. Although neural stem cells have the capability to grow in culture and repopulate the murine or rat brain, there is no easy way to obtain brain or other neural tissue for the derivation of stem cells. Therefore the derivation of neural and other specific stem cells from ES cells may be a viable alternative.

Umbilical Cord Blood Hematopoietic Stem Cell Transplantation

Umbilical cord blood has been established as a clinical source of hematopoietic stem cells (HSC), which were first used for a successful bone marrow transplant in a patient with Fanconi's anemia in 1988 (Gluckman, Broxmeyer et al. 1989). In utero and at birth, hematopoietic stem cells are found in the fetal circulation. Within hours following delivery, the HSC migrate to the bone marrow where they provide the progenitors of all the blood-forming elements, including erythrocytes, leucocytes and platelets. In addition to the fetal circulation, HSC are also found in the 100 ml or so of blood in the placenta and umbilical cord, which are

typically discarded after delivery. There are enough cells in the cord blood to repopulate the bone marrow of a child and in about 25% of cases, there are enough cells to transplant an adult (Rogers, Sutherland et al. 2001). The umbilical cord blood is easily collected at birth with no risk to mother or baby. The hematopoietic stem cells contained in the umbilical cord blood are naturally a perfect HLA match for the donor and have a high likelihood of being a perfect or very close match for siblings and other relatives.

Hematopoietic Stem Cell Expansion

There are two types of repopulating hematopoietic cells; progenitor cells, which have limited renewal capacity, and stem cells, which are contained within the progenitor population and have a much greater capacity for self-renewal (Bhatia, Wang et al. 1997). Increasing the number of stem cells available for a transplant is critical for broadening the uses of these cells. Hematopoietic stem cells will divide producing one daughter stem cell and a progenitor cell, which will then produce mature blood cells. Reproducing this division process *in vitro* will only result in maintenance levels of the starting population. The key barrier to *in vitro* cell expansion, therefore, is the loss of self-renewing stem cells that occurs during induced cell proliferation. In order for one stem cell to give rise to two new stem cells it is necessary to block the differentiation pathway by triggering proliferation prior to the onset of the cells internal differentiation program. Most attempts to cause hematopoietic stem cells to proliferate leads to differentiation since growth factors have both mitogenic and differentiation properties (Huber, Zhou et al. 1998). We believe that embryonic stem cell lines, established in both murine and human, can provide us with a good model on which to base adult stem cell research. The study of ES cells can give us insight into controlling proliferation and differentiation of HSC or other adult derived stem cells.

Hematopoietic Stem Cells

It is likely that both stromal and blood hematopoietic cells can give rise to ES-like cell effects. Hematopoietic stem cells (HSC) purified from bone marrow and transplanted into recipient mice were capable of differentiation into hepatocytes (Lagasse, Connors et al. 2000) and to rescue a liver defect. In another study, hematopoietic stem cells (HSC) were purified and clonal populations were examined to determine their self-renewal and differentiation potential. Long-term repopulation (LTR) of irradiated hosts was used to show that these cells migrate to the bone marrow but can also differentiate into epithelial cells of the liver, lung, GI tract, and skin (Krause, Theise et al. 2001).

Mechanism of Adult Stem Cell Transdifferentiation

It is not surprising that ES cells are multipotent due to their source, but it is surprising that adult stem cells have the ability to be multipotential. It is assumed the lineage restriction that cells develop protects the organism from unauthorized tissue development. Evolution may have developed a redundant system where restriction of competent cell fate occurs due to regulation by surrounding signalling cells. The generalized potential manifested by adult stem cells is fascinating but we must proceed with caution in determining a possible mechanism. Recent evidence that donor cells may fuse with surrounding cells and adopt their fate suggests that although the stem cell is contributing to the repair of the tissue, the mechanism may not be by transdifferentiation but rather fusion (Terada, Hamazaki et al. 2002; Wurmser and Gage 2002; Ying, Nichols et al. 2002). A study by Clarke et al, (Clarke, Johansson et al. 2000) demonstrated the expanded potential of neural stem cells by co-culture with ES cells. The success of the experiment was dependent on the ES cells having direct contact with the neural cells. In light of the recent reports on fusion, the multitissue potential of neural stem cells may be a result of fusion of these cells with the ES cells. In another study, neural stem cells co-cultured with C2C12 cells, a muscle cell line, resulted in the conversion of the neural stem cells to muscle, but required contact with the C2C12 cells. Clusters of neural cells separated from C2C12 cells within the culture failed to generate muscle characteristics. Moreover, once the neural stem cells had been induced to differentiate towards mature muscle cells they lost their ability to differentiate back into neural cells (Galli, Borello et al. 2000). At the present time, therefore, one possible explanation for the transdifferentiation of adult stem cells into various tissues may be fusion of the adult cells with stem cells.

Another possible mechanism is that of cell signal transduction. Although the development of the embryo to an adult follows a linear process of differentiation this does not mean that the cells have a restricted potential. Proper embryo development is dependent on spatial and temporal differentiation cues suggesting that in many cases the cells respond to instructive signals. Xenopus embryo studies have indicated that a window of time occurs in which individual cells can respond to specific signals. The competency of a cell to respond to inductive signals is fairly flexible when cells are tested in a disaggregation or cell transplantation system. In contrast, lineage-marking studies, which retain the cells *in situ*, indicate a more limited potential for the blastomeres (Gimlich and Gerhart 1984; Dale and Slack 1987; Kageura 1990; Kimelman, Christian et al. 1992; Vodicka and Gerhart 1995; Horb and Slack 2001) (Heasman 1997).

Cell marking studies in mouse embryos (Lawson, Meneses et al. 1991) have

shown that epiblast cells of the early streak are limited in potency and that cells will reproducibly contribute to specific tissues, such that a mouse fate map can be made. In contrast, cell transplantation studies have shown that the cells take on the fate of the host tissue (Parameswaran and Tam 1995). Studies using late streak cells illustrate that the totipotency is lost with time and with ingression through the primitive streak. This paradox is explained by the fact that it is not a result of a cell autonomous predetermination, but the timing of the passage of the pluripotent cell through the primitive streak where they are exposed to signals from surrounding cells. (Lawson, Meneses et al. 1991) (Tam and Beddington 1987).

A similar situation may occur with adult stem cells. It is important to determine the developmental limits of these cells. For example, a hematopoietic stem cell in the bone marrow will receive lineage restricted signals so that it develops into a blood cell but placing it in the liver now allows it to receive liver specific signals and alter its differentiation program so it becomes a functional hepatocyte, as observed in human bone marrow transplant patients (Theise, Badve et al. 2000; Theise, Nimmakayalu et al. 2000).

Are adult stem cells truly a blank slate ready to respond to any signal or are they limited progenitor cells that need to fuse with a more embryonic cell in order to transdifferentiate? Is there a rare stem cell in adult tissues that is similar to an ES cell or are adult stem cells capable of being reprogrammed? *In vitro* studies have to be carefully interpreted because the presence of cell specific markers does not guarantee functionality, which is the important end point in these studies. *In vivo* studies, utilizing clonal cell populations or single cells strongly suggest the presence of a multipotent stem cell. It is important to verify the ability of adult-derived stem cells to replace damaged or diseased tissue with functional cells. In order to use these cells safely for tissue therapy, the mechanism(s) of stem cell transdifferentiation must be elucidated. One possibility is that stem cells are responding to signals involved in tissue healing. In the case of hematopoietic stem cell transplantation, the recipient mice are irradiated, resulting in tissue damage. The cell signalling that occurs in the damaged tissue (e.g. liver) that triggers the cell repair mechanism may also trigger the transdifferentiation of the HSC. There has recently been success in using hematopoietic cells to 'cure' a mouse model of Parkinson's disease. Parkinson's disease occurs due to the loss of dopamine expressing neurons due to apoptosis. It is possible that repair signals from the surrounding neurons may trigger transdifferentiation of HSC, which find their way to the substantia nigra after transplantion, into dopamine producing neurons (Li, Chen et al. 2001; Nagatsu 2002).

Transdifferentiation and Reprogramming of the Nucleus

The ability to reprogramme a terminally differentiated adult somatic cell supports the idea of transdifferentiation of adult stem cells. Recently, Hochedlinger and Jaenisch (Hochedlinger and Jaenisch 2002) managed to use B-cell nuclei for somatic nuclear transfer (SNT) into oocytes and for the creation of functional ES cells. Previously, the most widely recognized report of SNT resulting in the reprogramming of the nuclei in order to generate a live organism was the cloning of the sheep Dolly in 1997 (Wilmut, Schnieke et al. 1997). These two studies prove that even an adult terminally differentiated cell can be reprogrammed to produce multiple cell types.

Are Umbilical Cord Blood Hematopoietic Stem Cells Multipotent?

Do other hematopoietic tissues have the same ability as bone marrow to be the source of multipotential cells? As mentioned above, human umbilical cord blood is a source of clinically useful hematopoietic stem cells supplying enough cells for pediatric bone marrow transplants. The ability of umbilical cord derived stem cells to produce non-hematopoietic cells has not so far been extensively documented.

In preliminary experiments in our laboratory, human umbilical cord blood CD34+ cells were tested for non-blood gene expression by PCR. Interestingly, cells grown in 10% serum showed a mesenchymal cell morphology and were positive by PCR for bone (TRAP), muscle (desmin), neural (nestin), and astrocyte (Gfap) markers (figure 1). The numbers of positive cells remained low but these results confirm the findings reported by Erices (2000) and demonstrate the potential of UCB cells to under go transdifferentiation.

It will be important to isolate the key cell and identify growth conditions that will promote its proliferation while maintaining the cells ability to be multipotential. In addition, having enough cells to make clinical therapeutics possible will be a major hurdle to overcome. This is similar to the problem encountered for the use of HSC from UCB cells for bone marrow transplantation (Madlambayan, Rogers et al. 2001). The HSC expansion studies will serve as a paradigm for the expansion of multipotential progenitor cells from UCB.

Conclusion

Stem cells represent a complex cell type not easily defined. The derivation of embryonic stem cells has allowed us to develop models of differentiation that have assisted our understanding of embryo development and tissue formation. The therapeutic use of embryonic stem cells is an obvious goal but for human ES cells is very controversial. To some extent, the properties of embryonic stem

cells are found in adult stem cells. We believe that the establishment of human embryonic stem cell lines for research purposes is important in providing a model from which to extend the studies of adult-derived stem cells. For example, it will be fascinating to determine if adult stem cells have the ability to acquire the same embryonic markers in culture as ES cells. The goal for this research should be the establishment of adult stem cells as a source for tissue therapeutics thereby obviating the need for the use of human ES cells in the future.

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Is LH needed for optimal ovulation induction?

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Summary

The role of LH in the natural menstrual cycle is not disputed. However, there are a variety of opinions regarding the potential role of exogenous LH in ovulation induction and whether it is actually needed. The recent introduction into clinical practice of recombinant gonadotropins has been paralleled by recent advances in knowledge of the endocrine and paracrine mechanisms that regulate human folliculogenesis. On this basis, we analize here: i) whether or not all patients need LH for follicular growth stimulation; and ii) new opportunities for improved treatment as a result of the availability of rhLH both in patients with ovulatory disorders and those undergoing multiple follicular development for assisted reproduction.

Is LH needed for ovulation induction in WHO group I anovulation?

Clinical data have shown that exogenous LH is strictly necessary for ovulation induction in hypogonadotropic women. rhLH thus appears as an ideal adjunct therapy to rhFSH in such patients. In a pioneering dose-finding study where patients were randomized to receive rhLH (0, 25, 75 or 225 IU/day) in addition to a fixed dose of rhFSH (150 IU/day), it was shown that a daily dose of 75 IU rLH was effective in most women in promoting optimal follicular development and enhancing the ability of these follicles to luteinize when exposed to hCG [1]. An even more recent multicenter study [2] provided further evidence supporting this contention. Interestingly, the latter study clearly suggested that hypogonadotropic patients having very low levels of endogenous LH (<1 IU/l, i.e., below the threshold for normal estradiol biosynthesis and full follicular maturation) would necessitate higher doses of gonadotropins as compared with women having basal LH levels 1 IU/l to reach the criteria necessary for hCG administration [2].

Additionally, the early study [1] showed that increasing the dose of LH (up to 225 IU per day) during the follicular phase reduced the number of growing follicles.

Both studies thus confirmed that there is individual variation in the dose of LH (but also FSH) required to promote optimal (mono)follicular development. The use of HMG containing fixed proportions of FSH and LH for ovulation induction in WHO group I patients has been linked to high prevalence of multiple folliculogenesis which is considered as a major drawback to its use. Further refinement of dosing schedule of both FSH and LH to minimize the likelihood of multiple ovulation occurring in these patients is now possible with the availability of monotherapeutic recombinant gonadotropic agents [3,4]. Thus, enhancing the LH environment would provide a means of inducing atresia in secondary follicles and promoting growth of a minimal number of pre-ovulatory follicles ('LH ceiling concept). In fact, a pilot study has shown that treatment with high-dose rhLH during the late follicular phase promotes emergence of a dominant follicle and the regression of the secondary follicles [5].

Is LH Needed for inducing multiple follicular development in assisted reproduction cycles?

At present, it is well established that successful IVF and embryo transfer requires both stimulation of the ovary and suppression of the pituitary. Thus, exogenous gonadotropins and GnRH analogues are the key hormones required to maximize IVF success being the long protocol the most commonly adopted protocol for assisted reproduction treatment (ART) cycles worldwide. The low endogenous LH levels achieved with GnRH agonists in some cases may amplify the differences, if any, in treatment outcome seen with the use of HMG and FSH-only preparations. The recent availability of GnRH antagonists, which can cause more profound LH suppression than GnRH agonists, adds further interest to the subject.

Considerable debate exists as to whether the LH activity contained in HMG preparations could affect the outcome of ART in GnRH agonist down-regulated women [6-10]. Some authors have postulated a negative impact of using 'LH free gonadotropins for ovarian stimulation in ART [11] but on the opposite side, the idea persists that elevated concentrations of LH (endogenous or superimposed through the use of HMG) during follicular development and in the periovulatory phase are unnecessary and may not be desirable because of their potential detrimental effects on oocyte health and subsequent fertilization and implantation rates [12]. Several facts support the concept that LH administration is not needed in the vast majority of patients undergoing ART in down-regulated cycles where it has been shown that: a) The use of urinary FSH-only

preparations is associated with a significantly higher clinical pregnancy rate than HMG which contains both LH and hCG [12]; b) There is a statistically significant increase in clinical pregnancy rate with rhFSH compared to urinary FSH [13]; c) The switch in stimulation regimens to a more widespread use of FSH-only preparations, without LH supplementation, has been associated with an increased rate of overall programme success [14-17]; d) LH serum measurements in the mid-follicular phase during ovarian stimulation with rhFSH cannot predict ovarian response and ART outcome [18]; and e) rhLH supplementation to rhFSH does not improve ovarian stimulation and ART outcome [19].

Therefore, it seems clear that normally ovulating women with pituitary down-regulation are not comparable to WHO group I anovulatory patients since that, in most cases, an absolute LH deficiency really does not exist as demonstrated by a very different steroidogenic response to FSH alone. Notwithstanding this, a need for some LH supplementation may be evidenced in some women depending on the extent to which the endogenous serum LH is suppressed by concomitant GnRH agonist therapy, the direct effect of the latter on the ovary, and the protocol of gonadotropin administration used. Different criteria for defining a severe LH deficiency represent an important additional confounder. Thus, evidence of LH deficiency has been identified in a fraction of ART population ranging between 6% [5] to 26% [20] and even 50% [21]. However, in these studies a potent GnRH agonist (buserelin) was given, the threshold values for LH were established according to the detection limit of LH assays used, a fixed starting dose of rhFSH was administered for 7 days, an despite that lower concentrations of estradiol in the mid-follicular phase and at HCG administration were found in patients with 'suppressed LH, the gross ovarian response was not influenced.

Using daily doses of an appropriated GnRH agonist (leuprolide or triptorelin having lower potency than buserelin) and a step-down regimen of rhFSH administration we found that the proportion of LH suppressed women is lower than previously reported [18] and we need to add some LH in no more than 1-2% of patients in our ART general programme. These are patients usually having a low estradiol response and/or an apparent discrepancy between estradiol serum levels and developing follicles. A recent study suggests that LH supplementation in IVF patients having a poor initial response to rhFSH may improve the ovarian outcome [22]. In that study, however, it is possible that a too low daily starting dose (150 IU) of rhFSH was administered mainly considering that a depot GnRH agonist preparation (having a more profound suppresive effect on the pituitary and ovaries than daily doses) was used. We prefer a tapering (step-down) regimen after pituitary suppression, wherein the highest dose of rhFSH is given on

stimulation days 1 and 2 (450 IU and 300 to 450 IU [depending on the patient body mass index], respectively) and is then reduced to 150 IU daily once follicular recruitment has been achieved. It should be noted that follicular recruitment takes place in the early follicular phase and thus both the timing and dose of FSH administered determine the number of follicles recruited and selected. Importantly, early studies in poor responders showed that neither increasing the intial dose nor doubling the dose of gonadotropins in the course of stimulation are effective to increase the ovarian response in ART cycles.

The use of a step-down regimen of rhFSH administration may be also important with respect to ovarian paracrine signalling. FSH activates a paracrine mechanism that up-regulates LH-responsive androgen synthesis, and hence estradiol synthesis; thus, it is tempting to postulate that higher doses of FSH used at a critical period of ovarian stimulation during the early follicular phase can overcome too low 'residual LH concentrations existing in some women once pituitary-ovarian suppression has been achieved. In vitro studies showing dose-dependent stimulation by FSH of paracrine regulators (inhibin and IGFs) production by granulosa cells from immature human ovarian follicles support that contention [3]. This is important taking into account that: i) LH isohormone profile may alter following GnRH agonist administration resulting in differences in biopotency not reflected in immunoassays; ii) measurements of serum LH either before or during ovarian stimulation are not useful to predict ovarian response; and iii) circulating LH measurement do not accurately reflect LH administration [1,2,22].

In our experience and that of others [23] the use of HMG in previous *poor responders* to FSH-only preparations is (usually) associated with an increase in estradiol levels but oocyte recovery and overall IVF results are still poor. A preliminary uncontrolled study suggesting that rhLH may improve IVF outcome in poor responders to rhFSH alone deserves a larger and more detailed investigation [24].

GnRH antagonists are administered during the last days of gonadotropin ovarian stimulation. This leads to a new profile of endogenous LH characterized by physiological LH concentrations during the early follicular phase followed by a sharp reduction in these levels within a few hours after treatment initiation. The need for compensating for the acute depletion in LH is unknown. We (unpublished observations) and others [25] have observed a lack of increase, and even dramatic drops, in estradiol levels when using GnRH antagonists (mainly in the form of a high-dose single injection) in spite of maintained ovarian stimulation with gonadotropins. If this effect is due to an extreme suppression of LH concentrations, the use of rhLH could be potentially useful. However, if the effect is explained by the direct action of the antagonist on the

follicle, then the use of rhLH would not be useful. A recent study investigating follicular dynamics during ovarian stimulation with rhFSH and GnRH antagonist supports the latter contention [26]. Randomized controlled studies utilizing rhLH are needed to properly examine its contribution to FSH-only stimulation regimens.

Is LH needed for ovulation induction in PCOS patients (WHO group II anovulation?

There is evidence that developing follicles have finite requirements for exposure to LH, beyond which normal development ceases leading to atresia ('LH ceiling concept). Accordingly, a pilot study has shown the clinical efficacy of high-dose rhLH (up to 450 IU per day) for inducing atresia of secondary follicles and promoting mono-ovulation in WHO group II anovulatory women [5].

Conclusions

LH play an essential physiologic role in follicle steroidogenesis and development and oocyte maturation. Thus, exogenous LH is an essential tool in ovulation induction. Current concepts on gonadotropic control of ovarian function have established that both a 'threshold and a 'ceiling for LH levels exist during the follicular phase of menstrual and induced cycles. Therefore, levels of LH should be neither too high nor too low during ovulation induction in order to not compromise reproductive performance. This implies that: i) treatment with exogenous LH is strictly necessary in WHO group I anovulatory patients; ii) resting levels of serum LH are sufficient in the vast majority of women receiving ovarian stimulation with FSH-only products in association with GnRH agonists for ART. The potential role of adding exogenous LH to ART cycles where GnRH antagonists are used remains to be established; and iii) it is inappropriate to use LH containing products for ovarian stimulation in PCOS patients. However, it should be noted that, for years, HMG (containing FSH and LH-like activity in the form of hCG) has been the only gonadotropin available for clinical use and most current available data on ovulation induction come from clinical experience with this urinary preparation. Recombinant gonadotropins (FSH, LH, hCG) are now available and they have higher biopotency and bioactivity than their urinary counterparts. Therefore, results with HMG in different clinical conditions cannot be fully extrapolate to recombinant drugs and thus further refinement in terms of recombinant gonadotropins requirements may be necessary to optimize ovulation induction protocols.

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Ovarian cryopreservation — are we progressing? Reimplantation or IVM

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Introduction

In the United States alone there are more than 50000 women under the age of 40 diagnosed with cancer each year. Four thousand pre-adolescent females are treated with chemo or radiotherapy annually for a variety of childhood malignancies (1)

In recent years the survival rates for many of the cancers that affect young adults and children have improved markedly. Indeed for many of the common malignancies affecting children and adolescents survival rates well in excess of 60% have been reported. From 1960 until the mid 1990's the mortality rate from childhood leukemia has fallen by over 70% in the developed world (2).

These improvements in outcome have not however been without cost. The late effects of cancer treatment is an issue that has recently gained a significant amount of interest (3).

One of the potential consequences of cancer treatment is premature gonadal failure both in males and females. In particular the use of radiotherapy or alkylating chemotherapeutic agents are particularly associated with the development of gonadal failure.

For many of these patients consideration of future fertility is an issue that should be discussed prior to the commencement of any therapy. An awareness of recent developments in the area of fertility preservation among health professionals is essential in order that these patients (and in many situations parents or guardians) are given every opportunity to make an informed decision.

As a science,cryobiology has a long history essentially dating back to the early part of this century with the significant discovery of cryoprotectant agents (4). With the development of assisted reproduction over the past 25 years, cryopreservation has become an integral component, primarily to avoid wastage

of excess embryos produced as a result of treatment. Refinements in the technique of cryopreservation have not only led to improvements in embryo survival following thawing, but have also allowed the expansion of the technology to other cell types. Cryopreservation of gametes (sperm or oocytes) and ovarian tissue has now become a reality, potentially offering the hope of fertility preservation for many individuals facing treatment that would otherwise render them sterile. This review will discuss the current state of fertility preservation with a predominant emphasis on the options available for maintaining future female fertility.

Embryo Cryopreservation

As mentioned previously, cryopreservation of embryos is commonplace in the field of assisted reproduction. The first recorded pregnancy from a frozen-thawed embryo was in 1983 (5) and the first live birth in 1985 (6). Embryos may be cryopreserved at any stage of development although embryos at the pronucleate stage are more resistant to cryoinjury (7). Theoretically at least, cryopreserved embryos can be stored indefinitely and although pregnancy rates are somewhat lower than for fresh embryos there is no evidence of any increased risk of congenital or developmental problems in the offspring (8).

Embryo cryopreservation may be an option for some women as a method of fertility preservation prior to any treatment. It is the most successful option at present in terms of the subsequent chance of pregnancy, however for a variety of reasons it may not be an appropriate option. It is applicable only to adult females who have a partner from which sperm can be used to fertilize the collected oocytes. For many cancers, therapy is commenced as soon as possible after diagnosis. This often does not allow sufficient time for a cycle of in vitro fertilization (IVF) to be performed as a necessary requisite for obtaining the mature oocytes. In addition, the use of ovarian stimulation may potentially have a deleterious effect on the malignancy itself. This is a particular concern for breast cancer where estrogen receptor positive tumours may potentially be stimulated by the high estrogen levels concomitant with ovarian stimulation during IVF.

Where time and disease permit, embryo cryopreservation is an attractive option for fertility preservation and should be offered to all appropriate patients.

Gamete Cryopreservation

Cryopreservation of sperm is the most widely applied aspect of cryobiology in reproductive medicine. It has also been used for many years to preserve the fertility potential of men undergoing chemo- or radiotherapy for a variety of

malignancies. Following thawing of cryopreserved sperm survival rates in the order of 50% are expected. The introduction of intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of male infertility and has also meant that immature spermatozoa surgically obtained from either the epididymis or the testis can be used to fertilize oocytes. Cryopreservation of immature testicular sperm is a potential fertility sparing option for men with already impaired fertility. To date there have only been a few reported pregnancies as a result of using frozen thawed testicular sperm (9).

However, cryopreservation of sperm is not an option for pre-pubescent boys and there is only a finite source of sperm available for future use. In addition the couple will require fertility treatment to conceive, as natural conception is not possible.

The cryopreservation of human oocytes has gained a lot of interest in recent years however the first births as a result of frozen oocytes were reported back in 1986 (10). For women wanting to preserve their fertility it is an attractive option as it does not require that the women is in a stable relationship and with new techniques such as in vitro maturation (IVM), oocytes may be obtained without the need for ovarian stimulation. At present the results of oocyte cryopreservation are generally poor and the expected livebirth rates is less than 1% (11). One of the main issues is that mature oocytes appear to be particularly susceptible to cryoinjury. The large size of the oocyte and its relatively high water content mean that it requires a considerable length of time to achieve adequate dehydration to minimize the risk of intracellular ice formation. More importantly in the metaphase II oocyte the chromosomes are arranged on a meiotic spindle. This spindle is very sensitive to both low temperatures and cryoprotectant toxicity. There is a significant risk of aneuploidy resulting from damage to this spindle (12) (13). Other problems such as damage to the zona pellucida or premature release of the cortical granules preventing normal sperm penetration may also occur (14).

Because of these difficulties associated with cryopreservation of mature oocytes the focus of oocyte cryopreservation shifted to immature oocytes at the germinal vesicle (prophase I) stage. The chromosomes at this stage of development are within a nuclear membrane and not arranged on a spindle. As a result they are less susceptible to chromosomal disruption during cooling or with the use of a cryoprotectant. Initial results demonstrated variable rates of survival, maturation and fertilization of frozen-thawed immature oocytes (15). In vitro maturation (IVM) of immature oocytes has developed separately as a form of assisted reproduction with significant success. Although used primarily in women with polycystic ovaries it is potentially an option as a fertility sparing treatment. Oocytes can be collected from unstimulated ovaries and either cryopreserved as immature or mature oocytes or indeed fertilized and stored as

embryos depending on the individual situation. IVM will be discussed further in a later section of this review.

Because of the problems associated with the traditional slow-cooling cryopreservation technique there has been recent interest in the process of vitrification (16). Although vitrification is not a new concept, the development of newer less toxic cryoprotectants has led to some encouraging results with both embryos and oocytes (17) (18) (19).

Vitrification is essentially a process that induces a glass-like solidification of a cell without any formation of intracellular ice. It involves exposing the cell to high concentrations of cryoprotectants at room temperature before being rapidly frozen in liquid nitrogen. As a technique it also has advantages in being technically easier, less expensive and does not require a programmable freezer that is mandatory for the slow-cooling method. In particular, vitrification of immature human oocytes leads to a dramatic improvement in development rates of subsequent embryos compared to the slow-cooling technique traditionally used.

Cryopreservation of Gonadal Tissue

Technically it is possible to cryopreserve gonadal tissue from both males and females with a view to restoration of fertility in the future. Tissue may be harvested from a planned surgical procedure or during unrelated surgery and can be done so from both pre-and post puberty patients.

It terms of ovarian tissue cryopreservation it is known that primordial follicles can be cryopreserved within an ovarian tissue sample (20). These follicles are significantly less susceptible to cryoinjury compared with both mature and immature oocytes on account of their relatively small size, their low metabolic rate, the absence of a zona pellucida and the fact that the cell cycle stage is arrested at the prophase of meiosis I.

In general, as the outer cortical layer of the ovary is rich in primordial follicles, this is the only part of the ovary required for cryopreservation. The sample of ovarian cortex is normally removed via laparoscopy or laparotomy and then prepared into strips of tissue around 1mm in thickness and up to 1cm^2 in total area. These small strips are to allow adequate penetration of cryoprotectants. A traditional slow-cooling technique is generally employed using a dimethlsulphoxide (DMSO)/ sucrose cryoprotectant solution. A sample of ovarian cortex should routinely be analysed histologically primarily to assess follicle concentration but also to evaluate for the presence of any tumour cells (21). It has been shown that the concentration of follicles varies significantly with age ranging from 350-400/mm^3 at less than 10 years of age to 30-35/mm^3 from 15 to 34 years of age. This implies that the size of the ovarian sample

should vary with the age of the patient. As a rule, the aim is to cryopreserve an ovarian cortical sample with =1000 follicles. Follicle survival rates of 74% have been reported using this technique, therefore > 700 viable follicles per sample would be expected (22).

Having cryopreserved ovarian tissue, at some point in the future the options are either to transplant the tissue back to the donor (autograft) or to culture the follicles in vitro. In vitro follicular culture is in its infancy as a technique and in one murine study less than 2% of the follicles that matured and fertilized reached the blastocyst stage of development, with only one reported livebirth (23).

Transplantation of ovarian tissue has proved more promising, although to date there have been no recorded human pregnancies as a result of transplanted frozen-thawed ovarian tissue. Animal studies have shown more success with return of ovarian function and indeed pregnancies and livebirths in a number of animal species (24) (25) (26).

In humans transplantation of ovarian tissue has resulted in evidence of return of ovarian function albeit for a limited duration. There have been essentially two methods of transplantation used. Orthotopic or pelvic transplants involve grafting the tissue near the infundibulo-pelvic ligament with the hope that natural pregnancy may occur. There is a single human case report of evidence of ovarian endocrine function following transplantation but no ovulation occurred (27). Heterotopic transplantation is the second method whereby the ovarian tissue is transplanted to a site outside the pelvis. In the human cases the forearm has been the preferred site. It is a technically easier procedure that allows easy monitoring of the ovarian tissue, although ovarian stimulation in the context of an in vitro fertilization treatment is required in order to attempt conception. A report of two cases using this technique showed a return of ovarian function with the development of a dominant follicle and resumption of menstrual cycles in both. In one case ovarian stimulation was performed but no embryo developed (28) (29).

Xenografting of frozen-thawed ovarian cortical strips into mice with severe combined immunodeficiency (SCID) has also shown success with healthy follicles present in the graft when removed 22 weeks after the initial transplantation (30).

There has been considerable interest recently in the possibility of cryopreserving and transplanting an entire intact ovary. Animal studies have shown that fresh whole ovaries can be successfully transplanted but the duration of subsequent ovarian function has been limited. This has in large part been due to ischaemic injury as a result of thrombosis in the vascular anastomosis (31). Improvements in surgical technique at grafting ovarian transplants have however led to improvements in graft duration (32).

Cryopreservation of intact ovaries has until recently proved somewhat problematic especially ensuring adequate and equilibrant perfusion of cryoprotectant solution throughout the ovary. By dissecting the ovarian vessels adequately during tissue harvesting and then utilising these vessels to perfuse the ovary with cryoprotectants has significantly improved tissue survival with similar rates of follicular viability and apoptosis compared to ovarian cortical strips (33). In fact, in a recent study, a successful pregnancy was achieved following transplantation of frozen-thawed rat ovaries (34)

In most situations where ovarian cryopreservation is being considered it is because the individual is facing fertility-damaging treatment for a malignancy. There are however a number of other situations where ovarian cryopreservation may be considered. Women with severe endometriosis may require oophorectomy where conservative medical or surgical treatment has failed. Normal ovarian tissue could be dissected and cryopreserved for future use. Systemic lupus erythematosus (SLE) is an autoimmune condition that most commonly affects young women and in severe cases, cyclophosphamide therapy may be advocated (35). This is a potentially sterilizing medication and therefore, prior ovarian cryopreservation may benefit these women. Women who carry either BRCA-1 or BRCA-2 gene mutations are at high risk for the development of ovarian cancer and prophylactic oophorectomy is often advocated. Cryopreserving ovarian tissue with a view to temporary transplantation in order to conceive could be considered in these patients (36).

The safety of cryopreserving ovarian tissue is an important issue that warrants consideration particularly in patients with a malignancy. The risk of transmitting metastatic cancer cells via ovarian transplantation appears to be highest for the blood borne cancers such as leukemia and lymphoma (37). This is an important reason why histological assessment of harvested ovarian tissue is essential. The detection of neoplastic cells can also be aided by molecular biology techniques such as polymerase chain reaction (PCR), which is able to detect these cells in even minute quantities (38).

Nevertheless, the progress in this field means that cryopreservation and banking of ovarian tissue for preserving fertility should be discussed with all women facing potentially sterilizing treatment. It should also be a consideration for pre-pubescent females who may particularly gain future benefit from such a procedure. It should be discussed in an informative and sensitive manner with the parents or guardians so they can be fully informed of any decision.

In Vitro Maturation of Oocytes

The collection of immature oocytes from unstimulated ovaries with subsequent in vitro maturation and fertilization is receiving increasing interest as an

alternative to traditional IVF in assisted reproduction. Recently pregnancy rates in excess of 25% have been reported particularly in women with polycystic ovaries (PCO) undergoing IVM treatment (39) (40) (41).

Additionally it has been advocated as a means of obtaining oocytes for cryopreservation. It is particularly attractive in women who are suffering from malignancy as it can be performed at relatively short notice and does not require ovarian stimulation, the effect of which may be a concern in some cases.

Patients undergoing an IVM cycle in the context of treatment for infertility have a baseline ultrasound scan performed between days 2 to 4 of their cycle. At this point the total number of antral follicles of greater than 2 to 4mm is assessed. It has been demonstrated that pregnancy rates following IVM depends upon the number of immature oocytes obtained which in turn can be predicted on the basis of the antral follicle count (42). Pregnancy rates comparable to conventional IVF maybe obtained when 10 or more immature oocytes are collected. Oocytes can be retrieved from about 50% of visible follicles. This implies that patients who have PCO (> 10 follicles per ovary) visible on ultrasound could be offered IVM treatment as an alternative to IVF (43).

The patient is normally scanned again on day 8 and oocyte retrieval is planned for between days 10 to 14. Thirty-six hours prior to oocyte collection a subcutaneous injection of hCG 10000iu is administered. This has been shown to improve the rate of maturation of the immature oocytes and the final number of mature oocytes obtained (44).

The cycle is normally cancelled if a dominant follicle (>10mm) is present on day 8 because initial reports have suggested a reduced pregnancy rate. The collection of immature oocytes is performed under ultrasound guidance with a reduced aspiration pressure. Although similar to IVF, it is a more technically challenging procedure.

Following collection, the immature oocytes are cultured in a specialized medium for 24 hours prior to fertilisation (by intracytoplasmic sperm injection, ICSI) of the then metaphase II oocytes. The media is essentially standard TC-199 supplemented with 20% patient's own serum, pyruvic acid and 75 mIU/ml of menotropins. Some oocytes will require up to 48 hours to mature and overall maturation rates of approximately 80% have been reported. Embryo transfer is performed 2 to 3 days later.

The endometrium is also primed with estradiol valerate from the day of collection, with a dose that depends on the endometrial thickness and progesterone luteal support is given from the day of ICSI.

Although the above process describes IVM as applied to patients receiving fertility treatment, the process can be applied to those desirous of fertility preservation. It allows the opportunity to cryopreserve either immature or

mature oocytes or proceed further to embryo cryopreservation if there is a stable relationship.

Conclusion

A diagnosis of cancer is a devastating one for any person but especially so in young adults and children. The reality is that for many of the common cancers seen in this group the advances in cancer treatment have led to significant improvements in overall survival. This has meant however that the longer-term effects of treatment have become significant issues. One of these issues is the potentially sterilizing effect of radio-or chemotherapy as it is used to treat individual malignancies.

Health professionals dealing with these patients often overlook discussing alternatives for preserving future fertility. However, recent advances especially with regard to cryopreservation of gametes and gonadal tissue has meant that fertility preservation is fast becoming a viable option. At present many of the techniques remain experimental but as many of these patients would not consider utilizing this preserved fertility for many years, the potential improvements in this area may lead to higher success rates in the future.

It is incumbent on health professionals dealing with patients who face a loss of fertility that these options are fully discussed prior to any therapy so that an informed decision can be made.

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Induction of ovulation and ovarian cancer — Is there an association?

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Summary

During the last decade, a possible risk of ovarian cancer following treatment with ovulation inducing drugs has been suggested. Epidemiological studies exploring this possible association have been criticized for their methodology and especially the magnitude and quality of data regarding infertility treatments. The objective of the current study was to evaluate the association between infertility, ovulation induction treatment and ovarian cancer incidence in a cohort of 5788 women who were treated for infertility in Israel during the period 1964-1984. Twenty ovarian cancer cases were observed as compared to 15.1 that were expected (SIR=1.33; 95% CI 0.8-1.1). Women with hormonal infertility were

significantly protected from ovarian cancer as compared to women with non-hormonal infertility. No statistically significant excess risk of ovarian cancer was observed among women who were treated with ovulation induction as compared to those who were not treated.

Introduction

More than 15% of couples during their reproductive years will require treatment for infertility (1). Infertility itself has been found to be a risk factor for ovarian cancer (2). Ovarian cancer represents the sixth most common female cancer in the western world and is the most fatal gynecological malignancy with 5-year survival of 40% (3,4). A number of identifiable factors have been associated with an increased risk of ovarian cancer, including environmental (5), hormonal (6,7), and genetic (8); particularly the presence of BRCA1 and BRCA2 gene mutations (9). Parity and oral contraceptive (OC) use have well-documented protective effects in ovarian cancer (10). The rationale for these protective effects is unclear and possibly involves decreased ovulation during these periods (11).,

Fathalla, in 1971 (12), was the first to suggest the relationship between ovarian cancer and ''incessant ovulation''. With each ovulation the ovarian epithelium was thought to incur minor trauma. The cumulative effect of repetitive surface injury was hypothesized to contribute to the development of ovarian neoplasm. The concern that there may be a causal relationship between ovulation inducing drugs and ovarian cancer was first raised in a number of case reports (13-22). However, most of these reports included women with short exposure to ovulation induction and limited latency period, suggesting that treatment may have been coincidental in women with pre-existing ovarian tumor. The growth of the tumors may have been enhanced by the treatment, or the presence of the tumor may even have been the cause of infertility.

A series of articles published by Whittemore et al (23-25) and Harris et al (26) in 1992, led to renewed interest in the potential carcinogenic effects of infertility and ovulation inducing drugs used to treat infertility. Several cohort and case control studies (27-35) that were published regarding this possible association have been criticized for their sample size, methodology and especially the magnitude and quality of data regarding infertility treatments (36-38).

In vitro fertilization (IVF) programs utilize even higher dosage of ovulation inducing drugs for ovarian stimulation in addition to repeated minor traumas to the ovary for ovum pickup. Although multiple case reports observed ovarian cancer among women treated with IVF (39-42), the majority were diagnosed during or shortly after the treatment, therefore can be attributed to the close surveillance of these women during IVF. Cohort studies of women undergoing IVF (43-45) failed to observe an association to ovarian cancer development. The

research results related to the possible association between infertility treatments and ovarian cancer development are still controversial. The overall objective of the current research was to evaluate the association between infertility, ovulation induction treatment and ovarian cancer incidence.

The specific aims are: 1) to compare ovarian cancer incidence in a cohort of infertile women to the expected cancer rates in the general population, and 2) to evaluate ovarian cancer incidence among infertile women who were treated with ovulation induction drugs as compared to infertile women who were not treated.

Material and Methods

The study used a historical prospective design and was conducted by abstraction of medical records of 5788 women who were treated for infertility at various medical centers in Israel during the period 1964-1984. The study cohort computerized database was linked to the National Population Registry and the National Cancer Registry updated to 31.12.1996, using identification variables. For every positive link with the Cancer Registry, the original pathology report was abstracted and verified.

The dependent variable was incidence of ovarian cancer while the independent variables were a history of infertility and ovulation induction treatments. Confounding variables included age, continent of birth, medical center, age at menarche, years of infertility, type of infertility and specific diagnosis of infertility. For the assessment of infertility as a risk factor for cancer development, the observed number of ovarian cancer cases in the cohort of infertile women was compared to the expected number of ovarian cancer cases in the general population in Israel matched for age and continent of birth, using standardized incidence ratios (SIR). In addition, a multivariate Poisson regression analysis with the inclusion of the general population cancer rates was calculated. For the assessment of ovulation induction treatment as a possible risk factor for ovarian cancer development, cancer incidence was further compared within the cohort among women who were treated with ovulation induction drugs and those who were not treated using relative risks (RR) and a multivariate Poisson regression analysis to evaluate the contribution of confounding variables.

Results

The study cohort comprised 5788 women who attended infertility clinics in five medical centers throughout Israel during the period 1964 — 1984. The mean age at first visit to the infertility clinics was 28.6 $\pm$ 5.6 years and the age at the end of follow-up was 49.9 $\pm$ 6.6 years, yielding 120,895 person years of follow-up.

Three hundred and seven cancer cases were observed in the cohort through linkage with the Cancer Registry including: 131 cases of breast cancer, 20 cases of ovarian cancer, 32 cases of endometrical cancer, 16 cases of cervical cancer and 108 cases in non-gynecological sites.

When evaluating the role of infertility itself, 307 all-site cancer cases were observed in the cohort as compared to 270.3 cases that were expected in the general population (SIR=1.14; 95% CI 1.0-1.3) after controlling for age and continent of birth. Twenty ovarian cancer cases (all invasive) were observed as compared to 15.1 that were expected in the general population (SIR=1.33; 95% CI 0.8-2.1). In multivariate analysis, infertile women who suffered from hormonal infertility (abnormal ovulation) were significantly protected from ovarian cancer (hazard ratio 0.5; 95% CI 0.32-0.76) as compared to women with non-hormonal infertility (normal ovulation).

Comparison between infertile women who were treated with ovulation induction drugs and those who were not treated within the cohort revealed similar ovarian cancer incidence in both groups. Multivariate analysis revealed no excess risk for ovarian cancer among women who were treated with ovulation induction, and women en who were diagnosed with hormonal infertility (abnormal ovulation) were again significantly protected from ovarian cancer (hazard ratio 0.5; 95% CI 0.33-0.84).

Discussion and Conclusions

Our findings of increased ovarian cancer incidence in infertile women with normal ovulatory cycles (non-hormonal infertility) are in accordance with the long-standing theory of ''incessant ovulation'' (12) as a possible risk factor for ovarian cancer.

The role of ovulation induction treatment as a risk factor for ovarian cancer has been debated in the scientific literature over the last decade. It was hypothesized (46,47) that exposure of the ovarian epithelium to high circulatory concentrations of gonadotropins may increase the likelihood of malignancy. Several animal experiments supported this hypothesis (48,49); however, the experimentally induced tumors originated from the stromal cells of the ovary and are either uncommon or non-existent in humans (50,51).

Ovarian cancer incidence was not found to be elevated in women who were treated with ovulation induction in our cohort. This finding contradicts some other studies that observed increased risk of ovarian cancer in infertile women, especially those who were treated with numerous cycles of C.C. (29). However, these studies received extended criticism as to the lack of adequate information on the reason for infertility, type of medication and the histopathological diagnosis of ovarian cancer (36-38). A recent pooled analysis of 8 case-control

studies on ovarian cancer (35) also did not observe a significant association between infertility treatments and this type of malignancy.

The infertile women who were followed in our cohort did not yet reach the age of peak incidence for cancer in general and gynecological malignancies in particular. Nevertheless, most women are in their perimenopausal period, and the cohort size has sufficient power to detect an association with relative risk of at least 2.5, for ovarian cancer development between infertile women who were exposed to ovulation induction drugs and those who were not exposed. Therefore, an excess risk of ovarian cancer of this magnitude that could be related to ovulation induction was excluded. However, our findings could not rule out an association less powerful.

Additional studies are needed to disentangle some of the limitations of the present cohort analysis. Given the fact that most cohort studies, including our own, are based solely on medical records (where valuable information on reproductive factors (parity), use of OC and family history of cancer is missing), and in view of the results of the sensitivity analysis, a nested case-control study within the cohort is necessary. A systematic nationwide registry of infertile women treated with ovulation induction and IVF would provide data on dosage of ovulation induction and on the outcome of each treatment cycle, which is missing, even from the medical records. Due to the fact that our cohort is not yet mature (median age 49 years), it is imperative to repeat similar data analysis with additional 5 and 10 years of follow-up.

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Does environmental pollution affect human reproduction?

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Summary

Environmental pollution is known to be associated with several adverse health effects, including a possible negative effect on reproductive health. We have focused on determining the impact of polycyclic aromatic hydrocarbon (PAH) components of cigarette smoke on male and female gametes. PAHs are known carcinogens and have previously been implicated in adverse reproductive effects in both men and women. For example, it is known that women who smoke undergo menopause 2-4 years earlier than non-smokers and that there is a presumptive link between cigarette smoking and osteoporosis. In men, we have demonstrated an increase in sperm DNA fragmentation in smokers compared to non-smokers.

In recent projects, we found that administration of PAHs to mice resulted in decreased epididymal sperm concentration. Using immunocytochemistry, we determined that PAHs resulted in the induction of DNA adducts and apoptosis in sperm. In addition, we determined that a natural aryl hydrocarbon receptor (AhR) antagonist, resveratrol, found in some red wines and in the root of polygonum cuspidatum, could reverse these harmful PAH effects. Zenzes and colleagues in our Division have discovered similar adverse effects of PAH on sperm in men, including the presence of DNA adducts which can be transmitted to the embryo. Similarly, using a murine xenotransplantation model, we have demonstrated direct negative effects of PAHs on human oocytes that involve the induction of cell death genes such as *bax* and *p53* and lead to apoptosis. These data suggest that environmental exposure to PAHs, either through inhalation of cigarette smoke or air pollution, has potential adverse effects on reproduction.

Introduction

Halogenated aryl hydrocarbons, polychlorinated biphenyls (PCB), polyaromatic hydrocarbons (PAH) and other industrial chemicals that bind to the aryl hydrocarbon receptor (AhR) are environmental contaminants that are collec-

tively referred to by the term **AhR ligands**. AhR ligands, especially the halogenated ones, have attracted much attention and concern recently because of their resistance to degradation, resulting in a long biologic half-life (>10 years) in soil (1) and between 4 and 12 years in human blood and fat (2). Every person on earth is continually exposed to AhR ligands, which are present in cigarette smoke, in exhaust fumes from both gasoline and diesel engines, in furnace gases, in cooked meat and fish, in dairy products, and even in mother's milk (3). There is sufficient evidence to link exposure to AhR ligands to the development of many diseases including atherosclerosis (3, 4), cancer (5-7), immunosuppression (8, 9) and skin disorders (10, 11).

The biological effects of AhR ligands have been shown to be mediated by the aryl hydrocarbon receptor, a 110 kDa protein of the basic helix loop helix/PAS family of transcription factors. AhR is present in the cytosol of mammalian cells of almost all organs and tissues bound to heat shock protein 90 (1). Upon binding to ligand, AhR dissociates from HSP-90 and the ligand-AhR complex is translocated to the nucleus through association with a structurally related protein, the AhR nuclear translocator (Arnt). Inside the nucleus, the heterodimeric AhR/Arnt complex regulates gene transcription by binding to DNA at dioxin-responsive enhancers (DRE) located within, or upstream of, a number of genes for phase 1 (cytochrome P-450) enzymes such as CYP1A1, 1A2, and 1B1 and phase 2 (detoxification) enzymes such as glutathione S-transferase Ya, aldehyde-3-dehydrogenase, and NAD(P)H:quinone oxidoreductase. The most important phase 1 enzyme is CYP1A1 which is responsible for the production of aryl hydrocarbon hydrolase (AHH) and leads to the oxidative metabolism of AhR ligands, often to more pro-carcinogenic compounds (12, 13). Phase 1 enzymes also increase the production of reactive oxygen species (ROS), which have been shown to be associated with lipid peroxidation, oxidative DNA damage and other pathologic effects (14, 15). AhR ligands also induce phase 2 enzymes responsible for detoxification and excretion of environmental toxicants. Therefore, a complex activation of gene transcription occurs following exposure to AhR ligands, which may initiate toxic effects as well as attempts to clear the ligand or its active metabolites from the body.

The prototype AhR ligand is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin) that binds to the AhR with the highest affinity of all known compounds (Kd = $10^{-10\ to-11}$ M in mice and 10^{-9} M in humans) (1). Benzo[a]pyrene (BaP) and 7,12-dimethylbenz[a]anthracene (DMBA) are two polyaromatic hydrocarbons present in cigarette smoke, furnace gases and exhaust fumes and, although their affinities to the AhR are lower than TCDD (16), are likely more important AhR ligands in terms of human exposure. These related environmental pollutants are not physiological AhR ligands. At the present time, the AhR is considered to be an orphan receptor since no endogenous ligand has been

identified. Up to now, the only known natural ligands for the AhR are indolo[3,2-b]carbazole and other methyleneindole condensation products formed after ingestion of cruciferous vegetables such as cabbage, broccoli and cauliflower (17, 18). The biologic activities of these natural compounds are complicated, since they appear to be mixed agonists and antagonists of the AhR (14, 17).

Cigarette smoking as a model of chronic AhR ligand exposure in man

Cigarette smoke contains BaP, DMBA and other AhR ligands such as polychlorinated dibenzo-p-dioxins (PCDD) in the tar fraction. It has been estimated that the amount of BaP in mainstream cigarette smoke is between 40 and 100 ng per cigarette and that up to 460 ng of BaP per hour can be inhaled by non-smokers in a smoking environment (19). In heavy smokers, daily exposure to BaP could be as high as 0.05 mg/kg in addition to other unquantifiable AhR ligand exposure from the environment. The AhR ligands in cigarette smoke are present in high enough concentrations to induce CYP1A1 and AHH activity in the lungs(6, 7), placenta (20), kidney (21), ovary (22) and in the endothelial cells lining blood vessels (4). AHH metabolism of BaP results in reactive carcinogenic intermediates, which bind to DNA forming predominantly covalent (+) trans adducts (23). Cigarette smoking has been identified as the direct cause of at least eight different human cancers including lung, bladder, GI tract, and leukemia, as well as ischemic heart disease (24).

Discovery of a natural AhR antagonist ligand

AhR ligands have enormous health and economic implications that have only recently been recognized. However, attempts to reduce exposure to these environmental toxins have not been successful, nor are they likely to be until internal combustion engines, the use of fossil fuels, and cigarette smoking are eliminated. It is, therefore, important to develop a method to antagonize the adverse effects of toxic AhR ligands. In an attempt to identify other natural or pharmaceutical AhR ligands, we utilized a breast cancer cell line (T47D), stably transfected with DNA containing one DRE coupled to a thymidine kinase (TK) promoter and a chloramphenicol acetyl transferase (CAT) reporter gene, as a method to screen for novel ligands. We discovered that a plant antifungal agent or phytoalexin, present in some red wines and in the root of polygonum cuspidatum, was capable of blocking the binding of dioxin-like chemicals to the AhR and subsequent phase 1 enzyme induction, by which AhR ligands exert their toxic effects (25). This substance, resveratrol (3,5,4'-trihydroxystilbene), inhibits the induction of DRE-driven transcription by TCDD and prevents the ability of dioxin to initiate the transcription of CYP1A1 and other phase 1 enzymes.

We confirmed this observation by western blotting of protein from T47D cells for CYP1A1 production in response to a toxic concentration of TCDD (10^{-9} M). We showed that resveratrol at a concentration of 10^{-6} M can block induction of CYP1A1 protein by approximately 50% (25). The inhibitory activity of resveratrol was slightly less than that of the synthetic dioxin antagonist, -naphthoflavone (NF). However, in contrast to NF, which has both agonist and antagonist, effects, resveratrol appeared to be a pure AhR antagonist ligand.

Direct toxic effects of AhR ligands on ovary

There is compelling evidence for a direct toxic effect of AhR ligands on the ovary. Women who smoke undergo menopause at an earlier age (between 1 and 4 years earlier) than non-smokers (26-30). Computer simulations from animal studies have suggested that destruction of 25% of oocytes at birth is required to see a 2-year advance in the age of menopause (27). Therefore, although the epidemiologic studies are not very sensitive in detecting oocyte damage by environmental toxicants, the consistent finding of early onset of menopause in smokers is important. These findings raise the possibility that AhR ligands or other components of cigarette smoke have direct toxic effects on ovarian follicles or on oocytes. Experimental data in animals are available to support these epidemiological studies. Miller et al (31) treated mice with single intraperitoneal injections of BaP (0-500 mg/kg) in corn oil, and followed ovarian volume and histology over 4 weeks after treatment. They found a transitory decrease in the number and total volume of corpora lutea at doses of BaP between 1 and 50 mg/kg and irreversible follicle destruction at higher doses (100 and 500 mg/kg BaP), with complete inhibition of ovarian activity. Other studies by this group have shown direct toxic effects of BaP and other AhR ligands on oocytes (32, 33) as well as evidence that induction of CYP1A1 and AHH by BaP is correlated with ovotoxicity (22, 34). These toxic effects of AhR ligands are likely mediated by the AhR since concomitant administration of the AhR antagonist, NF is able to prevent murine oocyte destruction (22). In an IVF program, Zenzes et al (35) showed that women who smoke, or who are exposed to second-hand smoke through living with partners who smoke, have a significant increase in diploid oocytes (failure of first polar body extrusion).

Apoptosis is a physiological process that entails the programmed death of a cell. It plays a critical role during development and in the maintenance of tissue and organ homeostasis. Imbalance of gene expression controlling apoptosis often contributes to the development of neoplasia. Apoptosis is also the process by which many genotoxic and chemotherapeutic drugs exert their cytotoxic effects. Recent work has linked the induction of apoptosis mediated by cell death genes with ovotoxicity and follicular atresia induced by environmental

toxicants. Specifically, the cell death promoter gene, *bax*, is upregulated in ovaries as a result of toxicant exposure (36, 37). Creation of *bax* knockout mice has resulted in the development of granulosa cells resistant to apoptosis (38) with increased numbers of primordial follicles compared to wild-type littermates (39). Bax:bax homodimer formation is thought to directly promote cell death while bax heterodimer formation with bcl-2 or bcl-X_{long} is capable of preventing apoptosis (40, 41). The well known tumour suppressor, *p53*, acts as a transcription factor inhibiting the expression of *bcl-2* and increasing expression of *bax* (42), thereby altering the balance between cell death suppressors and cell death inducers in favour of cell death. We have examined the sequence of the human and murine *p53* gene and found one dioxin response enhancer (DRE) in the mouse gene but 5 copies of the DRE in the human *p53* gene sequence (Casper and Savouret, unpublished). p53 was found to be abundantly expressed in granulosa cells (43), and we have shown that both immature and mature oocytes contain high levels of *p53* and *bax* mRNA (44). Recently, our group has demonstrated that follicle and oocyte damage by the AhR ligand, DMBA, in both the mouse and human is mediated by upregulation of p53 and bax, resulting in apoptosis of oocytes (45). Additionally, the co-administration of resveratrol is capable of preventing the increased expression of Bax and p53 protein determined by imuunohistochemistry of ovarian cortical slices (Jurisicova et al, unpublished). Since the ovary also contains CYP1A1 and AHH (22, 34), it is possible that AhR ligands induce ROS in the ovary with subsequent DNA damage, secondarily activating *p53* and programmed cell death.

Direct toxic effects of AhR ligands on the testis

Increased environmental pollution and cigarette smoking are potential explanations for an apparent decline in sperm counts over the last 50 years. Skaekkebaek reviewed data from 61 publications and reported that the average sperm count has decreased from 1,130,000 to 660,000 in the last half-century (46). Deleterious effects of cigarette smoking on male fertility have been reported to include reduced sperm concentration, increased abnormal cells, and chromosomal abnormalities (47-49). Although cigarette smoking is associated with a reduction in sperm quality and viability, these standard measurements of sperm do not provide information on the genetic integrity of sperm cells. We have recently demonstrated increased DNA damage (50) and Zenzes et al (51) observed increased DNA adducts in sperm from smokers compared to non-smokers. This observation raises the possibility that sperm DNA damage, besides affecting fertilization directly (52), may possibly result in later toxic effects on the embryo or genotoxic effects postnatally. Prenatal exposure may cause effects that are not detectable until puberty when the

sperm count, epididymus weight and seminiferous tubule diameter can all be decreased (53).

Apoptosis has been proposed as a mechanism by which testicular germ cells are removed during various pathological conditions (54, 55). Apoptosis may, therefore, be involved in the occurrence of oligospermia after exposure to various toxicants. Moreover, apoptosis of damaged premeiotic germ cells may serve a critical role in protecting subsequent generations from the diverse genotoxic effects of environmental toxicants.

We recently showed increased apoptosis in male germ cells in mice exposed to BaP (56). Detection and exclusion of apoptotic spermatozoa cells may improve the ability to select normal sperm for use in the treatment of male infertility. Soon after initiating apoptosis, most mammalian cell types translocate phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS was found to be specifically detected by staining with fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC), a protein with a strong, natural affinity for PS (57). Annexin V has been successfully applied to the evaluation of sperm (58) and oocyte (59) quality after cryopreservation. In a murine model of BaP exposure, we found that annexin V can be used to sort sperm into those undergoing early apoptosis and those late in the apoptotic process. The highest fraction of annexin V positive and propidium iodide negative sperm, implying early apoptosis, was observed in the intermediate (5 mg/kg) range of BaP exposure over 5 weeks (56). Higher doses of BaP appeared to result in sperm cell necrosis as seen by an increase in the PI positive fraction. These results suggest that flow cytometry with exclusion of annexin V staining apoptotic sperm could be used for choosing normal sperm for ART. Nevertheless, the safety of using annexin V antibody and fluorescent flow cytometry would require further study.

BaP is metabolized by CYP enzyme systems (oxidases) to reactive hydrophilic intermediates arising from epoxidation. The major diol epoxide, BPDE, binds covalently to the 2-amino group of guanosine in DNA and forms adducts designated BPDE-I-dG-DNA (60). These adducts are premutational lesions that, if not repaired, constitute a potential source of carcinogenic damage. Both baseline and BaP-induced CYP1A1 can contribute to metabolism of BaP to BPDE and augmentation of BPDE DNA adducts. Zenzes et al have demonstrated that BaP diol epoxide-DNA adducts are present in human sperm (51) and granulosa-lutein cells (61) obtained from cigarette smokers and have found that the sperm contributes DNA adducts to human preimplantation embryos formed through in vitro fertilization (51). Our recent study shows that injection of BaP in vivo in a mouse model leads to the significant formation of BPDE-DNA adducts in sperm cells (56). The formation of DNA adducts in spermatozoa can lead to DNA damage and to initiation of apoptosis. In addition,

sperm DNA adducts are a potential source of transmissible prezygotic DNA damage. In a few studies, the childhood risk of cancer associated with paternal smoking was shown to be higher than with maternal smoking (62). Reported smoking habits for the parents of 1549 children who died from cancer, compared by matched pairs analysis, found a positive correlation between paternal daily consumption of tobacco and the risk of childhood cancer ($P = 0.001$). About 15% of all childhood cancers in this series could be attributable to paternal smoking (63, 64). Thus, evidence from both human and animal studies demonstrates fetal genotoxic effects and the presence of DNA adducts from exposure to metabolites of tobacco smoke. These findings should lead to strong recommendations to protect fetuses, newborns and infants from tobacco smoke and environmental pollution.

Our findings also indicate a possible protective role for resveratrol in the testis as demonstrated by the significant reduction of BPDE DNA adducts and apoptosis in male germ cells of mice exposed to BaP and resveratrol (56). Further clinical research is necessary to confirm whether these findings will also be pertinent to humans.

Conclusion

In summary, exposure of the gonads to the polycyclic aromatic hydrocarbon AhR ligands BaP and DMBA results in a toxic effect on oocytes and on spermatogenesis. The result is a decrease in oocyte and sperm concentrations. In the testis, BaP exposure activates CYP1A1, which in turn results in the formation of BaP BPDE DNA adducts during spermatogenesis. These adducts cause DNA damage, apoptosis and necrosis. In the ovary, DMBA exposure upregulates cell death gene transcription, leading to apoptosis of oocytes. Resveratrol, a naturally occurring AhR antagonist, present in some red wines and in the root of polygonum cuspidatum, was able to significantly decrease the occurrence of apoptosis in both testis and ovary. These data suggest that environmental exposure to polycyclic aromatic hydrocarbons, either through inhalation of cigarette smoke or air pollution, have potential adverse effects on reproduction, and that resveratrol may be able to reverse the toxic effects of environmental exposure to AhR ligands on reproductive function.

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The role of different progestins beyond endometrial protection

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Beneficial effects of estrogen replacement in relieving climacteric symptoms (hot flushes, night sweats, genito-urinary atrophy, alterations in sexual function, insomnia, skin dryness and psychological symptoms, such as irritability, and depressive mood) might be counteracted, at least in part, by the addition of a progestogen. Unpleasant effects of progestins depends on doses, type and route of administration. In addition, different women present different susceptibility to progestins effects mainly related to the effects of these different molecules on central nervous system.

For women with a uterus, the addition of progestin protects against overgrowth of the uterine lining from estrogen stimulation, a risk factor for endometrial cancer. Benefit is derived through either inducing the periodic shedding of the uterine lining, thereby causing a menstrual-like bleeding (cyclic regimen: daily estrogen combined with cyclic exposure, usually 12 to 14 days of progestin) or through the attenuation of impact of estrogen on the lining over time resulting in amenorrhea (continuous combined regimen: daily estrogen combined with daily low-dose progestin). Women will experience different patterns and amounts of vaginal bleeding and spotting depending on the dose and number of days each month the progestin is taken (1). Despite the efficacy for ERT/HRT in the management of many short- and long-term consequences of estrogen deprivation, HRT is discontinued by more than 50% of all women initiating hormone therapy within the first year and is rarely used for longer than two years. (2, 3). The process of a patient's choice to begin and to continue HRT it has been reported that women's age, physician's opinion, reports in the media, previous oral contraceptives, experiences and opinions of friends and a prior hysterectomy are critical to patients' decisions about HRT (4-7). The higher prevalence of hormone treatment in hysterectomized women, underline that both the bleeding issue and the added progestin may play a critical role The optimal choice of the progestogenic component and issues such as the type, dose and duration of treatment are still widely debated. Besides the estrogen dose and route of administration, progestogens can make the difference in terms of the ultimate clinical and metabolic effects, as well as of compliance.

When used in low dose combinations progestins can act with a positive synergism with estrogens on vasomotor symptoms as well as on bone density and metabolism (8-10). While low dose HRT may represent the choice for many women (11,12), standard HRT requires doses of estrogens that induce an endometrial stimulation requiring an adequate progestin supplementation. Progestins used for HRT include natural progesterone (micronized) and synthetic derivatives: the 19-nortestosterone derivatives, such as norethisterone acetate and levonorgestrel, and the 17-hydroxyprogesterone derivatives, such as the unappealing medroxyprogesterone acetate and the cyproterone acetate (CPA). The progestogens presently available for prescription interact with other steroidal receptors, including the androgen receptor (AR), the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), and the human estrogen receptor (ER). According to their selectivity profile, these molecules will exert other actions than the expected progestational activity. In high dosages androgenic 19-nortestosterone derivatives as well as medroxyprogesterone acetate can have adverse effects on lipid metabolism. This is not observed with CPA, free of androgenic effects, which exerts some anti-androgenic effects. The favorable changes in lipids, lipoproteins and vasomotion patterns observed with estrogen supplementation are not significantly modified by the addition of CPA (13-15). When considering other estrogen/progestogen combination regimens, the anti-androgenic activity of CPA may confer benefits in comparison with those progestational compounds that have androgenic properties on blood pressure or upon the renin-angiotensin/aldosterone system. The early post-menopausal period is associated with an increase in body weight (16-20), that was not evident in HRT treated women (21-24) . The postmenopausal increase in body weight parallels an increase in body fat and a change in body fat distribution. Central body fat distribution has been associated to a series of endocrine and metabolic consequences (25-28) related to an increased risk of cardiovascular disease. Particularly, the HRT preparations containing CPA can blunt the increase in body weight, and prevent the shift to a more central, android fat distribution observed in normal women throughout their early postmenopausal period. In this view, besides the controversial results of trials conducted in elderly and infirm women (29,30), the observed stabilization of body weight and body fat distribution can be seen as a one of the mechanisms that can explain the protective effect of HRT against cardiovascular disease (31). However, CPA is a unique antiandrogen progestin, and the effects on body fat could be ascribed to this specific replacement regimen. The HRT preparations containing CPA are effective in relieving subjective symptoms, preventing postmenopausal bone loss and the impairment of lipid profile that characterize the postmenopausal years (13-15). Several new progestogens have been synthesized in the last decade. The new progestin derived from 17 alpha-

spirolactone(32), drospirenone is an innovative and unique progestin related to spironolactone, recently developed. Drospirenone has potent progestational activity along with the antimineralocorticoid and antiandrogenic properties of spironolactone. The drospirenone characteristics are useful in management of patients with premenstrual syndrome, premenstrual dysphoric disorder, acne and sebum production, with effects similar to those observed with CPA-containing products. The actions of drospirenone are more similar to natural progesterone than other progestins, showing also a direct antiandrogenic activity. Drospirenone is able to counteract the ''weight-increasing'' tendency of estrogens, which is due to aldosterone-induced sodium and water retention (through activation of the renin-angiotensin-aldosterone system). Importantly, the drospirenone induced sodium-wasting effect does not result in any significant increase in serum potassium levels. Based on the current reasons that women discontinue or avoid HRT, a new product containing drospirenone may improve women well-being and compliance. In addition, a combined HRT with antimineralocorticoid effects could offer a novel potential mechanism for reducing cardiovascular end points in postmenopausal women.

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Is diagnostic laparoscopy ever indicated?

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A diagnosis of unexplained infertility is usually made only after it has been shown that the woman ovulates regularly, has patent fallopian tubes, shows no evidence of peritubal adhesions, fibroids, or endometriosis and has a sexual partner with normal sperm production and function (Simon et al., 1993). Only when all standard clinical investigations yield normal results should the diagnosis of unexplained infertility be raised (Speroff et al., 1999). It has also been suggested (Rousseau et al., 1983) that the diagnosis should be reserved until two years after a normal laparoscopy, based on the observation that during the initial two years period of expectant management, a cumulative pregnancy rate of 65% was found. Moreover, approximately 60% of couples with unexplained infertility of less than three years of duration would become pregnant within three years of expectant management (Crosignani et al., 1993, Verkauf, 1983, Collins and Rowe, 1989).

Because the rates of spontaneous pregnancy are still considerably lower than those observed for the normal population (Simon et al., 1993), it is essential to complete the standard diagnostic work-up to avoid overlooking a treatable factor. The generally accepted investigation protocol to establish the diagnosis of unexplained infertility includes: semen analysis, a properly timed postcoital test, assessment of ovulation and demonstration of tubal patency (Speroff et al., 1999).

Hysterosalpingography, laparoscopy or both can be applied to demonstrate tubal patency. Hysterosalpingography is also essential to exclude mullerian tube malformations and other uterine cavity defects (Simon et al., 1993). However, even when tubal patency has been demonstrated by hysterosalpingography, laparoscopy has been suggested as a mandatory step to preclude the existence of peritubal adhesions as well as endometriosis as causes of infertility (Simon et al., 1993, Pepperell and McBain, 1985). Classically, diagnostic laparoscopy was considered as the gold standard in diagnosing tubal pathology and other peritoneal causes of infertility. Traditionally, laparoscopy was the final diagnostic procedure of any infertility investigation, included in the basic infertility work-up outlined by the American Fertility Society in 1992 and World Health Organization (WHO) guidelines.

It has been estimated (Drake et al., 1977) that using laparoscopy as a standard test of tubal function would reduce the apparent incidence of

unexplained infertility from 10% to 3.5%. In 24 cases of otherwise unexplained infertility they found abnormal finding in 18 (75%). Of these 18 subjects, unsuspected endometriosis was found in 11 (46%) and peritubal adhesions in 7(29%). They concluded that laparoscopy is, therefore, an essential final step in an otherwise negative work-up for infertility. Since then, laparoscopy has traditionally been suggested to be an integral diagnostic procedure of most infertility investigatory protocols (Simon et al., 1993, Speroff et al., 1999). It was considered a complementary procedure following normal hysterosalpingogram (HSG) precluding endometriosis or peritubal non-obstructing adhesions. Several reports have documented the shortcomings of HSG in establishing the diagnosis of peritubal adhesions (Gutmann, 1992). The sensitivity of HSG in detecting peritubal adhesions has been reported to be 34-75% (Rice et al., 1986). It was found (Henig et al., 1991) that in 21%, adnexal adhesions and pelvic endometriosis were identified during surgery in spite of normal HSG. They suggested that ''the recommended interval of six months between normal HSG and diagnostic laparoscopy can be shortened if the HSG is normal and the etiology of the infertility is obscured ''. The superiority of laparoscopy over HSG in assessing extratubular pathology was shown in other studies as well (Rajah et al., 1992; Opsahl et al., 1993; Cundiff et al., 1995; Swart et al., 1995; Belisle et al. 1996; al Badawi et al., 1999; Corson et al., 2000). Overall, abnormal laparoscopic findings were observed in 21-68% of cases in infertile couples after normal HSG. In addition, patient's history was found to be correlated with the presence of tubal disease and pelvic adhesions. Opsahl and Klein (Opsahl and Klein, 1990) found peritoneal adhesions in 22.7% of patients with negative history, compared to 71% of patients with positive history. Other reports confirm this observation (Holst et al., 1983; Taylor et al., 1977; Cumming and Taylor PJ, 1979). Snowden et al. (Snowden et al.,1984) similarly concluded that historical clinical data identifies patients more likely to have peritoneal abnormalities.

It has been suggested that endometriosis, regardless of its severity, rarely causes radiographic abnormalities on hysterosalpingography and therefore can be diagnosed only by laparoscopy (Johnson et al., 1994).

The diagnostic value of HSG in patients undergoing basic infertility work-up was evaluated in a meta-analysis of twenty studies that included over four thousand infertile women [Swart et al.,1995]. The authors concluded that tubal obstruction on HSG is a reliable test and would not necessarily need confirmation by laparoscopy, but a negative HSG is not sufficient proof of normality of the fallopian tubes and peritoneal factors, so tubal patency on HSG requires further investigation using laparoscopy. Other investigators have emphasized the importance of performing laparoscopy after HSG because of the high incidence of pelvic pathology overlooked by HSG [Feyez et al., 1988, Corson et al., 2000]

On the other hand, discussing the cost- effective infertility care, it was suggested (Gleicher, 2000) that in the case of a normal gynecoradiological procedure the probability of clinically relevant tubal disease or endometriosis is so low that laparoscopy does not seem warranted. He states that relevant diagnostic information can be obtained less expensively by performing a gynecoradiological procedure instead of doing a laparoscopy. In his view, proper utilization of surgical procedures, usually endoscopic procedures, represents the single most significant factor in providing cost effective infertility care. In addition, laparoscopy in normal infertile patients will ordinarily show a degree of endometriosis not requiring treatment (Speroff et al., 1999). In a recent investigation (unpublished data of Lavy et al.,), eighty-six infertility patients in whom both HSG and laparoscopy were performed were included. HSG results were compared with laparoscopic findings and suggested treatment based on HSG results was compared with the treatment plan based on laparoscopic findings.

Among 63 patients with a normal HSG or suspected unilateral tubal pathology which were assigned to ovulation induction and intrauterine insemination (IUI), 60 patients were found to have laparoscopic findings that did not necessitate any change in the original treatment plan. In three patients (4.8%) abnormalities discovered at laparoscopy were of such an extent that a change in the original treatment regimen and referral to in-vitro fertilization (IVF) was needed. These results demonstrate that the probability of clinically relevant tubal or pelvic disease in infertile patients with a normal HSG is very low, and that laparoscopy does not seem justified in such cases.

Among 23 patients with suspected bilateral tubal occlusion on HSG, 16 patients (69.6 %) were found to have an abnormal laparoscopy with bilateral tubal adhesions, 6 (26%) had unilateral tubal adhesions, and 1 patient (4.3%) had pelvic adhesions with no obstruction. These latter findings led to changes in the original treatment plan of these seven patients from IVF to ovulation induction and IUI. In conclusion, this data suggests that laparoscopy may be omitted in women with normal HSG or suspected unilateral distal tubal pathology on HSG, since it was not shown to change the original treatment plan indicated by HSG in 95% of patients. However, laparoscopy should be recommended in cases with suspected bilateral tubal occlusion on HSG, since it altered the original treatment plan in 30% of patients from IVF to induction of ovulation with IUI.

When HSG is normal, a laparoscopy, if performed, would be normal in most cases and the diagnosis of ''unexplained infertility'' made. The patient would be referred to ovulation induction by gonadotropins combined with intrauterine insemination (Speroff et al., 1999; Simon et al., 1993) for 3-6 cycles. When this treatment fails, assisted reproductive technologies (ART) are then offered to the couple. In the minority of cases who have normal HSG study, laparoscopy might

reveal minimal or mild endometriosis or peritubal adhesions. In the case of minimal or mild endometriosis, either surgery or medical treatment can be applied. However, this approach is not proved to be of benefit for infertility associated with minimal and mild endometriosis (Portuondo et al., 1983; Olive et al., 1985; Hull et al., 1987; Speroff et al., 1999). Expectant management in case of mild endometriosis is usually rewarded with reasonable pregnancy rates that are comparable to those obtained with treatment. It was reported (Badawy et al., 1988) that the cumulative pregnancy rate was 90% after 5 years in women not treated for minimal or mild endometriosis. However, treatment by either gonadotropins in combination with intra uterine insemination (IUI) or assisted reproductive techniques (ART) will be still warranted in order to achieve the goal of conception. Others have advocated the laparoscopic resection or ablation of endometriotic lesions to treat infertility associated with this condition. In a randomized controlled trial (Marcoux et al., 1997), it was found that laparoscopic resection or ablation of minimal and mild endometriosis enhances fecundity in infertile women. They reported that one in eight women with minimal or mild endometriosis should benefit from resection or ablation of endometriosis. Nevertheless, the monthly fecundity rate among women who underwent laparoscopic surgery (6.1 percent) was much lower than the rate expected in fertile women (20 percent). Furthermore, Parazzini (Parazzini, 1999) in his study of 100 infertile patients with minimal and mild endometriosis randomized to laparoscopic surgery or expectant management, demonstrated no difference in fecundity rate between the treatment and no treatment group (24% versus 29%, respectively) after a follow up of one year. Recognizing these numbers, turning promptly to ART might offer a higher success rate per cycle than in expecting for a relatively low pregnancy rates yielding for such laparoscopic surgeries. The minority of patients with normal HSG study who will be found to have peritubal adhesions by laparoscopy, might benefit from laparoscopic adhesiolysis followed by combined gonadotropins treatment and IUI to achieve pregnancy as soon as possible after diagnosis. For those who still fail to achieve conception after several cycles of attempt, ART is called upon as a second line of treatment. In our own practice we have defined 6 cycles of treatment as the number of attempts before offering ART for these patients.

In a comprehensive debate article published recently (Balasch, 2000) the role of laparoscopy as a diagnostic procedure used to investigate the infertile couple was addressed. In this debate Balasch (2000) raised the point that as the success with ART improves, clinicians increasingly believe that turning to the ART is appropriate even without laparoscopy. In addition, he stressed the difficulties in persuading a woman with normal HSG to undergo an invasive procedure such as laparoscopy. According to Balasch (2000) this attitude towards laparoscopy from both the clinicians and patients, represent in part the

move from ''diagnostic work-up'' to ''prognosis oriented approach'' in the investigation and treatment of the infertile couple.

In our daily practice, with the improved success rates of assisted reproductive technologies and the relatively low contribution of diagnostic laparoscopy in the decision as to the next step in treating patients having normal HSG, we found it logical to offer these women a treatment by combined gonadotropins and IUI for 3-6 months and switch to ART if such a treatment fails. This is a reasonable and appropriate suggestion since even if one finds an evidence of tubal or peritubal disease by laparoscopy, in most hands in vitro fertilization will be more successful than treating significant tubal disease through the laparoscope (Speroff et al., 1999).

The impatience of treated couples and considerations of healthcare cost are of utmost importance and influence the type of diagnostic tools or treatments selected by couples. Omitting laparoscopy from the infertility work-up when HSG is normal can reduce the cost without compromising the success rates. We suggest therefore, that couples diagnosed as suffering from unexplained infertility should undergo ovulation induction for several cycles before ART. Such an approach will prove to be cost effective and the fastest way to achieve conception in these infertile couples. The only indication for diagnostic laparoscopy and laparoscopic surgery for adhesiolysis or ablation of endometriotic lesions should be reserved for cases for whom ART is not easily available or covered by health care services.

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Sophisticated sperm analysis ever required?

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Semen analysis is of pivotal importance for the evaluation of the infertile couple.

If sexual and ejaculatory functions are normal, it is the result of the semen analysis that will determine whether or not a so-called ''male factor'' is involved.

There are, however, several issues that hamper the interpretation of semen analysis.

First, there is no clear-cut demarcation between ''fertile'' and ''infertile'' semen. Second, the techniques of semen analysis are insufficiently standardized, and often poorly performed. The coefficient of variation within and, certainly, between laboratories is high, rendering comparison of results generated by different laboratories difficult, even hazardous. Computer assistance can be of (limited) help in semen analysis, but some of existing programs and systems are hard to implement on human semen. Third, evaluation of sperm morphology in particular is extremely difficult since the limits of normality are poorly defined. Finally, there are a number of tests that may contribute to the diagnostic process, but these are rather time consuming and — therefore — are not performed in most routine labs.

It should be clear that a man can only prove his fertility through the intermediate of his female partner. It is well known that about half of the partners of ''subfertile'' men are subfertile themselves, which makes it particularly difficult to assess the intrinsic fertility of the male partner. In addition, fertility rather is a matter of degree, in so far that it is the probability of conception per cycle of the wife that can vary. A fertile men has attained pregnancy with his partner within 12 months of unprotected intercourse. The 5^{th} percentile of the conventional sperm characteristics of such a fertile men are: a sperm concentration of 35 to 40 million per ml, total progressive motility of 50% and rapid linear progressive motility (grade ''a'') in 25%.

Using the criteria for normality described in the WHO manual of 1999, at

least 15% of the spermatozoa will have ''normal'' morphology. In addition, the ejaculate volume should be 2.0 ml or more, viscosity should be normal, the number of white blood cells must be no more than 1 million per ml, and there should be no antisperm antibodies on the spermatozoa. If the semen characteristics of a particular man fulfil these criteria, he is 95% certain of being fertile.

At the other hand, the 5th percentile of the semen characteristics of subfertile men, who did ultimately attain spontaneous pregnancy, though needing more than 12 months to do so, are much lower. These values are: sperm concentration of 3 million per ml, total progressive motility of about 15% with grade ''a'' of about 5%, and normal morphology of 4%. Any man whose semen variables are below these values is 95% certain of being completely infertile, so sterile.

In the practice of the male infertility clinic, the majority of men have sperm variables that are situated between the limits enumerated above. These men are neither optimally fertile, nor completely infertile. They are classified as subfertile, meaning that they still may succeed in attaining spontaneous pregnancy, but that the time needed to do so may be very long, since the probability of conception per cycle is decreased. The probability of conception or ''fecundity'' of semen presents a linear correlation with the level of progressive motility, and with the proportion of morphologically normal spermatozoa. However, the correlation between fecundity and sperm concentration is parabolic. This means that e.g. doubling of sperm concentration from 4 to 8 million has a much stronger influence on fecundity (x 2.58) than doubling from 8 to16 million per ml. (x1.34).

The data cited above emphasize the importance of correctly performing conventional semen analysis. In addition, such analysis must include the detection on motile spermatozoa of antisperm antibodies of the IgG class, and if these are present, also of the IgA class. This can conveniently and accurately be performed using the direct (Sperm)MAR test. Furthermore, the staining of ''round cells'' for the presence of peroxidase is necessary, whenever 1 million or more round cells are present in the ejaculate. Peroxidase-positive round cells are neutrophyle granulocytes or ''pus cells'' that generate reactive oxygen species, and are associated with *inflammatory reaction* at the level of the accessory sex gland (prostate, seminal vesicles, epididymis).

We recommend performing sperm culture on all semen samples. Provided the semen has been collected in agreement with strict instructions, avoiding contamination with bacteria from the skin or urinary tract, sperm culture gives important information concerning possible *infection* along the genital tract.

Biochemical analysis of seminal plasma should equally be part of the routine. The total output per ejaculate of citric acid and of gamma glutamyl

transferase are markers of prostate function, and alfa-glucosidase activity reflects epididymal function. The ejaculate volume gives information about the secretory function of the seminal vesicles. These biochemical analyses permit the evaluation of the functional state of the accessory sex glands, and do contribute to the localization of e.g. inflammatory/infectious damage of these glands, or the site of a possible obstruction along the channels of sperm transportation in case of azoospermia.

Sophisticated, also called advanced, methods of semen analysis have been advertised for the purpose of increasing its accuracy in terms of assessing the fecundity, or to discriminate between ''fertile'' and ''infertile'' semen. Clearly, there is not one single test that can accurately do so, since fecundity is relative rather than absolute. Certain advanced tests can reveal functional defects of the spermatozoa that may not be detectable with the conventional methods, explaining the absence of conception in spite of apparently ''normal'' routine sperm variables. In a case where all the spermatozoa lack acrosin, spontaneous conception is impossible. Acrosin is stored in the acrosomal cap, and such cases most commonly lack an acrosome, or present an abnormally small acrosome. Measurement of acrosin, or assessment of the proteolytic activity of spermatozoa using a photographic plate will confirm the acrosomal defect.

Another test that has been popular some time ago is the hamster oocyte test. Zona-free hamster oocytes are placed in culture medium containing capacitated spermatozoa. After incubation, the number of adhering spermatozoa, as well as the number of decondensed sperm heads are counted. This test is very time consuming and subject to numerous artefacts. It has some value in assessing the in vivo fertilizing capacity of human spermatozoa, but has lost most of its interest since the introduction and wide-spread use of ICSI. The zona-free hamster oocyte test remains a useful tool in assessing the possible beneficial or deleterious effects of respectively medical treatment of the subfertile men, or the negative influence of certain environmental or toxic substances. In order to yield useful information in these settings, the test must be performed under rigorous circumstances by a specialised laboratory.

The hemi-zona test uses human zonae pellucidae and quantifies the binding of acrosome reacted (capacitated) spermatozoa to these. The number of binding spermatozoa in the sample to be investigated is compared to that of donor spermatozoa of a men with proven fertility. While this test has been excessively publicised by a few laboratories in the 1980's, being the early period of IVF, it has lost practically all of its interest since the introduction of ICSI ten years ago.

More interesting sophisticated tests on spermatozoa include the assessment of reactive oxygen species (ROS), the detailed analysis of the fatty acid composition of phosfolipids of the sperm membrane, and the estimation of the damage to the DNA in the sperm head.

Elevated ROS is associated with several diseases causing subfertility, such as male accessory gland infection, varicocele and immunological infertility. Elevated ROS causes imbalance between oxidative and antioxidant effects on spermatozoa, resulting in changes in the fatty acid composition and, subsequently, decreased fluidity of the sperm membrane. The latter reduces the fusogenic capacity of the membrane, with impairment of the inducible acrosome reaction and decreased fusion of the membranes of spermatozoa and oocytes. Increased ROS can be counteracted by antioxidant treatment, which will improve the functional state of the sperm membrane and enhance the probability of conception, either spontaneous or by means of IUI.

Oxidative damage to sperm DNA is detected by means of the comet test, or by measuring the concentration of oxidised DNA, namely of 8-hydroxy-2-deoxyguanosin (8-OH-dG). The latter induces mutagenesis and it may play a pivotal role in poor embryo development after ICSI. Adequate and appropriate antioxidant treatment (see further) can reverse the oxidative damage to DNA, and it does improve the outcome of IUI and of IVF, as well as of ICSI in those cases.

Antioxidants are naturally produced in high amounts by the healthy epididymis. The secretion of both antioxidants and of alfa-glucosidase decreases in parallel when epididymal function is impaired. Therefore, measurement of alfa-glucosidase in seminal plasma can help in selecting those subfertile patients who will probably benefit from antioxidant treatment.

In summary, accurate and reproducible semen analysis should include correct assessment of the conventional characteristics of the spermatozoa, as well as complementary tests to detect immunological infertility, or signs of inflammation/infection of the accessory sex glands, or deficiencies in the secretory capacity of these glands. Continuous internal and external quality control of the performance of the laboratory is mandatory in order to ascertain the high quality of these conventional analyses.

Few, if any, of the sophisticated tests, which were called ‘‘advanced’’ in the past, are still considered useful in the era of IVF and ICSI. The measurement of ROS may be worthwhile because of the deleterious effect of excessive ROS on the functional capacity of the sperm membrane and on the quality of the sperm DNA.

Salpingoscopy, Microsalpingoscopy or HSG, do we need them all?

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The evaluation of the uterine cavity is mandatory prior to IVF in order to improve outcome. Cervical pathology, congenital malformations, acquired pathology like myomas, polyps or cavity obliteration could impair the results. But also subtle lesions like diffuse polyposis, hypervascularisation or mucosal elevation are important. An endometrial biopsy will allow histological examination, genomic fingerprinting and pre-treatment assessment of endometrium receptivity. The incidence of abnormal findings during pre-IVF hysteroscopy is higher as expected (28.5%). Congenital disorders are found in 13.2% and acquired disorders like polyps, myomas, Asherman and synechiae in 15.3%.

Hysteroscopy is superior to transvaginal ultrasound, hysterosalpingogram and HyCoSy. Compared with hysteroscopy, transvaginal ultrasound shows a sensitivity of 81% and a specificity of 95%, hysterosalpingogram a sensitivity of 98% and a specificity of 15%, HyCoSy a sensitivity of 87.5% and a specificity of 100%. Only hysteroscopy shows small submucosal myomas, minimal adhesions, diffuse polyposis, subtle mucosal lesions and allows endometrial biopsy for histology, genomic fingerprinting and receptivity assessment.

Microhysteroscopy requires no cervix dilatation, no insertion of a probe, no portio tenaculum, no anesthesia or analgesia. The procedure is easy to perform, visualization is excellent, patient compliance is optimal and it is a safe procedure. The cost benefit for the surgeon is generally poor but the advantages for the patient enormous as microhysteroscopy is a real minimal invasive diagnostic procedure.

Is the evaluation of tubal, ovarian and peritoneal disease important before IVF?

The diagnosis and treatment of a hydrosalpinx, ovarian endometriosis and peritoneal endometriosis will certainly improve the pregnancy rate after IVF.

Transvaginal Hydrolaparoscopy is in our hands the gold standard for the evaluation of these factors. Transvaginal Hydrolaparoscopy represents a culdoscopic approach of the pelvic cavity, takes advantages of the micro-

endoscopic technology without grasping or manipulation, uses saline for distension and it performed under local anesthesia or patient sedation.

Transvaginal Hydrolaparoscopy offers the possibility to explore tubo-ovarian structures early on in infertile patients, combining the advantages of laparoscopy, HSG and ultrasound, but, at the same time, eliminating their disadvantages.

The patient is placed in a horizontal decubitus position, no general anesthesia is required. After routine Hysteroscopy, a Foley catheter is placed in the uterine cavity and a tenaculum is placed on the posterior lip. The CIRCON THL system is inserted, it consists of three parts: a Veress needle, a dilatating obturator and an outer cannula. The THL system is placed in the midline, approximately 15 mm below the posterior cervical lip. A 2.7 mm 30 endoscope is inserted, a continuous irrigation with warm saline is used. The tubo-ovarian complex can be explored without any problems. The scope and outer cannula are removed. The procedure takes maximally 10 minutes.

What about the significance of tubal patency and tubal wall morphology?

Tubal factors are estimated to account for 10-20% of infertility. Evaluation of tubal function is largely restricted to the appraisal of its patency. Treatment of tubal infertility is primarily focused on the restoration of tubal patency. But in spite of restored patency, infertility frequently persists and tubal pregnancy frequently occurs, as underlying disease is not diagnosed. It is increasingly evident that evaluation of peritubal adhesions and tubal patency is an incomplete evaluation of tubal function. Patients with patent tubes could be infertile because of tubal mucosal damage, not screened during traditional laparoscopy or HSG.

Changes of the major mucosal folds, adhesions, epithelial desquamation, reduced percentage of ciliated mucosal surface, presence of inflammatory cells and fibrosis of the tubal wall could be a reason for infertility, even in patients with patent tubes. Endoscopic evaluation of the tubal mucosa and especially tubal adhesions appears more and more to be the most important prognostic tool for evaluating the outcome of pregnancy.

THL offers the optimal axis to enter into the tube without grasping or manipulation. Distension of the ampulla is obtained by water pressure, no additional scope is required, no additional instruments.

Microsalpingoscopy allows examining the number of dye-stained nuclei on the tubal epithelium, which are either intermediary cells on the surface of the epithelium or inflammatory cells residing inside the tubal folds.

In conclusion, it can be stated that the identification of tubal pathology during salpingoscopy or microsalpingoscopy is of great importance when deciding whether IVF or expectant treatment is the better option for patients with unexplained infertility.

Hormones, antioxidants and food supplementation in the treatment of the infertile male: benefit to the patient or to the industry?

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In the 7th and 8th decade of the previous century, much attention was given to the alleged causes and associations of male infertility. A specific task force of the World Health Organization launched large-scale multi-center trials. These resulted in a diagnostic approach and standardized classification of male infertility and in the publication of manuals for the standardized techniques of semen analysis (WHO manuals, 1980, 1987, 1999).

The best methods for the diagnosis of varicocele were determined and the efficacy of treatment of this disease was established in a prospective randomized trial. Placebo controlled trials did not reveal any benefit in terms of improving the spontaneous conception rate using antibiotic treatment of male accessory gland infection, and when treating men with idiopathic oligozoospermia with clomiphene citrate or with mesterolone. In contrast, treatment of oligozoospermia with Tamoxifen has been proven effective.

The introduction of assisted reproductive technology (ART), namely IVF and intracytoplasmic sperm injection (ICSI) caused a true revolution in reproductive medicine, while also revealing the magnitude of the ''male factor'' contributing to couple infertility. Conventional treatment of the infertile male was considered outdated by some, but others have continued unraveling the mechanisms involved in man's defective reproductive capacity. In recent years concerns have been raised about economical and ethical aspects and negative side effects in offspring of ART. It seems, therefore, that the wheel has turned a full circle, and that clinical andrology will recapture its well-deserved place in the armatorium for the treatment of couple infertility.

Similar to other diseases, male infertility comes to expression as a result of

the synergistic coincidence of 4 major factors: genetic defects or constitution, life style factors, professional and/or environmental exposure, and diseases of the urethro-genital region or endocrine system. These conditions cause oxidative overload by reactive oxygen species (ROS) changing the phospholipid composition of the sperm membrane, reducing its fluidity and fusogenic capacity as well as the induced acrosome reactivity.

Men with infertile semen were found to consume less omega-3 fatty acids than fertile men, and a significant correlation was established between the food intake of alfa linolenic acid (18:3 omega3) at the one hand and sperm concentration and type ''a'' motility at the other hand. Also, exposure to environmental agents with hormone disrupting effects, mainly pseudo- or xeno-estrogens and anti-androgens, has caused serious concerns recently.

Food supplementation: Fatty acids

Since there is a positive correlation between the intake of alfa-linolenic acid and sperm concentration and motility, and since the food intake of essential fatty acids of the omega-3 group was found to be sub-optimal among subfertile men, it seems logical to supplement these patients with a source of 18:3 omega3, namely linseed oil also called flaxseed-oil. When given in association with the co-factors Zinc and

Vit. B6, which enhance the elongase and desaturase enzymes, the alfa-linolenic acid will be converted into the long-chain, highly unsaturated omega-3 fatty acids, namely Ecosapantaenoic acid (EPA) and docosahexaenoic acid (DHA). The latter increase the fluidity of the sperm membrane, improving the induced acrosome reaction and fusogenic capacity of the spermatozoa.

Supplementation with fish oil can also be considered as a source of the highly unsaturated long chain fatty acids, EPA and DHA. These fatty acids are, however, highly susceptible to oxidative damage, initiating an undesirable chain reaction of lipo-oxidation. If fish oil is given for food supplementation, it is mandatory to ascertain a favorable antioxidant internal environment at the same time.

Antioxidants

Subfertile patients were found to present an imbalance between excessive oxidative stress as compared to a reduced antioxidant capacity in serum. Food supplementation with an antioxidant preparation can significantly and persistently correct this imbalance. Also, treatment with either acetylcysteine (600 mg per day orally) or an antioxidant mixture of beta-carotene (30 mg per day orally) and alfa-tocopherol (180 mg per day orally) significantly reduces the level

of reactive oxygen species (ROS) in semen. When given in combination with fish oil intake, providing 1g of DHA per day, antioxidant treatment increased sperm concentration, and significantly reduced the concentration of oxidized DNA (8-OH-2d-Guanosine) of spermatozoa of subfertile men. In the same time, the fatty acid composition of the phospholipids of the sperm membrane shifted toward the long chain highly poly unsaturated EPA and DHA, increasing membrane fluidity. This then resulted in an increased calcium-ionophore induced, but not spontaneous, acrosome reactivity. The spontaneous pregnancy rate during the treatment period was to 7.2% per month in the partners of (ex)-smokers, but remained at baseline low value among the partners of non-smokers (1.6%).

Astaxanthin is a lipophilic carotenoid produced by the algae haematococcus pluvialis, that has a strong antioxidant capacity. In a double blind randomized trial 16 mg per day of the natural Astaxanthin (AstaCarox, Astacarotene AB, Gustavsberg, Sweden) was given to the male partners of infertile couples, whose semen characteristics were below the WHO recommended reference values. This food supplementation resulted in a significant reduction of seminal ROS among treated cases, but not in the placebo controls. Rapid linear progressive motility significantly increased and sperm morphology also improved in the Astaxanthin group, but sperm concentration remained unchanged. In the treated group the total and monthly pregnancy rates were 54.5 and 23.1% respectively, as compared to 11.1 and 3.6% in the placebo group.

Carnitine

L-carnitine plays a pivotal role in the transport mechanisms that are necessary for the translocation of longer-chain-length fatty acids from the cellular cytosol into the mitochondrial matrix, where these can be oxidized and generate energy and stimulate respiratory chain complexes. The free carnitine concentration in seminal plasma was significantly correlated with sperm concentration and motility and sperm motility could be stimulated by addition of acetylcarnitine in vitro.

Treatment with a food supplement containing a combination of L-carnitine (2gr per day) and acety-L-carnitine (1 gr per day) together with fructose and citric acid (Proxeed, Sigma-tau Health Science, Rome, Italy) was found to significantly increase sperm concentration and forward progressive motility in both open label trials and a double blind cross-over trial. In the open label trial a total spontaneous pregnancy rate of 6.7% in 3 months was registered.

Folic Acid and Zinc

Folic acid (5 mg per day) and zinc sulfate (66 mg per day) have been given orally to both men with normal sperm quality and to patients with moderate oligozoospermia in a placebo controlled trial. This combination was found to

significantly increase sperm concentration (by an average of 60%) and morphology in the subfertile men. These changes occurred in spite of the absence of deficient blood levels of folic acid or zinc before supplementation in the subfertile men. It was hypothesized that the supplementation with lower, physiological doses of micronutrients may even have a larger beneficial effect, since these have a stronger influence on absorption, transport and metabolism processes. It remains, however, to be established whether the administration of the combination of folic acid and zinc will result in improvement of fertility.

Seed oil and lignans

Aside from alfa-linolenic-acid (see above) linseed or flaxseed oil contains several lignans, which are converted in the intestine into enterodiol and enterolactone. Enterolacton is a rather strong aromatase inhibitor reducing the conversion of androgens (androstenedione and testosterone) into the potent oestrogens: estron and estradiol. Hence, food supplementation with linseed oil will decrease the level of endogenous oestrogens, which were commonly found to be increased in men combining oligozoospermia with normal serum concentrations of FSH and Inhibin B.

Plant extracts

Using immune histochemical techniques, it was recently demonstrated that the Cyclo-oxygenase (COX) iso-enzyme 2, converting arachidonic acid (20:4omega6) into the inflammatory prostaglandin E2, is present in the testicular interstitial tissue of patients with idiopathic oligozoospermia, but not in men with normal spermatogenesis. The extract of the bark of the Pinus Maritima (Pycnogenol) contains substances which inhibit the COX enzyme, reduce the m-RNA of the inflammatory cytokine Interleukin 1 beta, and protect the effects of VitE on endothelial cells. In an open label study including 4 subfertile men, oral administration of 200 mg per day of this extract improved sperm morphology by an average of 99%.

The extract of Lepidium Meyenii (also called Maca), a plant growing in the central Andean region of Peru, between 4000 and 4500 m altitude, increases sexual function of male mice and rats, and invigorated spermatogenesis at the mitotic stages. When given to 8 men with normal spermatogenesis, this extract significantly increased sperm production (+85%) and motility (+15%) without interfering with endocrine regulation.

Though these plant extracts may show promise for the future, complementary studies are needed before their use can be recommended for the treatment of male infertility.

Considerations

Several controlled and well-validated trials provide evidence that food supplementation with particular substances can improve semen quality and function of subfertile men. These include carnitine, zinc, folic acid, and astaxanthin. There is suggestive evidence that certain of these supplements, when given as a complement to the WHO recommended conventional treatment (Rowe et al, 2000), can improve male fertility.

On a deductive basis, but without convincing data from clinical trials, certain other food supplements, such as linseed oil and plant extracts, may favorably influence sperm quality.

Although the exact mechanisms of action of these supplements on spermatogenesis and sperm function remain to be unraveled, a direct effect on the cells of Sertoli and via epididymal function seems conceivable.

It makes sense to further explore the therapeutic potential of food supplementation in the management of couple infertility due to the male factor.

(references upon request to the authors)

Should We Treat Klinefelter's Syndrome Patients with IVF-ET?

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I. The sex chromosome ploidy of spermatozoa extracted from testicles of patients with non-mosaic Klinefelter's syndrome.

Klinefelter's syndrome is one of the most common chromosomal abnormalities found in newborns. Its incidence is approximately 1 in 500 phenotypic males (1). The vast majority of patients with Klinefelter's syndrome have a non-mosaic 47;XXY karyotype originally described as a syndrome with hypergonadotrophic hypogonadism, gynecomastia and azoospermia(2). Once considered eternally infertile, these individuals can reproduce now to bear their own children, using intracytoplasmic injection of spermatozoa extracted from their testicles (3-6).

Although few normal babies have been born so far following IVF in couples with 47;XXY karyotypes, routine clinical IVF in such couples is far from being safe (7-13). In order to address this question we have determined the ploidy of spermatozoa extracted from the testicles of such patients during ART cycles.

A total of 20 patients with non-mosaic Klinefelter's syndrome were prepared for testicular biopsy and IVF. Prior to the ART cycle all 20 patients completed an evaluation including testicular volume estimated by Seager orchidometer, serum levels of LH, FSH and Testosterone. In addition they underwent Human Chorionic Gonadotrophin (hCG) stimulation test, which was performed by intramuscular injection of 5000 IU of hCG and determination of Testosterone levels at time of injection and 72 hours after injection (14, 15).

In 8 patients (40%) mature sperm were found in the wet preparation and have been used for ICSI. In five patients who consented to donate spermatozoa for analysis, individual spermatozoa were collected and fixed on a glass slide and analyzed for chromosomes X, Y and 18 by FISH.

The mean age of all patients was 32.2 ± 5.0 years (range: 24-43 years). The mean testicular volume was 7.8 ± 2.5ml in patients with spermatogenesis (n=8,

Group-I) and 5.6±1.2 ml in patients without spermatogenesis (n=12, Group-II, P<0.05, Table-I). The mean basal serum testosterone level in group-I and -II were 3.5±1.2 and 1.7±0.8 ng/ml respectively (P<0.005, Table-I).

Table I: *Clinical and laboratory parameters of the patients with non-mosaic Klinefelter's syndrome (N=20).*

	Group I Sperm found (N=8)	**Group II Sperm not found (N=12)**
Mean age (years)	32.5±4.7[a]	32.0±5.5
Testicular volume (ml)	7.8±2.5[b]	5.6±1.2
Serum FSH (mIU/ml)	29.0±13.0[a]	37.4±14.0
Serum LH (mIU/ml)	17.3±6.0[a]	19.2±6.0
Basal Serum Testosterone (ng/ml)	3.5±1.2[c]	1.7±0.8
Serum Testosterone post hCG administration (ng/ml)	16.0±6.3[c]	6.7±5.6

[a]NS; [b]P<0.05 ; [c]P<0.005

The mean serum FSH and LH levels were 29.9±13.0 mIU/ml, 17.3±6.6 mIU/ml in group-I respectively and 37.4±14.0 and 19.2±6.0 mIU/ml in group-II respectively. The mean serum FSH and LH in both groups were not significantly different (Table-I).

A total of 132 testicular spermatozoa were analyzed from the five consenting patients. One hundred and twelve sperm cells (84.8%) underwent successful hybridization and FISH analysis. One hundred and five (93.7%) of the analyzed sperm cells had normal karyotype (Table II). Fifty out of them (47.6%) had a normal X,18 karyotype and the rest 55 (52.4%) had a normal Y,18 karyotype (P= 0.62, NS). Seven sperm cells had abnormal karyotypes. Five out of the 7 abnormal cell had sex chromosome abnormalities resulting from errors in the first (XY,18) and the second (YY,18) meiotic divisions.

Out of the 8 couples treated 5 pregnancies were achieved. One set of triplets, two sets of twins and two singleton pregnancies. All 9 babies born (4 males and 5 females) were normal.

Mature spermatozoa that are found occasionally in Klinefelter's syndrome patients, have been used successfully for clinical IVF and lead recently to the birth of small number of healthy babies (3-5). Nevertheless, routine clinical IVF in such couples may impose a significant risk for sex chromosome aneuploidy.

The origin of the meiotic products in the testicles of patients with presumably non-mosaic 47,XXY variant is not clear. It is believed that 47;XXY germ lines are unable to proceed through mitosis and meiosis, probably because of the presence of two functional X chromosomes. Therefore, any

sperm found in such patients originates probably from normal germ lines. These germ lines may occasionally populate the seminifrous tubules during gonadogenesis. Despite these considerations several authors (6-8) have proposed that 47,XXY germ cells are, in fact, able to undergo meiosis. Their suggestion is based on the increased rates of sperm sex chromosomes hyper- and hypo- haploidies found among ejaculated sperm from such patients in compare to the normal population. It appears however, that the aneuploidy rates found in these studies were only slightly increased and usually do not exceed 1%. This rate is significantly lower than expected assuming that 47,XXY spermatogonia undergo meiosis.

Table II: *The incidence of chromosome abnormalities among the sperm cell study population.*

Patient	Sperm analyzed	Normal cells	Sex chromosomes distribution		Abnormal cells
			X,18	Y,18	
1	21	18	11	7	3
2	21	20	9	11	1
3	28	27	10	17	1
4	20	18	8	10	2
5	22	22	12	10	0
Total	112[a]	105[b] (93.7*)	50 (47.6**)	55 (52.4**)	7 (6.3*)

* Per cent from [a]; ** Per cent from [b]

In a single study on a small number of sperm a relatively higher rate of (25%; 6/24) hyper hapliody was reported in a patient with the non-mosaic variant (16). These observations, however, may not necessarily be the result of meiosis in abnormal germ lines but rather as a result of meiotic errors in normal germ line population. In the mouse XXY model the few germ lines found in the adult testis are exclusively of the XY karyotype. Therefore, meiotic aneuploidies that are found in the sperm of these mice most probably relate to a compromised testicular environment rather than to abnormal spermatogenic cell lines (17).

A recent study has demonstrated a significantly higher rate of sex chromosome abnormalities among infertile males undergoing ICSI in compare to fertile donors (18). In other study we have demonstrated a linkage between gonadal failure (High serum FSH levels) and an increased incidence of chromosomal abnormalities (about 19% when screening for chromosomes X, Y and 18 only) found among testicular spermatozoa retrieved from patients with non-obstructive azoospermia (19). In these patients the meiotic errors were particularly confined to the sex chromosomes.

These studies and our observations may suggests, therefore, that sperm

found in Klinefelter patients are probably the products of normal germ lines and that spermatogenesis in these patients probably follows the pattern found among patients with gonadal failure due to other reasons

Over 90% of the sperm cells analyzed by FISH in the present study were normal. The favorable clinical outcome in our patient group also correlates with these findings. Among the seven abnormal cells that were detected here there is evidence for non-disjunction errors during meiosis-I (24,XY karyotype) and meiosis-II (24,YY karyotype). The origin of such errors is probably from normal 46,XY germ cells since 47,XXY line would have yield higher aneuploidy rates.

The production of mostly normal sperm in the testis of these non-mosaic Klinefelter's syndrome patients by presumably normal 46,XY germ lines raises the question about the origin of these germ lines. It may be speculated that during multiplication of the primordial germ cells in the primordial testicles of such patients ''correcting mitotic errors'' might give rise to isolated testicular mosaicism i.e. normal germ lines that survive to produce sperm later during adulthood.

II. Predictors of spermatogenesis in patients with Klinefelter's syndrome

The sexual development of 47,XXY males is normal during the prepubertal years and includes the initiation of normal puberty changes and normal pituitary gonadal function. Following an early adolescent rise in serum testosterone, its level begin to fall by the age of 15 years and after that it is usually within the low or low to normal range (23).

In virtually all these patients FSH, LH, and estradiol concentrations are elevated significantly above normal, and by midpuberty Klinefelter's syndrome males have usually overt hypergonadotrophic hypogonadism. It appears, however, that despite these abnormal hormonal values there are occasionally patients with normal appearing secondary sex signs. It has been found that such patients occasionally respond to gonadotrophin administration with a modest rise in testosterone levels (24-26).

The purpose of this section of the study was to search for clinical and laboratory parameters which may predict sperm presence during testicular sperm retrieval in this group of patients (27).

The hCG test in our patient groups (Table-I) showed significant increase in the mean serum testosterone levels in group-I, 16.0 ± 6.3 ng/ml compared to 6.7 ± 5.6 ng/ml in group-II ($p=0.002$; Table-I).

The present results shows that the mean testicular volume in patients with sperm following TESE (7.8 ± 2.5 ml, Group-I) was significantly higher than in patients without sperm (5.6 ± 1.2 ml, Group-II). It is well established in previous

publications (19) that there is direct correlation between testicular volume and spermatogenesis. This may explain our observation that patients of group-I had larger testicles than patients in group-II. Unfortunately our number of patients is too small to determine a cutoff value of testicular volume for better prediction of sperm presence.

To determine the functional capacity of the testis we used the hCG stimulation test that assesses the function of the Leyding cells component of the testis. Several authors including Okuyama et al, Tapanainen et al, Forest and Roulier, (24-26) also reported that In hypogonadal males testosteron rise was observed in all patients within 48-96 h (mean 72h) after hCG administration. The testosterone produced after hCG stimulation may also affect the seminal vesicles, the epididymis and the prostate, increasing their excretory products in the seminal fluid. Increase of plasma testosterone and lack of change in any of the physiological markers of the accessory glands may indicate either mechanical block, or dysfunction of the secondary sex glands. Thus, measurement of plasma testosterone following hCG stimulation may help in the diagnosis process in hypogonadal males.

Our hCG test results showed a significant increase in the mean serum testosterone levels in group-I, 16.0 ± 6.3 ng/ml compared to 6.7 ± 5.6 ng/ml in group-II ($p=0.002$; Table-I). These results prove that these patients have some functional testiticular tissue, which is responding to hCG administration. Therefore, Klinefelter's syndrome patients with relatively high response to hCG test may have favorable prognosis in finding sperm during TESE.

Testosterone administration in these patients during adolescence and there after is important for secondary sexual development and characteristics. Exogenous testosterone administration, however, suppresses the hypothalamic-testicular axis and cause spermatogenic arrest. Therefore, in our opinion, all Klinefelter's syndrome patients should receive hCG treatment whenever fertility is considered for at least six months before TESE.

Our findings should encourage and reassure physicians and patients with Klinefelter's syndrome to pursue the use of their own sperm while treated for infertility. In our small series of unselected patients with non-mosaic Klinefelter's syndrome mature sperm were found in 40% of the patients and presently about 60% of them achieved pregnancy. Any increase in the risk of producing pregnancies with sex chromosome abnormalities when using sperm from men with Klinefelter's syndrome is still to be adequately determined. Few previous studies using pre implantation genetic diagnosis on embryos produced from such men yielded normal results (20, 21). According to the literature and our present findings it appears that the clinical outcome of most of these pregnancies is favorable although one case of 47,XXY karyotype have been reported recently (22).

Therefore, in advising these patients a cautious approach is warranted and pre implantation or prenatal diagnosis should be carefully considered. We believe that analysis of spare sperm cells during IVF may help the patients in making their own decisions regarding treatment. Our studies also demonstrate that testicular volume, basal serum testosterone levels and hCG test are important predictive factors of spermatogenesis in patients with non-mosaic Klinefelter's syndrome. hCG therapy prior to IVF attempts may improve the prognosis of these patients.

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Round and elongated spermatids injection in assisted reproduction: should it be used?

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Summary

Spermatids are the earliest male germ cells with one set of haploid chromosomes. After experiments, mainly in rodents, the spermatid injection was introduced in human assisted reproduction to the treatment of men with non-obstructive azoospermia. Spermatid injection is a technique with particular difficulties that may negatively influence the outcome. The identification, isolation and the assessment of viability, especially for round spermatids, require intensive work and considerable experience. Up to date, it appears that the rates of fertilisation, implantation and delivery reached with round spermatid injection are dramatically low and significantly less compared to the use of elongated spermatid injection. The extremely low fertilisation potency of the round spermatids led to attempts for their in-vitro culture and maturation. The immaturity of round and elongated spermatids has raised concerns regarding the potential genetic risk for the offspring. Under these facts, a reconsideration of the use of spermatids in assisted human reproduction is necessary.

Introduction

Forty years have passed since the first intracytoplasmic sperm injection. It was 1962 when sea urchin eggs were successfully fertilised by microinjection of live spermatozoa (Hiramoto, 1962). Thirty years later this technique was introduced for the treatment of human infertility (Palermo et al., 1992).

Today, ICSI is ‘‘the state of the art’’ in assisted reproduction technologies. It gives reliable solutions in cases of severe male factor infertility and not only on these cases. However, it is proved that ICSI is not a panacea. There are still

cases of azoospermia where ICSI is not possible even after MESA or TESE. In such cases, spermatid injection is considered as a promising alternative.

Spermatids are the youngest male germ cells with a single set of haploid chromosomes (complete meiosis). Once they have completed meiosis they undergo a complex cellular differentiation and maturation process known as spermiogenesis. Spermiogenesis starts at puberty and continues throughout the reproductive life of males. During spermiogenesis, round spermatids (Sa) have approximately 7μm size and they are transformed into mature spermatozoa. One of the most important changes that takes place in this process is nuclear DNA packaging. DNA condensation is associated with biochemical alterations such as the replacement of lysine-rich histones, first by transistion proteins and later by arginine-rich protamines, as well as with the formation of disulphide bonds that stabilize the chromatin structure (de Kretser and Kerr, 1969; de Kretser et al., 1998; Nikolettos et al., 1999). As a result of DNA condensation the cell size is reduced and thereby less energy is required to support its mobility and the cell is better protected against mechanical and chemical damage (Nikolettos et al., 1999). Other important changes during spermiogenesis include the process of genomic imprinting, the disappearance of the distal centriole and the formation of the acrosome.

Spermatids as the only finding of TESE

It is known that various pathological conditions can lead in spermatogenetic abnormalities with resultant subfertility or sterility (Martin-du Pan and Campana, 1993). In cases of non-obstractive azoospermia with a lack of spermatozoa in the ejaculate, TESE is considered as the next step of the treatment. For most of these cases, TESE results to the retrieval of enough spermatozoa to proceed in ICSI cycles, while in a few mature spermatids are completely absent. In the latter cases, round and elongated spermatids may be present, indicating spermatogenesis arrest after the meiosis stage. In a large series of TESE (N=364) performed in the Medical Univ. of Lübeck, the incidence of such cases was 3.9%; in 2.2% elongated spermatids were found, whereas in 1.7% only round spermatids were observed (Al-Hasani, unpublished data). Although it is still controversial, it seems that in most of the cases the spermatogenesis arrest happens in elongated spermatid stage. According to Cremades et al. (1999) the recovery of only round spermatids is a frequent finding in non-obstructive azoospermic patients with complete absence of spermatozoa. On the other hand, Schulze et al. (1999) reported that from 1418 testicular biopsies (766 subfertile men), only in 26 samples (seven patients — 0.9%) spermatogenesis arrest was in the round spermatid stage. There are also other investigators supporting that maturation arrest is extremely rare in the round spermatid stage (Silber and Johnson, 1998; Silber et al., 1997; 2000).

Isolation and identification

In wet preparation, the identification of round spermatids has many difficulties mainly due to their morphological similarities with small lymphocytes (Vanderzwalmen et al., 1998; Silber et al, 2000). A considerable experience is necessary for reliable identification of round spermatids and avoidance of mistakes. Under the inverted microscope, without using any specific staining methods, four different stages of spermatids can be distinguished, according to their morphology: round spermatids (Sa, Sb1), elongating spermatids (Sb2), elongated spermatids (Sc, Sd1) and late elongated spermatids (Sd2) (Vanderzwalmen et al., 1998). Round spermatids appear as round cells with a diameter of 7μm and they are characterised by a dense, smooth and dark nucleus positioned centrally or inclining towards the cell membrane. The nucleus is surrounded by a continuous rim of cytoplasm. In some cells, the early acrosomal vesicle or acrosomal cap is visible as a bright white spot or sickle-shaped adjacent to the nucleus (Tesarik and Mendoza, 1996; Sousa et al., 1998; Verheyen et al., 1998). The distinction of elongating and elongated spermatids is made on the basis of their shape and the size of the tail. The mature spermatids (late elongated) appear to be similar to the ultimate sperm morphology (Vanderzwalmen et al., 1998). The appearance of abnormal spermatid forms in the preparation makes the identification procedure more difficult.

The assessment of spermatid viability is a hard task, particularly of the round ones (Schoysman et al., 1999). Aslam et al. (1998), using the Trypan blue exclusion test, found that 97% of collected round spermatids were viable. However, distinguishing the viable round spermatids from the non-viable ones, without staining or destroying the cells, is not an easy task. Usually, the viability of round spermatids is estimated during aspiration, according to their ability to undergo a reversible deformation. The dead round spermatids are usually subjected to lysis upon aspiration (Tesarik and Mendoza, 1996; Vanderzwalmen et al., 1998).

Spermatids injection in assisted reproduction

The concept of using round spermatids in ICSI cycles was born in 1993, when Ogura and his collaborators reported that the spermatids nuclei were able to duplicate their DNA and participate in syngamy when incorporated into hamster or mouse oocyte either by microsurgery or by electro fusion (Ogura and Yanagimachi, 1993; Ogura et al., 1993). A year later, Ogura et al (1994) reported the normal birth of four young mice after electro fusion of oocytes with round spermatids, while Sofikitis et al. (1994) reported successful pregnancy after injection of round spermatids nuclei into rabbit oocytes. In 1995, the same research centre reported that mouse oocytes developed into normal offspring

after injection with testicular round spermatids (Kimura and Yanagimachi, 1995). Based on these animal studies, Edwards et al (1994) put forward the question: ''are spermatid injections into human oocytes now mandatory?'' That suggestion along with the success of these animal experiments was the initiation for the introduction of spermatid injection in human assisted reproduction.

Vanderzwalmen et al (1995) reported the successful fertilisation of an oocyte after injection with late stage testicular spermatid and during the next years several papers presented pregnancies achieved after spermatid injection (Table 1).

Table 1. *Published papers reporting pregnancies achieved after round (ROS) or elongated (ELS) spermatid injection.*

	Type of spermatid	No of cycles	No of injected oocytes	No (%) of 2PN oocytes	No of transferred embryos	No of pregnancies	Outcome
Tesarik et al. (1995)	ROS		39	14 (36)	>7	2	Born (first world birth)
Fishel et al. (1995)	ELS	1	10	1 (10)	1	1	Born
Mansour et al. (1996)	ROS/ELS	15	105	40 (38)	32	1	Born
Antinori et al. (1997a)	ROS	29	211	117 (55.4)	81	4	2 Ongoing
	ELS	34	229	158 (69)	119	7	4 ongoing, 1 born
Antinori et al. (1997b)	ROS	7	48	26 (54)	19	2	1 Ongoing
Amer et al. (1997)	ROS	31	251	63 (25)	_	4	Biochemical
	ELS	3	34	19 (56)	_	2	Ongoing
Araki et al. (1997)	ELS	9	116	_	_	3	Born (1 twin, 2 singletons)
Vanderzwalmen et al. (1997)	ROS	32	260	57 (22)	_	1	Born
	ELS	5	21	15 (71)	_	4	2 born, 1 ongoing
Barak et al. (1998)	ROS	8	37	10 (27)	_	1	Born
Barros et al. (1998)	ELS	7	_	_	_	3	1 born, 2 ongoing
Bernabeu et al. (1998)	ELS	1	7	3 (43)	3	1	Born
Kahraman et al. (1998)	ROS	20	199	51 (26)	32	1	Biochemical

Table 1. — (cont.)

	Type of spermatid	No of cycles	No of injected oocytes	No (%) of 2PN oocytes	No of transferred embryos	No of pregnancies	Outcome
	ELS	3	31	22 (71)	11	2	1 born (twins)
Sofikitis et al. (1998)	ELS	13	79	52 (66)	41	2	Born
Al-Hasani et al. (1999a, 1999b)	ELS	2	18	10 (56)	6	2	Ongoing
Gianaroli et al. (1999)	ROS	1	5	2 (40)	2	1	Born
Tesarik et al. (1999)	ELS	1	6				Born (twin)
Saremi et al. (2002)	ROS					1	Born
Sousa et al. (2002)	ELS (cases with hypoplasia)	10	73	34 (49.3)		4	3 Born, 1 ongoing
	ELS (cases with maturation arrest)	16	140	79 (59.8)		5	2 born, 2 biochemical, 1 ongoing
	ELS (cases with Sertoli cell-only syndrome)	7	48	21 (51.2)		3	2 born, 1 ongoing

Recently, results for the development of blastocysts after testicular round spermatid injection were published. Balaban et al. (2000) reported that 34% of the embryos derived from round spermatids injections reached the blastocyst stage but none of them hatched. Urman et al. (2002) also presented results from the transfer of blastocysts derived from injection with testicular round spermatids: with a fertilisation rate of 19.7%, the blastocyst stage was reached by only very few embryos (7.6%) and these embryos did not manage implantation.

It is clear that the reproductive potency of round spermatids is inferior to that of elongated spermatids. So far, the fertilisation rate with round spermatids appears to be low ranging from 20% to 25% in most of the reported cycles, while the fertilisation rate with elongated spermatids is significantly higher, being between 40 to 60%. Correspondingly, the implantation rate is extremely low with round spermatids, while it is higher with the elongated ones.

In-vitro maturation attempts

Obviously, the aforementioned poor results reduced the previous enthusiasm for the use of spermatids in assisted reproduction procedures. However it is true that while the outcome of round spermatid-ICSI cycles is totally disappointing, the use of elongated spermatids resulted in better fertilisation and pregnancy rates. Consequently, attempts for the in-vitro maturation of spermatids were made in order to solve the problem. Tesarik et al. (1998, 1999, 2000) presented some encouraging results on in-vitro maturation of primary spermatocytes and round spermatids, especially using media supplemented with rFSH. Cremades et al. (1999) managed to obtain elongating spermatids and a few mature spermatozoa, after a prolonged co-culture on Vero cells, in four cases. Aslam and Fishel (1999) found out that although short term in-vitro culture of the spermatogenetic cells has a positive effect; it does not improve the incidence of fertilisation significantly. They pointed out that spermatid maturation, which takes about 16 days to complete in-vivo, cannot be accelerated in-vitro, especially within 48 hours (Aslam and Fishel, 1999).

Problems and concerns

Which are the main problems responsible for the poor outcome of spermatid injection, especially the round ones?

It is possible that the immaturity of spermatids may be the most important factor to impair fertilisation capacity in various ways. This can be due to an incomplete histone/protamine transition, since it can lead to chromatin instability and sensitivity making spermatids more vulnerable to denaturing stress. This can further lead to DNA fragmentation and apoptosis. In addition, a lack of protamines may enhance the cell cycle imbalance between spermatid and oocyte resulting in premature chromatin condensation, with a consequent failure of the transformation of spermatid nucleus into male pronucleus (Sousa et al., 1998; Aslam and Fishel, 1999). Aneuploidy is also a major consideration in spermatid injection. Oppedisano et al. (2002) studied the rate of aneuploidy/diploidy in spermatids from three different sterile mice strains showing pathological and histological similarities to human idiopathic non-obstructive males. They found that the round spermatids had elevated level of numerical chromosomal abnormalities in two out of three different sterile strains. These results support the hypothesis that abnormal testicular environment can adversely affect meiosis (Oppedisano et al., 2002).

Nevertheless, one of the most important considerations in spermatid injection is the incomplete or abnormal genomic imprinting. This process is an allele-specific modification of DNA which is developed essentially during gametogenesis. Genomic imprinting results in the expression or repression of

maternal or paternal alleles of certain genes (Reik and Walter, 1998; Brannan and Bartolomei, 1999; Sleutels et al., 2000). Disruption of the imprinting mechanism is associated with disordered growth and development, especially prenatal, as well as with certain clinical syndromes (Preece and Moore, 2000). The status of genomic imprinting has been studied in mouse embryos derived by round spermatid injection (Shamanski et al., 1999). In this study, the expression of imprinted genes did not differ significantly from controls, indicating that paternal genes underwent proper imprinting by the round spermatids (Shamanski et al., 1999). However, the problem still exists since there are no studies from primates or human embryos available. It is worth to note that defects on genomic imprinting process may be manifested relatively late in postnatal life (Preece and Moore, 2000; Cox et al., 2002; rstavik et al., 2003).

At the present time, there is not enough data on the rate of malformation of the children born after spermatid injection. It is fact, however, that an extremely small number of such births has been reported (Fishel et al., 1995; Tesarik et al., 1995; Mansour et al., 1996; Amer et al., 1997; Antinori et al., 1997a, 1997b; Araki et al., 1997; Vanderzwalmen et al., 1997; Barak et al., 1998; Barros et al., 1998; Bernabeu et al., 1998; Kahraman et al., 1998; Sofikitis et al., 1998; Al-Hasani et al., 1999a, 1999b; Gianaroli et al., 1999; Tesarik et al., 1999; Saremi et al., 2002; Sousa et al., 2002; Zech et al., 2002). A recent report described two out of four cases of pregnancies obtained through injection with elongated spermatids, in which congenital malformations were observed (Zech et al., 2002). The authors decided to postpone injection with spermatids due to unpromising results and to potentially high rates of malformation (Zech et al., 2002).

Last but not least, the methodology for isolation, identification and assessment of viability, especially of the round ones, are also important factors affecting the outcome of spermatid injection (Vanderzwalmen et al., 1998; Silber et al., 2000).

Conclusion

Spermatids injection, as an assisted reproduction technique, concerns a small number of the cases of male infertility. It presents significant difficulties in methodology, regarding the isolation, identification and assessment of the viability of spermatids, in particular the round ones. The fertilisation and the implantation rate are extremely low with round spermatids, while they are higher with elongated spermatids. Until today, only a few pregnancies have been achieved. The risk for genetic abnormalities of the offspring has not been estimated yet but it could be high, mainly because of incomplete biochemical transitions in the nucleus and of possible high rates of aneuploidy. Unfortu-

nately, there is a complete lack of experimental studies in primates and there are no large clinical studies available.

Taking these facts altogether, it is a question whether spermatids injection should be considered even as a treatment of last choice. According to our opinion, for the time being, round spermatid injection should be faced as an interesting technique for animal experiments. In that way, the methodology will be developed, the fertilisation, implantation and pregnancy rates will be improved and there will be a better estimation of the possible genetic risks. The injections with elongated spermatids seems to be more efficient, theoretically more secure and consequently could be considered as a treatment of last choice in cases of azoospermia and only after the couples have received appropriate counselling for the possible risks of that procedure.

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Gender preselection for non-medical conditions

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Abstract

Examination of current methods of pre-conceptual gender selection revealed that in vivo methods such as timing of intercourse, the use of ovulation induction medications, and artificial insemination do not appear to affect the sex ratio to a clinically significant degree. In vitro separation of X- and Y- bearing spermatozoa by gradient techniques have been reported to alter significantly the sex ratio at birth. However, these trials were not controlled, and molecular biological techniques could not validate that these methods indeed change the Y- to X bearing spermatozoa ratio sufficiently for clinical use. Nevertheless recent scientific advances have made highly reliable pre-conceptual sex selection possible by using pre-implantation diagnosis (PGD) or sperm separation by flow cytometry combined with AIH or IVF. At present, these methods have been used to avoid sex-linked disorders. Both involve the invasive procedure of IVF and thus are held by most as inappropriate for non-medical indications. It may be that in the near future, an improvement in flow cytometry output of sexed spermatozoa will provide sufficient sorted gametes for artificial insemination. In such a case, the medical community will be forced to take a stand, whether this reliable non-invasive method of sexing will be allowed for social purposes and even if the practice of PGD should be allowed for non-medical indications.

According to Christian view, especially the one of the Catholic Church, gender pre-selection even for medical indications is forbidden. Jewish and Islamic legal viewpoint is that gender pre-selection is lawful when it is practiced on an individual basis, to fulfil the wish of a married couple to have a boy or a girl through available medical means.

Key words: Pre-implantation diagnosis; sex pre-selection; sperm separation; religion and human reproduction.

Introduction

Interest in sex pre-selection has its roots in ancient cultures (1). The Egyptians believed that women of a greenish cast of complexion were "certain to have boys." The early Greeks believed that tying off the left testicle would produce boys because male determining sperm were derived from the right testicle. Aristotle gave advice on positions for sexual intercourse, and desirable diet for the mother. Hippocrates deduced that male and female

children developed in different parts of the uterus. In the Jewish tradition, the Talmud suggests that the determination of sex have boys." The early Greeks believed that tying off the left testicle would produce boys because male-determining sperm were derived from the right testicle. Aristotle gave advice on positions for sexual intercourse, and desirable diet for the mother. Hippocrates deduced that male and female children developed in different parts of the uterus. In the Jewish tradition, the Talmud suggests that the determination of sex takes place at the moment of copulation. When the woman emits her semen before the man, the child will be a boy. Otherwise it will be a girl. Talmud said that placing the marriage bed in a north-south direction favours the conception of boys. Rabbi Ammi stated: If the woman emits her semen first she bears a male child; if the man emits his semen first she bears a female child; for it is said: If a woman emits semen and bears a man child (2). At first it used to be said that if the woman emits her semen first she will bear a male, and if the man emits his semen first she will bear a female, but the sages did not explain the reason, until Rabbi Zadok came and explained it: These are the sons of Leah, whom she bore unto Jacob in Paddan Aram with his daughter Dinah (3). Scripture thus ascribes the males to the females (i.e., sons of Leah) and the females to the males (i.e., his daughter Dinah).

And the sons of Ulam were mighty men of valour, archers, and had many sons, and sons' sons (4). Now is it within the power of man to increase the number of sons and sons' sons? But the fact is that because they contained themselves during intercourse, in order that their wives should emit their semen first, so that their children should be males, Scripture attributes to them the same merit as if they had themselves caused the increase of the number of their sons and sons' sons. This explains what Rabbi Kattina said: "I could make all my children to be males." A statement in the Talmud pronounced "he who desires all his children to be males should cohabit twice in succession."(5). This advice to the husband to repeat the coital act in order to increase the chance of a male birth is explained by Rabbi Rashi, that during the second intercourse the woman will certainly "emit seed first." That is if she did not reach orgasm first and did not conceive with the first ejaculation. She may be impregnated as a result of the second intercourse during which she should be allowed to reach orgasm first. Talmudic passage states, what should a man do that he may have male

children? He should marry a wife that is worthy of him, and conduct himself in modesty at the time of marital intercourse.

The Talmud raises the question of what happens if both man and woman emit seed simultaneously. Several possible answers are given: the offspring may be a hermaphrodite, one whose sex is unknown *(tumtum),* or twins, one male and one female (6).

French aristocrats of the eighteenth century are said to have had their left testicles removed so as to be sure of an heir (male). The French ensured the conception of males by reciting chants or by lying on one particular side while having sexual intercourse, by wearing boots to bed, by waiting for a certain phase of the moon or a rising tide, or by eating certain food. Several scientists prescribed special diets methods as a means of conceiving males (1). One custom suggested by Pacific Island women was dressing in men's clothes in order to conceive sons, and men in the United States believed that hanging their underpants on the right side of the bed conceived a male and the left side for a female.

In Vivo and In Vitro Methods

During the present century, interest has focused mainly on vivo methods of pre-conceptual gender selection such as timing of intercourse, the use of ovulation induction medications, and artificial insemination. Those methods do not appear to affect the sex ratio to a clinically significant degree. In vitro separation of X- and Y-bearing spermatozoa by gradient techniques have been reported to alter significantly the sex ratio at birth (7). The treatment for conception of a boy involves running the husband's sperm sample through columns containing human serum albumin solutions of different concentrations by which Y -bearing sperm can be separated through a filtering process.

There have been several reports in the scientific literature recently which suggest that sperm subjected to a swim up procedure and used for artificial insemination can increase the chances of conceiving a male by between 80 and 90%. Medical techniques tested so far are swim-up separations, and separation using Sephadex columns and Percoll gradients were tried. However, these trials were not scientifically controlled, and molecular biological techniques could not validate that these methods indeed change the Y- to X-bearing spermatozoa ratio sufficiently for clinical use.

Nevertheless recent scientific advances have made highly reliable pre-conceptual sex selection possible by using pre-implantation diagnosis or sperm separation by flow cytometry combined with AIH and /or IVF. At present, these methods have been used to avoid sex-linked disorders. Both involve the invasive

procedure of IVF and thus are held by most as inappropriate for non-medical indications.

Sperm Separation and AIH

Preconception sex selection has generated great interest and controversy over the years. Examination of current methods of pre conceptual gender selection revealed that in vivo methods such as timing of intercourse, the use of ovulation induction medications, and artificial insemination do not appear to affect the sex ratio to a clinically significant degree. In vitro separation of X- and Y -bearing spermatozoa by gradient techniques have been reported to alter significantly the sex ratio at birth (8, 9). Medical techniques tested so far are swim-up separations, and separation using Sephadex columns and Percoll gradients were tried. However, these trials were not scientifically controlled, and molecular biological techniques could not validate that these methods indeed change the Y- to X-bearing spermatozoa ratio sufficiently for clinical use.

Flow cytometry is the only technique that produces a clinically enrichment of X- or Y- bearing spermatozoa (7). However, concern has been raised about the methodological implications of the flow technique because of the use of DNA stains and UV light. Births of normal daughters after sperm separation and intrauterine insemination, in-vitro fertilization, or intracytoplasmic sperm injection was already achieved. Offspring was of the desired female gender in 92.9% of the pregnancies that were obtained after use of flow cytometric separated human sperm cells (MicroSort) for preconcep- tion gender selection. Most of these pregnancies and births were achieved after simple intrauterine insemination(10). However, improvement in flow cytometry output of sexed spermatozoa might provide in the near future sufficient sorted gametes for artificial insemination.

Pre-implantation Sex Determination

In vitro fertilization techniques, which were developed originally for the treatment of infertility, recently have been combined with new technologies that allow gender determination of IVF embryos and selective transfer of the desired male or female embryos (11,12). The main objectives of PGD include an effort to offer the widest possible range of choices to women at risk of having children with genetic abnormalities, to provide reassurance, and reduce the anxiety associated with reproduction.

These objectives are especially for women at high risk; and to enable women at high risk to continue a pregnancy by confirming the absence of certain

genetic diseases. The emphasis is placed on the provision of life for new children who may otherwise never have been born.

The advantage that enhances the acceptance of PGD by social groups is that it avoids the

implantation of defected embryos, and this process of selection eliminates the need for future termination of pregnancy. PGD avoids all the debates related with the issue of abortion in society and in individual cases, reduces or prevents the suffering for the affected family, foetus, and society and also protects the society's resources (13). One of the goals of PGD is to enhance the couple's ability to make informed reproductive decisions, even though sometimes such a decision is influenced by pressure applied by society.

Pre-implantation gender selection, another issue of social conflict, is already practiced in some centres. Gender selection can be used in order to avoid the almost 300 X-linked recessive diseases that are known today. So far, sex selection in pre-implantation embryos has been reported only for the avoidance of X-linked disease, carried by the mother, by the se- lection of female embryos for transfer. In this approach, after traditional IVF, a biopsy of one or two blastomeres from each of he cleavage stage 3-day-old embryos provides the material for gender determination by single genome DNA analysis. The genetic diagnosis is carried out within the day of embryo biopsy, allowing selected embryos to be transferred the same evening. In vitro development of human embryos to a blastocyst does not appear to be affected adversely by a one- to two-cell biopsy at the eight-cell stage and pregnancy rates appear to be unaffected, as long as embryos are transferred on the same day. Currently, two methods are used for gender pre-implantation diagnosis: either polymerase chain reaction (PCR) amplification of DNA from the biopsies blastomeres, or cytogenetic preparation of the single blastomeres for fluorescence in situ hybridization (7). Gender determination by PCR amplification of a specific sequence derived from the Y chromosome was used in the first clinical trials. This approach led to successful pregnancies after the transfer of fern embryos to mothers who were carriers of serious linked disorders (14).

An alternative method of sex pre- selection human embryos is by dual fluorescence in situ hybridization for simultaneous detection of X- and Y-chromosome- specific sequences. It allows simultaneous detection of the X and Y chromosomes each in a different colour. The major advantage dual fluorescence in situ hybridization over *PCR* is that it can assess the ploidy of the gonosomes. Mosaicism and aneuploidy such as XO and XXX have been detected

using dual fluorescence in situ hybridization, and the transfer of such abnormal embryos was avoided .Thus, fluorescence in situ hybridization is considered the method of choice for pre-implantation human embryo sexing.

Social Aspects

Family balancing through PGD remains an issue of debate. The subject raises such concerns and emotions that people generally have very polarized views: those wanting complete freedom to choose however they please the sex of their child and those that demand the total prohibition of sex selection for non-medical purposes. The objection to sex selection arises mainly from the issue of human rights which entails non-discrimination on grounds of sex and from social reasons on in the history of mankind. Man preference is still dominate in many countries for examples such as China, Korea, India and the countries of Middle East, in which boys are highly prized for economic, hereditary, or religious/cultural reasons. The financial hardship of raising girls in some of these countries has led to the abandonment of female children and the widespread use of abortion and infanticide in favour of boys, and this would ultimately alter the established sex ratio (14).

For example in India, the commonest methods employed by the vast majority of the populace, usually after two female children is ultrasound directed foetal sex determination at 13/14 weeks gestational age and in more affluent urban areas the chorionic villus sampling technique at 8/9 weeks. Women who discover that their foetus is female often opt for legal abortions. Estimates of the number of female foetuses being destroyed every year in India vary from two million to five million. This practice has reportedly skewed sex ratios from the natural 106 boys to 100 girls to as high as 130 boys to 100 girls. In China, the issue of gender preference was complicated by the introduction of the One Child Policy to control the population. There are an estimated 114- 118 males born for every 100 females in China, while the international norm is 106 males to 100 females. It is estimated that males may outnumber females by up to 30 millions in 2029. The imbalance in gender ratio will cause other social, economic, and political problem. (15)

Religious Perspectives

Judaism — Halacha

A strict association between faith and practical ruling characterizes the Jewish religion. In principal Jewish law has two divisions, written and oral. The foundation of the written law and the origin of authority is the Torah, the first five books of the Scripture. It is an expression of God's revelation reaching and guiding humanity. The Torah is viewed as a single unit, a divine text that includes moral values as well as practical laws. The oral laws interpret, expand, and elucidate the written Torah and regulate new rules and customs. Its authority is derived from the written Torah. The dominant parts of the oral law are as follows: Mishnah, Talmud, Post-Talmudic codes, Responsa.

Responsa. The various attitudes of rabbinical scholars about the way religion should be applied in the changing world is analyzed and discussed with regard to the legal codes, and written opinion is given by qualified authorities to questions about aspects of Jewish law. Responsa is the term usually confined to written replies given to questions on all aspects of Jewish law by authorities from the time of the later Geonim to the present day. About 1000 volumes, containing more than half a million separate Responsa, have appeared in print.

Halacha and Reproduction

Jewish attitudes toward infertility can be discerned from the fact that the first command from God to Adam was, "Be fruitful and multiply." This is expressed in the Talmudic saying from the second century, "Any man who has no children is considered as a dead man." This attitude arises from the Bible itself and refers to the words of Rachel, who was barren: "Give me children or else I die."

A rabbinic disagreement in the Mishnah deals with the number of children required to fulfil the divine command of procreation (16). It would have been impossible for the human race to propagate had not one of each sex been created. For the preservation of the race, however, it is necessary for every man to have children of **both sexes**. With two males, a man has not fulfilled the mitzvah of "be fruitful and multiply." So how is it possible to explain that it is written that in order to fulfil the obligation of "pru and rvu" you need to have two male children.

The Gemara explains that they learn this from Moses, who only had two sons (I Chron. 23:15), and withdrew from his wife. Beit Hillel says, a male and a female, as it is written, "Male and female He created them" (Gen. 5:2.) which they learn from the creation of the world, as it is written-regarding Adam and Eve "Male and female He created them " -and just as male and female were created, so is it necessary to fulfil the mitzvah of "be fruitful and multiply" by begetting a male and a female.

Their view was based on God's creation of the rib, with Adam and Eve as the first humans. It would have been impossible for the human race to propagate had not one of each sex been created. For the preservation of the race, however, it is not necessary for every man to have children of both sexes. As in most cases, Talmudic preference is in accord with the Hilled School (15).

Man is commanded to be "fruitful and multiply," but not woman. (15).

Although a man who accomplishes the basic command of procreation is not committed by the Torah to continue to procreate he is obligated to be married and not live in celibacy. Along these lines, the Mishnah raises an interesting question: Does the demand to procreate rest equally on men and women, or is it exclusive obligation of men while women who bear all of the risk of childbearing

bear no responsibility? Rabbi Yohanan ben Beroka says, Regarding both it states, "And God blessed them, and God said to them: 'Be fruitful and multiply'" (Gen. 1:28).

According to both Schools, Beit Shamai and Beit Hillel in order to fulfil the obligation of procreation at least one son is required. Therefore the application of pre-sex selection may be of practical importance using the method of sperm separation or sex selection of pre-embryo (PGD).

The application of new technology according to Jewish law is based on the following principles: The Mishnah emphasizes that only prohibitive, strict decisions require juridical substantiation while permissibility or leniency needs no supportive precedent. The absence of a prohibitive substantiation is to be equated with halachic permissibility. This implies that any technological innovation is permissible unless there is a halachic reason for prohibiting it. In order to be sure that there is no halachic prohibition against a new procedure, an accepted halachic authority must be consulted. Jewish law differentiates between the authority to abrogate a temporary prohibition and the authority to determine permanent permissibility. Faced with uncertainty or insufficient information, one is entitled to be strict with one- self; no special authority is needed for prohibition by the individual. On the other hand, in order to establish permissibility, there must be unequivocal information. When there is no clear precedent in halacha to decide the issue at hand, one must be thoroughly versed in all halachic sources before definitely con- firming that no halachic reason for prohibition exists.

There are well-known halachic rules for deciding controversial issues. If, for example, there is a doubt in a matter prohibited by the Torah, the ruling is prohibitive; if the doubt is related to a rabbinical ruling the decision is usually permissive.

AIH and Halacha

There is near unanimity of opinion that therapeutic insemination with a husband's sperm (AIR) is permissible according to Jewish Law, halacha, if no other method will allow the wife to become pregnant (17). However, certain qualifications do exist. First, the couple must have attempted conception for a reasonable period of time 5-10 years and medical proof must exist of the absolute necessity for AIH. Second, according to many authorities,

insemination may not be performed while the woman is in Niddah. The halakhot (religious laws) surrounding a woman's menstrual cycle form the basic backdrop for this discussion because they govern the normal sexual life of a religiously committed Jewish couple (16). Understanding their basic concepts is indispensable to professionals providing fertility therapy to an observant couple.

A menstruating woman is called Niddah in the Bible and in the Talmudic and post- Talmudic literature. As long as she is within the status of Niddah sexual contact with her is forbidden. The Bible differentiates Niddah menses, and Zavah, dysfunctional, uterine bleeding. **In** the case of Niddah, the duration of menstrual prohibition is 7 days, even if the bleeding is of much shorter duration. A ritual bath (Mikveh) is required to revert Niddah back to the regular status. In the case of Zivah, Mikveh is required after a period of 7 entirely "clean" days (Shivaah Nekeim). The laws concerning Niddah are some of the most fundamental principles of the halachic system, and the historical development of the relevant tracts through I the centuries is also extremely complicated (17). Most rabbis allow sperm to be obtained from I the husband both for analysis and insemination, but opinions differ about the best method of procuring" them. Masturbation should be avoided if at all possible, coitus interruptus and the use of a special condom are preferred. Artificial insemination of the husband's sperm is generally regarded as a halachically is permissible procedure through which paternity can be established and also for the mitsvah of *peru u-revu* "be fruitful and multiply." The biblical obligation to have children or at least *la-shevet* "to be inhabited," this rabbinical obligation to have children can thus be fulfilled.

In conclusion flow cytometry that produce a clinical enrichment of x-or y bearing spermatozoa can be applied for sex pre-selection according to Halacha.

Pre implantation Sex Determination and Halacha

Pre-implantation sex determination technique allows gender determination of IVF embryos and selective transfer of the desired male or female embryos. This reliable medically assisted sex selection does not involve abortion or

infanticide, but the post conceptual genetic diagnosis and implantation method would involve infanticide on an embryonic level. If a couple were to choose implantation of embryos that are of one sex, what would happen to the embryos of the opposite sex? They would be discarded. Discarding an embryo, infanticide on an embryonic level is not morally acceptable in the minds of many and doing so violates the right of the discarded embryo to live.

The foetus is not considered a person (Hebrew Nefesh,"soul") until it is born. It is regarded as a part of the mother's body and not a separate being until it begins to egress from the womb during parturition. In fact, until 40 days after conception, the fertilized egg is considered mere fluid (18, 19). The Judeo-biblical tradition does not grant moral status to an embryo before forty days of gestation. Such an embryo has the same moral status as male and female gametes, and its destruction prior to implantation is of the same moral import as the "wasting of human seed." After 40 days-the time of "quickening" recognized in common law-the implanted embryo is considered to have human hood, and its destruction is considered an act of homicide. Abortion on demand is repulsive to the ethics of

the Halacha; however, in many situations a pregnancy may be terminated. If the mother's life is in danger, each foetus is a Rodef an aggressor who may (or must) be killed to save the individual in danger. Rabbi Eliashiv, possibly the most influential *posek* in Israel today, has permitted PGD and destruction of affected zygotes to prevent cases of Fragile-X and even in a case of a woman with neurofibromatosis who only had skin lesions. Rabbi Feinstein has taken a similar view as to the permissibility of discarding "extra" pre-embryos. Pre-implantation diagnosis, which is already accepted by some Rabbinic authorities, is likely to be acceptable to most Jewish legal experts when used to prevent serious diseases in offspring. Although the Halacha generally takes a conservative approach regarding abortion, many contemporary rabbinical decisions maintain that un-transplanted embryos have no standing and may be discarded. Therefore, screening for genetic diseases is permitted.

The requirement for a man to procreate by having a minimum of two children-a boy and a girl is obligatory according to Jewish law. According to both schools, Beit Shamai and Beit Hillel in order to fulfil the obligation of procreation at least one son is required (20). Therefore the above analysis shows that the application of pre-sex selection may be of practical importance using the method of sperm separation or sex selection of pre-embryo (PGD).

Christianity and Reproduction

Christianity is cantered on Jesus, the Son of God and His supreme revelation. Christian beliefs are based on His teachings, as reported in the four officially accepted Gospels and on the Jewish Scriptures, which Christians call the Old Testament. Christianity comprises three principle divisions: the Roman Catholic Church, Protestant churches, and Orthodox Churches. Christianity is characterized by its universality and the command given by Jesus to proclaim the Gospel to all humanity. The most striking development in the evolution of Christianity from its Jewish origin has been the transition from a national religion (of the Jewish nation) to a universal religion.

Issues of sexuality, marriage, and parenthood are central to Christian values. In particular the Catholic Church intervention in the field of reproduction is inspired by the love it feels for humans, helping them to recognize and respect their rights and duties. Christianity, like other intersociety religions, is characterized by a great diversity of sects. It is difficult to find common elements, other than origin and the acceptance of common sacred writings and symbols among all Christian sects. There is, however, a core of common principles within the early Christian movement the western medieval church, and the modern Roman Catholic and major Protestant churches. But in so far as a core of European Christian principles exists, its relations to fertility are largely

indirect and cannot easily be formulated. According to Roman Catholic Instruction suffering of spouses who cannot have children or who are afraid of bringing a handicapped child into the world is a suffering that everyone must understand and properly evaluate. The desire for a child is natural: it expresses the vocation to fatherhood and motherhood inscribed in conjugal love. This desire can be even stronger if the couple is affected by sterility, which appears incurable. Nevertheless, marriage does not confer upon the spouses the obligation to have a child, but only the right to perform those natural acts, which are per se ordered to procreation.

The child is not an object to which one has a right, nor can he be considered as an object of ownership: rather, the most gratuitous gift of marriage, and is a living testimony of the mutual giving of his parents. For this reason, the child has the right, as already mentioned, to be the fruit of the specific act of the conjugal love of his parents; and he also has the right conceived through an act of love and, indeed of sexual intercourse.

Within marriage AIH cannot be accepted except for situations in which the procedure is not a substitute for the conjugal act but facilitates it so that the act attains its natural purpose. Therefore in vitro separation of X- and Y-

bearing spermatozoa by gradient techniques or flow cytometry is unacceptable. "The moral relevance of the link between the meanings of the conjugal act and between the goods of marriage, as well as the unity of the human being and the dignity of his origin, demand that the procreation of a human person be brought about as the fruit of the conjugal act specific to the love between spouses" (22).

The Eastern Orthodox Church supports medical and surgical treatment of infertility. However, IVF and other assisted reproductive technologies are absolutely rejected. The Baptist, Methodist, Lutheran, Mormon, Presbyterian, Episcopal, United Church of Christ, Christian Science, Jehovah's Witness, and Mennonite denominations have liberal attitudes to- ward infertility treatments. All denominations except Christian Sciences accept .IVF with spouse gametes and no embryo wastage. Christian Science poses no objection to AIH but oppose IVF because of use of drugs and surgical procedures. Assisted reproductive technology was developed in Great Britain and Australia. The Anglican Church is liberal on the use of IVF-ET and allows semen collection by means of masturbation for artificial insemination by the husband for IVF. However, it forbids the use of donor gametes, semen or oocyte, from a third party .

Christianity and Pre-implantation Gender Selection

The Vatican statement opposed pre-implantation diagnosis, "the Church remains opposed from the moral point of view to homologous in vitro fertilization. Such fertilization is in itself illicit and in opposition to the dignity

of procreation and of the conjugal union, even when everything is done to avoid the death of the human embryo"(22).

This reliable medically assisted sex selection by PGD would likely involve infanticide on an embryonic level. If a couple were to choose implantation of embryos that are of one sex, what would happen to the embryos of the opposite sex? They would be discarded. Discarding an embryo, infanticide on an embryonic level is not morally acceptable by Christianity and doing so violates the right of the discarded embryo to live. The long tradition of Christianity and mainly of the Catholic Church is that human live be- gins at conception and, therefore any use or destruction of pre-embryo that optimized the opportunity of birth, seriously discounts human life.

In conclusion, gender selection according to Christianity is forbidden.

Islam (23)

Islam founded about 1400 years ago by the Prophet Muhammad (A.C. 700-632). He was born in Mecca and spent his early life as a merchant. In middle life an inner conviction dawned on him that he was the prophet chosen by Allah to convey eternal messages to the Arabs.

There are two broad subdivisions of Islam-Shim's and Sunnis. Shi'ism originally referred to the partisans (Shia) of Ali and over the centuries developed its own body of law. This differed in minor ways (inheritance and the status of women) from that of the majority of Sunnis.

Islamic law, Sharia, is the heart of Islamic religion; it defines the path in which God wishes humans to walk. It not only deals with matters of religious ritual but also regulates every aspect of political, social, and private life. The main roots from which it is derived are the Quran and the Hadith, the tradition of the Prophet Muhammad. The Sharia is binding primary for Muslims, who are directly responsible for God, and it is not enforced by the state. According to orthodox Muslims, the law is founded in divine revelation, and since revelation ended with the death of Muhammad the Sharia is immortal. There are two sources of Sharia in Islam, Primary and Secondary.

Good Muslims resort to Secondary sources of Shiria, matters not dealt with in the Primary sources.

Islam and Gender

Most pre-Islamic urban women have lived in a male-dominated society in which their status was low and their rights negligible. They were continually under the thumb of either a male relative or a husband. Men's rights over their women were the same as their rights over any other property. Marriages were made by purchase or contract. The suitor paid a sum of money (the mohar) to the

guardian of the bride-to-be (and possibly another sum, the sadaq to the woman herself), thereby purchasing her and making her his exclusive property. The marriage contract, in other words, was a contract between husband and guardian, with the bride the sales object. Furthermore, neither conventions nor laws existed to limit the number of wives that a man could have simultaneously, so the only

restrictive considerations were economic ones. The marriage laws enjoin women not only to strict monogamy but also to marriage with Muslims only. Muslim men, on the other hand are free to marry Jewish, Christian, and Sabian women, although not an idolater. Both the social status and the legal rights of Muslim women were improved through Quranic legislation. Among the laws that effected such improvements were the following.

1. Laws that put an end to the pre Islamic custom of burying baby girls alive.
2. Sanctions of marriage as a meritorious institution that invests it with importance and dignity.
3. Laws that guarantee women the right to inherit and bequeath property.
4. Laws that guarantee women the right to have full possession and control of their health, including the dower, while married and after divorce.
5. The right of the wife to be properly fed and clothed at the husband's expense.

The general attitude toward women reflected in the Hadith is positive. The Hadith elaborates on the Quran's teaching regarding the spiritual equality of women and men. The nature of women as reflected in the Hadith spans the whole spectrum from the saintly to the evil and unclean. The Hadith gives un-Questionable evidence that the Hijab, which implies not only the face veil but also the sum of practices connected with the seclusion of women, was legislatively made obligatory for the wives of the Prophet. It also contains much evidence of women's visibility as well as full participation in communal matters in the early Islamic period. Later generations of pious scholars changed these patterns considerably; rather, they sanctioned such changes as occurred within Islam under foreign influence. Through the centuries, traditionalist commentators on the Quran emphasized restrictive norms with the distinct purpose of legitimizing the newly restricted status of women in Islam. The result was that restrictions increased with the progression of time.

Islam and Artificial Reproduction

Artificial reproduction was not mentioned in the primary sources of Sharia; however, these same sources affirmed the importance of marriage, family formation, and procreation. When procreation fails, Islam encourages treatment,

especially because adoption is not an acceptable solution. Thus attempts to cure infertility not only are permissible but also are a duty. The duty of the physician is to help a barren couple achieve successful fertilization, conception, and delivery of a baby.

According to Islam AIR can be applied as a method of infertility treatment. Therefore the procedure of intra-uterine insemination of sperm separated by flow cytometry can be attempted.

The procedure of IVF-Ff is acceptable, but it can be performed only if it involves only the husband and wife (24). The fusion of sperm and egg, a step beyond the sex act, should take place only within a legal marriage. Since marriage is a contract between wife and husband, during their marriage no third party can intrude into the marital functions of sex and procreation. A third party is not acceptable, whether providing egg, sperm, embryo, or uterus.

If a marriage has come to an end through divorce or death of the husband, artificial reproduction cannot be performed on the woman even with sperm cells from her former husband.

Islamic law strictly condemns the practice of AID on the grounds that it is adulterous. It enhances the risk for inadvertent brother-sister marriage and violates the legal system of inheritance. The procedure also entails the lie of registering the offspring of a man who is not the real father and therefore leads to confusion of lines of genealogy, the purity of which is of prime importance in Islam. If a man's infertility is beyond cure, it should be accepted.

Islam and Sex Pre-selection

In November 2000, a workshop organized by the International Islamic Centre for Population Studies and Research at Al-Azhar University in Cairo, Egypt, supported the practice of pre-implantation genetic diagnosis (PGD) (25).

Islamic view on the beginning of human life is close to Hebrew Law, that human life requiring protection commences 2-3 weeks from conception and implantation, contrary to Christianity that life begins at conception. Pre-implantation of gender selection was accepted with some reservations. Family balancing was considered acceptable, for instance where a wife had borne three or four daughters and it was in her and her family's best

interests that another pregnancy should be her last. Employing PGD to ensure the birth of a son might then be approved, to satisfy a sense of religious or family obligation and to save the woman from increasingly risk in future pregnancies. The workshop considered that an application for PGD for sex selection should be disfavoured in principle, but resolved on its particular merits. It should be pointed that during previous seminar on Human Reproduction on Islam in 1983 they had a different statement regarding sex pre-selection (26).

There was an agreement on the Islamic legal view- point that foetal sex selection is unlawful when it is practiced at a national level, while on an individual basis, some of the scholars participating in the seminar, believe there is nothing legally wrong with the attempt to fulfil the wish of a married couple to have a boy or a girl through available medical means, while other scholars believe it is unlawful for fear that one sex might outnumber the other.

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Oral contraceptive pills: should they become an over-the-counter medication?

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The oral contraceptive pill (OC) is one of the most thoroughly studied medications. Despite the fact that it is the most efficient contraceptive method, its use remains low among many countries worldwide. On the other hand, unwanted pregnancies pose a heavy socioeconomical problem in the community and a strong psychological burden in women. The question whether the OC should be transferred to the list of prescription-free medications remains a matter of debate. Countries wishing to control birth rates, such as India have since long accepted the over-the counter distribution of OC. Experts from industrialized countries express some concerns on this issue: if OC were prescription — free, then a considerable number of women with gynecological problems, such as pelvic inflammatory disease or other infections of the reproductive system would be left undiagnosed. Moreover, the misuse of OC would increase the rate of sexually transmitted diseases and the occurrence of OC related side effects, as self-screening would not be as efficacious as professional screening. Finally the rate of contraceptive failure of OC might be increased, because of improper use. A more rational approach to expand the use of oral contraceptives would be to reinforce the activity of family planning Clinics and to facilitate public access to such professionals, by instituting charge-free visits or by organizing visits to schools, universities or municipalities. Better access to the physician might be the best way to establish the OC as the main and most efficacious contraceptive method.

Oral hormonal contraception is the most efficacious contraceptive method available today, posing negligible health risks and controlling unwanted pregnancy rates in an easily compliable way. However, nearly one half of all pregnancies during the year 1995 in United States were unintended, and from those ''intended'', many of them were not planned or prepared for. 40% of women with unintended pregnancies reported no use of any contraceptive method, while the remaining 60% had used some form of contraception, which apparently had failed (Nelson 2000).

The currently employed schemes of oral contraception are divided in monthly schemes for primary prevention and in emergency contraception following unprotected intercourse. The main estrogen used is ethinyl estradiol in doses usually ranging from 20-35ã, combined with various progestins, the principal of which are gestodene, desogestrel, levonorgestrel and recently drospirenone. Currently emergency contraception schemes are the Yuspe regimen (100ã ethinyl estradiol + 500ã levonorgestrel, repeated in 12 h) and levonorgestrel alone 750g repeated in 12 h. (Grimes 2002).

In most countries oral contraceptives are available only by prescription. Women should first visit a family planning Clinic or a private gynecologist. The doctor records the woman's medical history and performs a physical examination, which includes manual breast examination and subsequently a pelvic examination. Thereafter, arterial blood pressure is recorded and a PAP smear is taken. Usually general counseling concerning issues on family planning, methods of contraception and issues on sexually transmitted diseases and public health problems, such as cervical and breast cancer, arterial hypertension and diabetes (Stone 1993).

In order to increase access to oral contraceptives (OC) and decrease unwanted pregnancy rates many authorities suggest that OC should be reclassified as over the counter (OTC) drugs. In that way many more women will have the opportunity to use OC directly. This applies mainly for young teenagers who are many times unwilling to visit doctors, for women in remote regions with difficult access to specialized Clinics or private physicians and for poor women with no insurance who are unable to afford frequent visits to health care facilities. Furthermore, many women, especially young teenagers may find the process of a physical and pelvic examination intimidating and thus avoid contact with the health care system. Transferring OC over the counter may by-pass these problems and increase OC utilization rates and subsequently decrease unwanted pregnancy rates and the socio-economic cost which derives from abortions, obstetric complications and from bringing up an ''unwanted'' child (Shah 2001, Folkes 2001).

The concerns of transferring OC over the counter relate mainly to safety and compliance issues. The safety of long-term use of OC has since long been documented (Trussell 1993). The problem which arises in transferring OC over the counter is whether women are able 1) to screen themselves with respect to absolute and relative contraindications and 2) to follow themselves up with respect to possible adverse effects. Absolute contraindications for the use of OC are undiagnosed unscheduled uterine bleeding, undiagnosed breast lump, history of reproductive system malignancy, history of venous thromboembolism and current liver dysfunction. With the help of a detailed questionnaire enclosed in the package, women may easily identify these medical conditions. The

problem arises with relative contraindications, such as migraines, arterial hypertension and diabetes mellitus. In these cases, the choice to prescribe an OC is met after considerations of other factors, such as age, body mass index, smoking habits, family history of cardiovascular disease and the possible estrogen dose in the OC. Regarding relative contraindications, the consultation of a physician before self-selecting an OC may be more desirable, in order to ensure long-term safety (Trussel 1993, Forman 1997).

Regarding compliance, even prescription administered OC are associated with major misuse. Possible types of non-compliance include 1) wrong time of initiation of the first package (late into the cycle, which may be associated with intake while being pregnant), 2) failure to start a new package on time, 3) quiting in midcycle (because of nuisance side-effects, which the patients perceives as important, 4) interrupting for one or more cycles, because the women thinks her body needs ''a rest from hormones'', 5) forgetting one ore more pills, 6) taking the pill the wrong time (more than six hours apart from the usual time), 7) taking triphasic pills in the wrong sequence and 8) not taking additional contraception if vomiting or diarrhea occurs or if drugs affecting hormone metabolism are taken concomitantly, such as antibiotics or anticonvulsants (Trussell 1993). If women are not instructed thoroughly how to use OC and which ''adverse effects'' are harmless, the compliance with OC may decrease dramatically, and subsequently the efficacy of hormonal contraception.

Another issue associated with the transfer of OC over the counter is that associated with the function of family planning Clinics. In such Clinics women are counseled about sexually transmitted diseases and major public health problems, such as cervical and breast cancer, arterial hypertension and diabetes mellitus and receive routinely breast examination and PAP tests. These Clinics, besides prescribing OC, offer a vast number of services: They provide primary medical care for a huge number of women, they refer patients for specialized medical care, they screen and treat sexually transmitted diseases and they screen for breast and cervical cancer, diabetes and anemia. If women can by-pass this process to obtain OC, then the opportunity for women to receive all these services might be lost and the survival of such Clinics may be threatened (Forman 1997, Hewitt 1997).

Emergency contraception, as stated earlier is administered after unprotected sexual intercourse, to prevent a subsequent pregnancy. The progestin-only formulation or the estrogen — progestin combination currently available in most countries are safe and do not require physical examination. Furthermore they are effective resulting in a 96-99% of pregnancy prevention in cases with high compliance. However, utilization of this method is very low: only 2.5% of women who attend Clinics for termination of an unwanted pregnancy report having used this method. This is either because of unawareness of this method,

or because of difficulties in obtaining these medications (Matheson 1998). Thus, regarding emergency contraception and taking into account the circumstantial and not systematic intake of these preparations, transfer over the counter may be more compelling than ordinary OC.

Concluding, the transfer of OC over the counter, although it poses an intriguing option to increase access to contraception and decrease unwanted pregnancy rates, has still limiting factors. Cost-benefit analyses should be performed in order to assess the percent of reduction in unwanted pregnancies versus the increase in adverse effects and the possible increase of sexually transmitted diseases and other conditions associated with primary prevention. Alternatives to reclassification of OC as over the counter medications include better education of the population via the media or via lectures in schools, universities and municipalities, increase of the number of family planning Clinics and possibly decrease of the prerequisites to prescribe an OC. In this way access to OC may increase while in parallel women receive primary health care and professional counseling on reproductive health issues.

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Contraceptive & Hormonal Needs in the Perimenopause

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The contraceptive needs of older women have never been more important than they are today in our changing society, nor have their choices ever been so great. Sadly many women over 35 are not having these needs met and unplanned pregnancy and termination rates are rising.[1]

Many women in their late thirties, and even more by their mid-forties, are experiencing effects of their fluctuating hormonal environment. Many of the hormonal contraceptive options available would help to stabilise this and ameliorate early symptoms. However most women are unaware of this, or indeed believe that they are ''too old to use hormones'' or that it would be in some way risky. Indeed for some it might, but not for many who could benefit massively from efficient contraception and hormonal stabilisation. It should be the role of the doctor or practice nurse to proactively initiate discussion at various stages in a woman's life — after childbirth and during her late 30s and 40s, when many women or couples may be considering sterilisation.

Changes in Society

With divorce rates in the U.K. running at around 41% of all marriages,[1] many women in their ''middle youth'' find themselves 'back out in the sexual market place'. Not only must they cope with the demands of their growing children, they often form new relationships and so must start dealing once more with the issues of contraception and sexual health. If, as so often is the case, their ex-husband has had a vasectomy, they

have not had to face these considerations for many years and are often unaware of modern choices.

The Perimenopause

It is becoming increasingly apparent that the transition into menopause, specifically the perimenopausal period, is a gradual process happening over many years. The age of onset and the duration of this perimenopausal phase can vary greatly[2]. Most women do not move from regular menstruation to sudden amenorrhoea but rather experience a time of menstrual irregularity often with shortened or irregular cycles. Many women report increased premenstrual

symptomatology, including headaches and migraine[3], increased menstrual flow and more painful periods.

This is a time a huge hormonal variability, with hormone levels fluctuating more intensely than at any other stage of a woman's life. For some this leads to a variety of problems including insomnia, emotional lability, forgetfulness, poor concentration, joint aches and tiredness. This is a time, above all other, when a woman may turn for advice to the medical profession. To the unwary, this constellation of symptoms could be misinterpreted as depression and it is certain that significant numbers of women are thus inappropriately labelled. Unfortunately, at the present time, clinical trial data are insufficient to establish evidence-based treatment standards and clinicians may need to rely on experience when considering management options[4]. The need to include the woman herself in the decision-making process is self-evident. The problem is to balance her need for contraception with that for hormonal support and to allow her to make an informed choice. The perimenopause as an entity has only been recognised recently and indeed little reference is made to it in standard gynaecological texts making its management challenging (see figure1).

- ↓ fertility
- ↑ hormonal instability
- ↑ TOP rate
- ↑ miscarriage rate
- ↑ risk foetal abnormality
- ↑ maternal morbidity
- ↑ perinatal mortality
- ↓ sexual frequency
- ↑ delaying first pregnancy

  ↑ women with new partners

Figure 1 *Challenges in the Perimenopause.*

Decreasing Fertility Rates

Many women assume it is impossible to conceive in their mid-forties, and although fertility falls with age this is certainly not the case. Reliable methods of contraception are still needed to avoid unintended pregnancy. At age 40, half of women are still fertile (see figure 2)

Age	<25	40	45
Fertility Rate	85	45	15

Figure 2 *Fertility rate =pregnancies per 100 women years*[5(adapted from)]

Termination of Pregnancy

Women aged over 40 in the U.K. have the highest TOP rate per number of conceptions (40%) of any age group of women, including teenagers.[5] This is an indication that most pregnancies in this group are unplanned and unwanted. It would be infinitely preferable to avoid this situation by allowing women sufficient knowledge of available contraceptive options.

What Methods do Older Women Use?

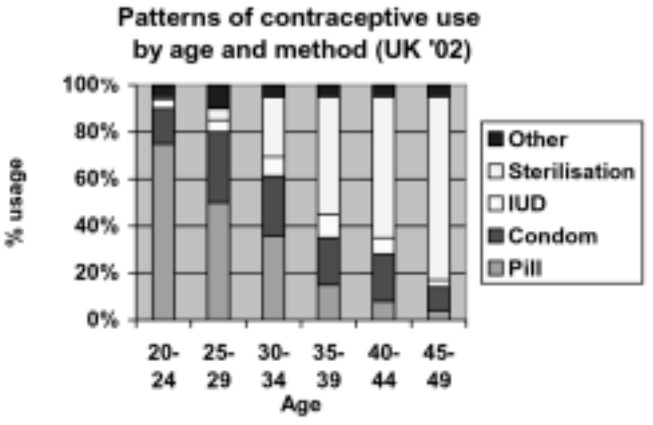

Figure 3 *(adapted from)*[6]

There is a steady transition away from hormonal methods and towards sterilisation with advancing age, in the UK (see figure 3). By the age 35, only about 1 in 3 women use hormonal methods. At least 40% of couples over the age of 40 rely on female or male sterilisation, yet there are now increasing numbers of highly effective, reversible methods available. The RCOG sterilisation guidelines[7] recommended that ''all couples should be fully counselled about all such alternatives, before proceeding to sterilisation''.

More Than Just Contraception: Positive Health Benefits

Many women would more than welcome the chance to eliminate some of their perimenopausal symptoms and these possibilities should be discussed with them. The ability to stabilise a woman's hormonal environment, perhaps by use of the combined pill, in a non-smoker with no other risk factors, may well enhance the quality of her life.

Combined Oral Contraceptive Use in the Perimenopause

Many women who stop the combined pill to be sterilised experience unwelcome hormonal and menstrual changes and conversely those who do continue the

COC into their 40s report maintaining feeling well. There are many fears and misconceptions about using the pill and duration of use in this older age, which need addressing.

Modern 20μg COCs offer first rate contraception with the benefits of regular, predictable light withdrawal bleeds[8] and masking of early menopausal symptoms. This can be of particular advantage to those women developing menorrhagia and dysmenorrhoea at this stage in their life, where previously hysterectomy might have been considered the best option. It can also help control worsening perimenopausal PMS and mood change, and give back a sense of control to many women whose lives are being made more difficult by the unpredictability of these perimenopausal years.

Our job is to ensure that they are not given to ''risky women''. So long as a woman is a healthy, migraine-free, non-smoker, with no other risk factors for arterial disease, the term ''contraceptive gap'' no longer applies, and at her request, the COC may legitimately be taken through to the menopause. Age alone is no longer considered a contraindication.[9>]

Arterial Disease Risk Factors: prohibiting COC use in over 35s:

- Cigarette smoking
- FH of CVD in parent or sibling <45 yrs
- Diabetes mellitus
- Hypertension >140/90
- Obesity: BMI > 35
- Migraine (including ''without aura'' in this age group)

The consensus of opinion is that lower oestrogen doses should probably be used be used over the age of 40 if the COC is to be continued[10]. It would appear that the modern progestogens (usually to be found in 20μg preparations) are indeed less likely to cause cerebro-thrombotic events than the older generations[11], thus ''risky women'' in this age group would also include those with risk factors for venous thromboembolism.

Risk Factors for Venous Thromboembolism:

- Previous DVT/PE
- FH of unprovoked VTE in 1st degree relative <45yrs
- Obesity BMI > 39
- Immobility
- Extensive varicose veins

NB. Smoking <10/day is not a risk factor for VTE

Although a recent study found no increased risk of breast cancer among women who were current users of low dose COCs in the 45-54 age group, the authors concluded that their findingd may not be conclusive, and further investigation of this question is merited[12]. It is no longer disputed that the COC provides

protection from cancer of the ovary and endometrium, whose incidence rises particularly above the age 40.

Overall, the balance would appear to be in the favour of COC use in older women being beneficial in risk-free older women, providing reliable contraception with ''add-on benefits'' with respect to cycle-control, symptom relief and protection from osteoporosis.

The COC/ HRT Overlap

After the menopause (or leading up to it if contraception is not an issue), ''natural'' oestrogen (oestradiol or conjugated equine oestrogens) are sufficient for symptom relief; they are not, however, contraceptive. If, then, women stay on the COC ''until the menopause'' how can this event be diagnosed, and infertility be assured?

One method is to try measuring the FSH at the end of the pill-free week in a woman of 50. If it is still normal, she cannot be advised to stop using contraception, but nor would it seem wise to continue the COC indefinitely, and so a switch to an oestrogen-free method might be advisable. If the FSH is at menopausal levels on 2-3occassions, she might be advised to switch if she chooses, straight to HRT.

Our aim as health professionals should be to guide each woman towards informed choices, dispelling myths along the way. We should not miss this golden opportunity to control the hormonal milieu and help improve the quality of life in the perimenopause.

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Levonorgestrel-IUS: The Modern Alternative to Sterilisation

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The **levonorgestrel intrauterine system** (LNG-IUS) has been hailed as one of the major advances in the field of contraception since the introduction of the pill. It is not only **a highly effective and reversible** method, but it also has other **non-contraceptive benefits**.

Local release of LNG produces an inactive and atrophic endometrium, so normal **menstrual flow is reduced**. This in turn leads to less endometrial prostaglandin production and therefore **less dysmenorrhoea.** Studies show an objective reduction in menstrual loss (86% after 3m and 97% after 12m[1]). 17% of users are amenorrhoeic after 1 year of use[2], 27% by the end of the first 5 years and up to 60% after a second 5 years. The number of bleeding days per cycle also gradually diminishes. Within 30 days of removal the endometrium has returned to normal and menstruation occurs[3]. It is thus easily and **completely reversible**. (see figure 1).

The systemic absorption of LNG is extremely low (= 2 progestogen-only pills/week)[4] thus minimising **side effects**, such as breast tenderness, greasy skin and hair, headaches and abdominal bloating. The **plasma oestradiol** of users remains within the normal range[5], important for perimenopausal women.

The **ectopic pregnancy rate** in users is exceptionally low — 10x less than the ectopic rate for Nova T users (0.02 cf. 0.25 per 100 women-years)[6]. The incidence of **pelvic inflammatory disease** is also much lower than for copper-IUDs, due to a combination of factors including thickening of the cervical mucus, endometrial suppression and reduced bleeding[7].

The **pregnancy rate** in LNG-IUS users has been shown to be exceptionally low (Pearl Index 0.16)[8]. In light of the recent report from the U.S. Collaborative Review of Sterilisation (CREST Study, 1996[9]) showing failure rates for female sterilisation considerably higher than expected (Pearl Index up to 0.5), this makes the LNG-IUS a viable alternative to this procedure in terms of efficacy alone. The gross cumulative pregnancy rate at 3 years is 0.3 per 100 users[6].

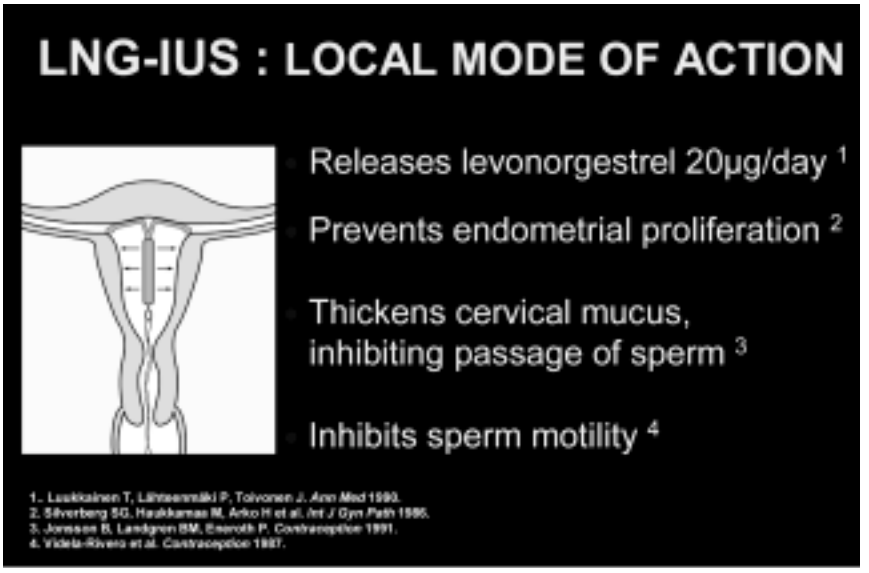

Figure 1: *Mode of Action of LNG-IUS*

The extremely low side-effect profile combined with the obvious benefits of reduced or absent menstrual flow, less dysmenorrhoea and an improvement in PMS, are all reflected in the extremely **high continuation rates**, with one study showing 81% at 3 years and 65% at 5 years[10].

LNG- IUS AS ENDOMETRIAL PROTECTION IN HRT

• Andersson et al	1992	oral
• Raudakoski et al	1995	transdermal
• Suhonen et al	1995	implant
• Suveanto-Luukonen et al	1999	percutaneous

Figure 2

Since user satisfaction is so high and given the economic cost of female sterilisation, should we not be considering LNG-IUS for all couples seeking highly effective contraception as the modern **alternative to female sterilisation** or indeed male vasectomy? Indeed The U.K. Royal College of Obstetricians and Gynaecologists, in their evidence-based guidelines for male and female sterilisation[11], state that comparative failure rates and advantages/disadvantages of alternative long-term methods should have been discussed. They conclude: ''non-operative methods of long-term contraception should have been specifically rejected before proceeding with sterilisation''.

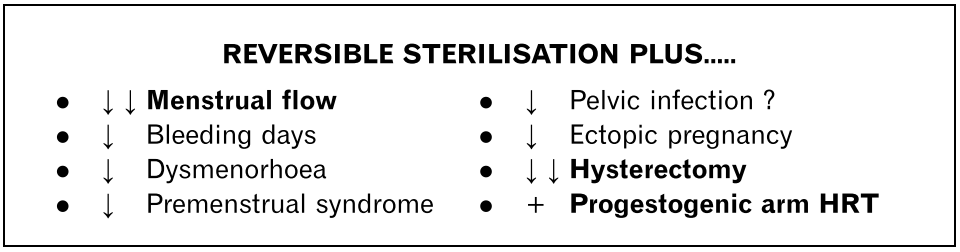

Figure 3: *Add-on Benefits of LNG-IUS compared to Female Sterilisation*

The LNG-IUS also provides an effective (although as yet unlicensed) method of delivering **progestogenic opposition** to oestrogen in hormone replacement therapy[12] (see Figure 2), especially in the perimenopausal age group where the incidence of dysfunctional uterine bleeding is high and there is still a need for contraception.[13]

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Prophylactic oophorectomy in BRCA carriers with or without hysterectomy

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Summary

Introduction: The purpose of this study was to determine whether the addition of a hysterectomy is beneficial for BRCA carriers undergoing prophylactic oophorectomy(PO).

Materials and Methods: An English language MEDLINE review of ''BRCA'', ''prophylactic oophorectomy'' and ''prophylactic hysterectomy'' was performed.

Results: PO is recommended for BRCA1/2 germline mutation carriers to prevent ovarian cancer. However, several studies have now recognized that fallopian tube cancers and uterine serous cancers are also associated with BRCA1/2 mutations.

Conclusion: Complete removal of not only the ovaries, but the fallopian tubes and uterus provides substantial cancer prophylaxis benefits for women with BRCA1/2 germline mutations.

Introduction

Inactivating mutations of the BRCA1 and BRCA2 genes are associated with inherited breast and ovarian cancers.[1] These cancers account for up to 10% of all breast and epithelial ovarian cancers. Unique founder mutations have now been identified in Ashkenazi Jewish, Asian as well as in Western European populations.[2] Carriers of BRCA1 mutations have been reported to have a lifetime risk for developing breast cancer of 60-85% and their lifetime risk for developing epithelial ovarian cancer has been reported to be 15-65%.[2,3] Carriers of BRCA2 mutations may have a similar risk of breast cancer but a 10-20% risk of ovarian risk.[1] The high risk for ovarian cancer in women with BRCA1/2 mutations and the lack of effective techniques for the early detection of ovarian cancer has led many centers to recommend prophylactic oophorectomy to avoid the development of ovarian cancer.[4,5] However, there are many questions regarding the role of prophylactic oophorectomy in the reduction of ovarian

cancer risk as well as it decreasing the possibilities of breast cancer.[6] Additionally, BRCA1/2 germline mutations have now been associated with fallopian tube, primary peritoneal and uterine serous cancers.[7-14] This article will concentrate on prophylactic oophorectomy in BRCA carriers and whether there is a benefit to performing a hysterectomy at the same surgery. A MEDLINE review of ''BRCA,'' ''prophylactic oophorectomy'' and ''prophylactic hysterectomy'' was performed to address this issue.

Timing of Prophylactic OophorectomY

Prophylactic oophorectomy has been recommended in women known to have BRCA1 or BRCA2 mutations once they have completed childbearing.[4,5] The ideal age to perform this surgery has not been identified. Some authorities have recommended that the best opportunity for achieving a reduction in ovarian cancer risk and prolonging life is to perform the surgery as close to 30 years of age as possible.[15] One recent study reported that the mean age of women with BRCA1 or BRCA2 mutations who underwent surveillance only and subsequently developed ovarian cancer was 50.3 years, suggesting that at least half of the patients would still have benefited from a prophylactic oophorectomy had they waited until their age was well into the late forties before they underwent the procedure.[1] However, another study reported that 70 percent of the women with a BRCA mutation had developed hereditary breast cancer when prophylactic oophorectomy was deferred until 47.5 years of age.[4] If prophylactic oophorectomy is beneficial in reducing breast cancer risk, an earlier age for this surgery is necessary.

The reason for delaying prophylactic oophorectomies in premenopausal women is related to the well-documented increased risk for cardiovascular events and osteoporosis associated with an early menopause and hot flashes, sexual dysfunction and cognitive changes associated with the menopause.[4,5] Some centers have routinely recommended administering hormone replacement therapy (HRT) following prophylactic oophorectomy until the patient reaches age 50.[4,5] By combining progestins with estrogen one can decrease the risk of endometrial cancer in the retained uterus. HRT in some series has not had an impact on the beneficial effect of prophylactic oophorectomy in patients with BRCA1 or BRCA2 mutations.[4,5]

The recently reported Women's Health Initiative (WHI) study revealed that women who take estrogen and progestin in combination have a statistically increased risk for the development of breast cancer.[16] Although the breast cancer risk for any one individual in that study was small (<1/1000), it was a statistically measurable risk that has frightened many American women into not taking HRT. Nevertheless, in the second of the two groups of patients

participating in the WHI study, i.e. women who previously had a hysterectomy and were randomized to either estrogen or placebo, there has been no reported increased risk for breast cancer in the group taking estrogen only. Removing the uterus at the time of prophylactic oophorectomy would allow the patient to take estrogen only postoperatively and avoid any deleterious effects due to the progestin.

Fallopian tube cancer in BRCA1/2 carriers

Recent studies have suggested that there is an increased risk for fallopian tube carcinoma associated with BRCA1/2 mutations.[7-10] In a genetic, epidemiological study of 44 women with fallopian tube cancer, 5 cancers were associated with BRCA1 germline mutations and 2 were associated with BRCA2 mutations.[8] The sites of these occult fallopian tube cancers have been reported to be in the distal end of the fallopian tube. When one performs a prophylactic oophorectomy only, the interstitial part of the fallopian tube is left *in vivo*. Performing a total hysterectomy at the time of the prophylactic oophorectomy will remove the entire fallopian tube.

Uterine serous cancer in BRCA1/2 carriers

The association of uterine cancers in women with germline mutations of the BRCA1/2 genes has been anecdotal. Recently a series of 20 Ashkenazi Jewish women with uterine serous cancer have been reported, four of whom had BRCA1 mutations.[14] Nine of the 20 had breast cancer diagnosed prior to the uterine serous cancer diagnosis. Serous cancers of the uterus tend to occur in older age women and tend to be hormonally insensitive tumors.[17] Their management is palliative when found in an advanced stage, but can be curative when identified in an early stage.

Tamoxifen is routinely recommended in women with breast cancer to prevent the development of cancer in the contralateral breast and to prolong the progression-free interval.[18] Tamoxifen has been shown to reduce the incidence of contralateral breast cancer in women with BRCA1/2 germline mutations.[19] Tamoxifen has been associated with both low grade and highly curable endometrioid adenocarcinomas of the uterus as well as with serous cancers of the uterus.[18,20] Nevertheless, some authors advocate prophylactic oophorectomy only and tamoxifen in BRCA1/2 positive breast cancer patients who do not elect to have prophylactic mastectomies.[21] Performing a hysterectomy at the time of prophylactic oophorectomy in breast cancer patients receiving tamoxifen who have a germline BRCA1/2 mutation would seem extremely prudent!

Should a hysterectomy be routinely performed in women who undergo prophylactic oophorectomy?

The identification of fallopian tube cancers in association with women who carry these BRCA1/2 germline mutations would strongly argue for the routine removal of the entire fallopian tube including the interstitial part of the tube when a patient undergoes prophylactic oophorectomy. This can be best accomplished by performing a hysterectomy. The removal of uterus would also remove the potential for developing a serous cancer of the uterus, particularly in women who already have been diagnosed to have breast cancer.

The majority of studies of women who have undergone prophylactic oophorectomy appear to include women at extremely high risk for developing ovarian cancer. In one study, 69 of 98 (70%)of the participants who underwent prophylactic oophorectomy had already experienced breast cancer as did similar numbers (45 of 72, 62%)of patients in the surveillance group.[4] In another study, 200 of 551 (36.3%) patients known to have BRCA1 or BRCA2 mutations had previously been diagnosed to have breast cancer.[5] Most physicians encounter patients who do not have family cancer histories comparable to those included in the latter studies. Alternative strategies to prophylactic oophorectomy, including the use of oral contraceptives and tubal ligations, can be employed for lower risk women who do have BRCA1 or BRCA2 mutations.[22] However, for those women who have experienced breast cancer at a young age, prophylactic oophorectomy in combination with a hysterectomy gives the best protection against experiencing additional gynecologic cancers and will allow patients to take estrogen replacement therapy.[4,5]

Should a prophylactic omentectomy be performed at the time of prophylactic oophorectomy?

Serous cancers of the peritoneum, i.e. primary peritoneal carcinomas, do occur in women who have BRCA1 and BRCA2 germline mutations. Indeed, one early ovarian cancer detection program study reported that 7 of the 10 so-called ''ovarian cancers'' detected were primary peritoneal cancers.[13] Three of four primary peritoneal cancers tested were associated with BRCA1 mutations. These cancers cannot be recognized early using detection techniques such as serum tumor markers and endovaginal ultrasounds and have been reported to occur after a prophylactic oophorectomy was performed.[12] The incidence of BRCA1/2 germline mutations in 68 Ashkenazi Jewish women with primary peritoneal cancers in a population based epidemiologic study was 28% compared to a 30% mutation rate among Stage III and IV ovarian cancer patients.[12]

Serous cancers of the peritoneum may be recognized in an advanced stage

by a CT scan revealing the presence of ascites, in association with the dominant mass being in the omentum and the ovaries not being obviously involved by cancer. Since the dominant mass is routinely the omentum in this disease, one could argue for prophylactic omentectomy at the time of a prophylactic bilateral salpingo-oophorectomy and hysterectomy. This may provide additional prophylaxis of another site at risk for malignant change in women with BRCA1/2 mutations. The benefits of such a surgical approach are theoretical only. The incidence of serous cancers of the peritoneum in patients with BRCA1/2 germ line mutations remains unknown. Short follow-up in many studies limits our opportunity to know how great a risk a patient truly is for a primary peritoneal cancer and who among the BRCA1 and BRCA2 germline mutation carriers is at high risk for this disease. Of course, one should routinely perform peritoneal washings at the time of prophylactic oophorectomy to identify malignant cells that might establish the presence of an occult primary peritoneal cancer.

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Second trimester miscarriage and delivery of a viable child — a controverse combination in delayed interval delivery

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Summary

Since 1991 we have performed delayed interval delivery in 30 twin and 7 triplet pregnancies.

Management of all procedures followed a protocol consisting of 4 periodes.

Gestational age of delivery of the first multiplet ranged between 16 and 31 weeks. Among twin pregnancies, there were 17/30, among triplet pregnancies there were 3/7 cases with a gestational age of < 25 weeks at the delivery of the first multiplet with a mean of 24.7 in the total group of twin and of 24.8 in the total group of triplet pregnancies. The mean interval between delivery of the first and second twin was 19 (range: 1 to 77) days, it was 11 (range: 2 to 34) days between the delivery of the first and second triplet and 3 (range:0-13) days between the delivery of the second and the third triplet. Perinatal mortality of all first born twins was 21/30 (70%), of first born triplets 5/7 (71%) and even 100% when the first-born multiplet was born before 26 gestational weeks. Perinatal mortality of the second twin was 8/30 (26.7%) in the total group and but only 3/21 (14.3%) if born after 26 weeks. However, mortality of the second and third triplet was 11/14 (78.6%).

In conclusion, delaying the delivery of a second or third infant has a positive effect on the short term outcome more evident for twin compared to triplet pregnancies.

Introduction

Up to now, many strategies have been attempted but failed to prevent premature birth in multiple pregnancies.

Increased maternal age but mainly artificial reproductive technologies have changed the demographics of multifetal pregnancies and thereby the incidence of preterm deliveries and cerebral palsy. The high perinatal mortality and

morbidity associated with multiple pregnancy are mainly due to the high rate of prematurity.[1] After immature or very premature birth of one baby in multiple pregnancies, arrest of labor can occur and be stimulated. In 1880, Carson was the first to report on an interval of 44 days between the birth of two babies from a woman with uterus didelphis and double pregnancy.[2] In 1957, Abrams was the first to report on an interval of 35 days between the delivery of two babies born from one uterus born alive.[3]

Since 1991, we have performed delayed interval delivery in multiple pregnancies complicated by the delivery of the first multiplet between 16 and 32 gestational weeks.

We here report on our findings.

Patients and Methods

Delayed interval delivery was performed in 30 twin and 7 triplet pregnancies.

Management of all procedures followed a protocol consisting of 4 periodes.[4]

Phase 1: After informed consent and exclusion of patients not appropriate for the procedure the management all attempts should be undertaken to prolong the delivery of the first baby (tocolytic treatment, possibly peridural anaesthesia and antibiotics) but to be prepared to an immature or premature delivery. Phase 2: During delivery of the first multiplet antibiotics and tocolytics are continued. Episiotomy is avoided. The corresponding placenta remains in utero.

A cultural swab is taken and the vagina desinfected. The umbilical cord is ligated ear to the cervix. In Rh negative women, anti-D is prescribed. Phase 3: After delivery of the first baby the cervix is monitored by transvaginal ultrasound. Monitoring of the maternal condition focuses on the detection of early signs of infection, premature delivery and abruptio.

Phase 4: After the last delivery, careful examination of the placenta prevents retention of one part of the placenta. Special attention should be paid to the emotional state of the parents, which is ambiguous in the situation when there is one baby dead and one alive.

Results

Gestational age of delivery of the first multiplet ranged between 16 and 31 weeks. Among twin pregnancies, there were 17/30, among triplet pregnancies there were 3/7 cases with a gestational age of < 25 weeks at the delivery of the first multiplet with a mean of 24.7 in the total group of twin and of 24.8 in the total group of triplet pregnancies. The mean interval between delivery of the first

and second twin was 19 (range: 1 to 77) days, it was 11 (range: 2 to 34) days between the delivery of the first and second triplet and 3 (range:0-13) days between the delivery of the second and the third triplet. Perinatal mortality of all first born twins was 21/30 (70%), of first born triplets 5/7 (71%) and even 100% when the first-born multiplet was born before 26 gestational weeks. Perinatal mortality of the second twin was 8/30 (26.7%) in the total group and but only 3/21 (14.3%) if born after 26 weeks. However, mortality of the second and third triplet was 11/14 (78.6%).

The complete results are summarized in Table 1 and 2.

Table 1. *Delayed interval delivery in twin pregnancies: Maternal age, gestational age of delivery, weight, sex, mode of delivery, outcome of the first or second twin, placentation and origin of pregnancy.*

f=female, m=male, V=vaginal, CS= Caesarean section, D= perinatal death, SB= stillborn, A=alive

n	age	delivery	delivery	interval	weight	sex	weight	sex	mode	outcome	placenta	origin
		1	2	days	1	2	1	2				
1	35	16+2	20+2	28	140	m	490	m	V/V	D/D	DC	spontaneous
2	26	19+0	19+5	5	250	v	300	v	V/V	SB/D	DC	ICSI
3	33	19+1	20+4	10	235	m	250	v	V/V	D/D	DC	spontaneous
4	31	20+1	20+2	1	320	v	360	m	V/V	D/D	DC	stimulation
5	37	20+1	21+6	12	300	m	390	m	V/V	D/D	DC	IVF
6	32	20+1	31+4	73	350	v	1890	m	V/V	D/A	DC	IVF
7	36	22+0	26+6	34	410	v	795	v	V/CS	SB/A	DC	spontaneous
8	31	22+0	37+1	106	465	v	2995	v	V/V	D/A	DC	ICSI
9	29	22+5	24+3	12	550	v	620	v	V/V	D/D	MC/DA	IVF
10	27	22+5	26+3	25	310	v	710	m	V/V	D/D	DC	stimulation
11	31	23+1	25+6	19	550	v	740	m	V/V	D/SB	DC	IVF
12	39	24+0	26+2	16	795	m	860	m	V/V	D/A	MC/DA	ICSI
13	23	24+2	26+2	14	615	f	805	v	V/V	D/SB	DC	spontaneous
14	27	24+4	25+1	4	680	m	765	m	V/V	D/A	DC	ICSI
15	26	24+6	25+4	5	670	m	750	v	V/V	D/A	DC	IVF
16	29	24+6	29+6	35	1000	m	1520	f	V/V	D/A	DC	spontaneous
17	28	24+6	27+3	18	690	m	1250	m	V/V	D/A	DC	spontaneous
18	29	26+1	26+4	3	875	m	715	m	V/V	D/D	MCDA	spontaneous
19	29	26+2	26+5	3	780	f	870	f	V/V	D/A	DC	stimulation
20	30	26+2	34+0	54	840	m	2310	f	V/V	A/A	DC	stimulation
21	39	26+3	28+1	12	835	f	1340	f	V/CS	A/A	DC	spontaneous
22	33	26+3	32+3	42	850	m	1690	m	V/CS	A/A	DC	spontaneous
23	35	26+4	30+4	28	950	f	1470	f	V/CS	D/A	DC	IVF
24	28	28+1	30+3	16	865	m	1470	m	V/V	D/A	DC	spontaneous
25	35	28+4	28+6	2	1150	f	1040	f	V/V	A/A	DC	spontaneous
26	31	29+3	29+3		1305	m	1580	m	V/V	A/A	DC	spontaneous
27	26	30+1	32+1	14	1450	m	1550	f	V/V	A/A	DC	spontaneous
28	34	30+2	31+4	9	1220	m	1945	m	V/V	A/A	DC	spontaneous
29	26	30+5	31+2	3	1660	m	1770	m	V/V	A/A	MCDA	spontaneous
30	28	31+0	32+2	9	1271	f	1625	m	V/V	A/A	DC	stimulation

Table 2. *Delayed delivery in triplet pregnancies: Maternal age, gestational age of delivery, weight, sex, outcome of the first, second and third triplet, mode of delivery, placentation and origin of pregnancy.*

f=female, m=male, V=vaginal, CS= Caesarean section, D= perinatal death, SB= stillborn, A=alive

n	age	delivery	delivery	delivery	interval (days)	weight (sex)	weight (sex)	weight (sex)	mode	outcome	placenta	origin
		1	2	3		1	2	3				
1	34	18+6	23+5	23+5	34	220 (m)	520 (f)	580 (m)	V/V/V	D/D/D	TC	stimulation
2	36	24+4	25+3	25+3	6+6	332 (f)	570 (f)	670 (m)	V/V/V	SB/D/D	TC	stimulation
3	31	24+5	25+2	25+2	4	329 (f)	660 (m)	580 (f)	V/V/V	D/D/D	TC	IVF
4	34	25+3	27+0	27+2	11+2	650 (m)	900 (m)	810 (m)	V/V/CS	D/D/D	TC	stimulation
5	27	26+0	26+6	26+6	6	830 (f)	900 (f)	850 (f)	V/V/V	A/A/A	TC	IVF
6	33	26+0	27+6	27+6	13+13	820(f)	1000 (m)	1200 (m)	V/CS/CS	A/A/D	TC	ICSI
7	41	28+0	28+2	28+2	2	675 (m)	870 (f)	900 (f)	V/V/V	D/D/D	TC	stimulation

Neonatal follow-up has been recently evaluated in 17 sets of twins and 3 sets of triplets [5]. The mean delay of 19.6 days accounted for a significant increase in birth weight and neonatal survival as well as a decrease in adverse outcome and number of disease; a decrease in long-term development could not be demonstrated. No serious maternal complications were observed.

Discussion and Conclusions

We have already published a large series of patients with delayed interval delivery.[4] Our results are comparable with the findings of Farkouh et al.[6] en Arias[7] with an approximate success rate of 50%. This success rate suggests that the high success rate of a summary of case reports of more than 90% represents a bias based on the fact that preferably successful results were published.[4] The so-called success criteria are: a postponement of pregnancy between the delivery of the first and the following multiples which can be useful for the administration of corticosteroids, the chances to survive comparing the chances of the first and the following multiples and the consecutive increase of intact survival. Thereby it is difficult to compare the long time morbidity of the second (or third) with the first multiplet due to the high mortality of the first multiples.

Besides of the technical and medical aspects the psychosocial and emotional aspects of delay interval delivery have to be considered.

If multiple pregnancies are complicated by the immature or very premature delivery of the first multiplet it seems worth while to try whether delay interval delivery may improve the outcome of the second or third fetuses. The fact that the parents are possibly simultaneously confronted with the delivery of an

immature (dead) or a very premature delivery of the first multiplet and the delivery of an older child has to be considered during the informed consent process and in dealing with these patients.

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Cervical length and funneling — false positive and false negative observations

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Summary

Transvaginal sonography (TVS) was performed longitudinally in singleton, twin and triplet pregnancies in a supine and upright position. An opening of the internal os was more pronounced in multiple compared to singleton pregnancies and earlier and more frequently observed in an upright position. In patients with preterm birth an opening of the internal os in an upright position combined with cervico-vaginal fibronectin was comparable to the cervical length to predict prematurity.

In risk populations, we emphasize longitudinal examinations by TVS to increase the chance to recognize symptoms in a reversible instead of an irreversible phase and to evaluate whether maternal postural challenge assists in predicting patients at risk for premature delivery.

Introduction

The continuing high perinatal mortality and morbidity associated with prematurity is a legitimate reason to reflect about early detection and prevention of spontaneous preterm birth (SPB). Cervical shortening as seen by transvaginal sonography (TVS), increased concentrations of maternal salivary estriol, inflammatory cytokines in amniotic fluid, fetal fibronectin expression in cervicovaginal mucus are all detected weeks to months before a delivery.

The recurrence rate of SPB is estimated to be 15% and depends on the number of premature deliveries but not on the gestational age of the previous SPB.[1] In twin pregnancies, there is a 6 to 8 fold risk to deliver prematurely. Up to now, there are no guidelines how to diagnose the risk in asymptomatic non-risk and not even in risk-groups. In addition, preventive measures are not standardized. With the exception of France, population-based studies and scoring systems have up to now failed to reduce the rate of SPB though it was proven that a systematic approach may reduce general costs.[2] Currently, it is

still common practice to wait until clinical symptoms such as uterine contractility, bulging or even premature ruptured membranes (PROM) are unpredictably" present often at an irreversible stage although the condition might have been earlier apparent.

Cervical incompetence is traditionally considered a cause of (recurrent) midtrimester abortion. Ultrasonographic cervical assessment during the course of pregnancy has suggested that there is a wide spectrum of the disease. Preterm ripening may be a result of a congenital disorder of the connective tissue, exposure to diethylstilbestrol in utero, traumatic damage to the structural integrity, uterine overdistension, repetitive bleeding or vascular lesions in the placenta leading to membrane destabilization and finally local or intrauterine infection inducing an increase of cytokines and prostaglandines[3]. This may lead to a condition in which there is activation of all components of the common terminal pathway with uterine contractility, membrane activation and cervical incompetence.[4]

Based on digital examination, Gream reported in 1865, that a soft cervix was a cause of premature labor.[5] One of the largest studies examining the relationship between cervical ripening and prematurity was conducted by Papiernik et al. in a high risk population of more than 8000 pregnant women.[6] Dilatation of the internal os was the most significant risk factor for SPB. Bishop defined features of the cervix by a semiquantitative tool.[7] However, routine pelvic examination was not shown to be predictive of SPB in a large European study comparing routine digital cervical examination versus no examination at each prenatal visit.[8]

Currently, it is accepted that transvaginal sonography (TVS) of the cervix is better than digital examination as a predictor of the likelihood of SPB.[9] It has the advantage that a shortening of the cervix and the opening of the internal os can be reliably diagnosed.

Patients and Methods

In our study group, the probe was introduced into the anterior vaginal fornix, the sonographic picture controlled in a sagittal view until the endocervical canal was visualized between external and internal os and then retracted to avoid compression of the cervix. The length of the closed portion of the endocervical canal is detected whereby the thickness of anterior and posterior cervix appear to be equally thick. The distance between internal and external os is not always a straight but in around 50% a curved line.[10] In patients with a short cervix <15 mm, no curvature was observed any longer, the ratio of a curved to a straight cervix decreases with decreasing length. Therefore, the disparity of a

curved or straight cervix does not seem to have essential implications. After serial measurements the shortest result are considered.

The cervical length, width and form of the external or internal os (funneling) and the position of the cervix in relation to a horizontal line were determined from ultrasound pictures.

Up to now, most physicians perform their examinations with the women in a supine position.

Maternal postural challenge has been performed by only a few groups.[11-15] W have conducted nearly every transvaginal sonographic examination in both a supine and an upright position whereby the patient is asked to place one foot on a footstool and to guide the transvaginal transducer into the lower part of the vagina until it can be directed by the examiner vertically until the cervical canal is visualized. The difference in length and width of the opening of the internal os seems to increase in patients with advanced gestational age, multiple pregnancy or in patients at risk for SPB. Thereby the width of the internal os may increase or in cases with cervical dilatation membranes may progressively prolapse. In severe cases, the dynamic changes in an upright position may even be documented on video tape or serial measurements. The risk of postural stress for the pregnant cervix is easily visible for the patient at the ultrasound screen. This may be useful if maternal lifestyle changes seem appropriate. Vice versa insignificant changes may motivate the patient to lead a normal life or the physician to avoid unnecessary interventions.

Results

A. Normal values in supine and upright positions for singleton and twin pregnancies

.Endocervical cervical length at 23 weeks shows a normal distribution with values of < 15mm in 1.7%, the differences based on parity have no essential implications.[16] Up to now, most reference values of the cervix are based on measurements of the cervical length in a semilying position.

In contrast to the digital examination, TVS allows to detect that the opening of the internal os is combined with a shortening of the closed endocervical canal length. In a pilot study, we could demonstrate that this is most evident in an upright position of mothers with twins.[15] In a larger data set we found that cervical length and width of the internal os are significantly different in singleton and twin pregnancies from around 25 weeks onwards. It is assumed that the pathophysiology of premature ripening of the cervix and premature labor may be different between singleton and multiple pregnancy. Therefore we have collected data of the cervical length, width of the internal os and a presumed angle with a horizontal line reflecting the degree of curvature

separate for singleton, twin and triplet pregnancies throughout pregnancy. Gestational age was determined by the last menstrual period or by early ultrasonography if the sonographic estimate disagreed with menstrual dating by > 1 week.

TVS was performed from 15 weeks onwards at 5 weeks' intervals in singleton, twin and triplet pregnancies in both a supine and upright maternal position. Thereby the cervical length, the width of the internal os and the angle of the cervical canal with a horizontal line were determined. Retrospectively, cases with premature birth < 36 weeks and all interventions to prevent premature labor were excluded.

Cervical length decreased significantly from 15 weeks to term in both a supine and upright position and in both singleton and multiple gestation. Values between the two positions were significantly different from 20 weeks onwards ($p<0.001$). In a standing position, significant funneling was observed in normal singleton pregnancy with a delivery > 36 weeks, in normal twin pregnancies from 30 weeks onwards and in triplet pregnancies from 20 weeks onwards. Differences in funneling between the supine and upright position were significant from 30 weeks in normal singleton and from 25 weeks in multiple gestation.

The presumed angle with a horizontal line taken from ultrasound pictures decreases significantly in both positions ($p<0.001$). Differences of the angle between both positions were not significant.

Our data have been published in more detail[17, 18].

B. Prediction of spontaneous preterm birth and use of TVS in high risk pregnancies

From 1997 onwards, singleton pregnancies with a history of SPB or other risk factors and twin pregnancies were followed by TVS in a supine and upright position from 15 weeks onwards and by tocography, fibronectin, digital examination and cervical culture. In these patients, interventions (e.g. reduction of physical stress, the application of a cervix pessary) were indicated if results of the TVS were outside the 50% normal values of the Box Whisker plots. The diagnostic values were calculated by ROC analysis and multivariate regression for examinations obtained between 20 and 24+6 and between 25 and 29+6 gestational weeks. Independent variables included age, parity, history of preterm delivery, voluntary abortion, miscarriage, conization, uterine malformations, the registration of contractions, the Bishop score, bacterial swab, cervicovaginal fibronectin after 24 weeks, the funnel width and cervical length both in a supine and upright position. Multiple logistic regression revealed that in both considered intervals funnel width and cervical length in an upright position

and the detection of fibronectin were the most significant independent variables to diagnose subsequent preterm birth in singleton and multiple pregnancies.

Sensitivity, specificity, positive and negative predictive values are summarized in Table 1 separate for singleton and twin pregnancies.

Table 1 a) and b): *Diagnostic value of the most significant combination to determine the risk of spontaneous onset of preterm birth (multivariate regression): Funnel width in a standing position (ST) without or with fibronectin, determined at different intervals during pregnancy*
a) in 102 singleton pregnancies at risk for preterm birth
b) in 106 twin pregnancies

Table 1a)

Parameter	Sensitivity	Specificity	Positive	Negative
			predictive value	
Funnel width ST 20-24+6 weeks	38,5%	88,7%	29,4%	92,1%
Funnel width ST +Fibronectin 20-24+6 weeks	61,5%	92,4%	50,0%	95,1%
Funnel width ST 25-29+6 weeks	58,3%	86,8%	33,3%	94,8%
Funnel width ST 25-29+6weeks +Fibronectin	66,7%	87,7%	38,1%	95,9%

Table 1b)

Parameter	Sensitivity	Specificity	Positive	Negative
			predictive value	
Funnel width ST 20-24+6 weeks	44,4%	84,3%	33,3%	89,6%
Funnel width ST 20-24+6 weeks + Fibronectin	61,1%	79,4%	34,4%	92,0%
Funnel width ST 25-29+6 weeks	76,2%	77,5%	36,1%	94,0%
Funnel width ST 25-29+6 weeks +Fibronectin	66,7%	77,5%	34,3%	92,9%

Using historic controls, the rate of SPB decreased in both singleton and multiple pregnancies with the application of early longitudinal TVS, including information to the patient and an increased indication of a vaginal pessary.

In patients with early progressive dilatation but intact membranes TVS is useful to detect the degree of protrusion (''ballooning''), the position of the fetus and the umbilical cord. As the pathogenetic reason is difficult to detect in a progressive state this is still decided on an individual basis. We have managed pregnancies even with fully dilatation but intact membranes <32 weeks expectantly if there were no signs of infection and/or fetal demise. In up to now 32 patients with ballooning < 28 weeks at admission, we could prolong the interval from admission to delivery for two weeks (mean) and a maximum of up to 4 weeks.

Discussion and Conclusions

In the future, obstetricians should be stimulated to care for evidence based data directed to evaluate how to reduce risks for SPB by ''non-invasive'' methods such as patient education as to the symptoms of premature labor, reduction of physical activities, TVS at regular intervals and mainly interventions based on results from TVS. Collaborative studies are needed to define and standardize the outcome parameters not only as gestational age at delivery but also as morbidity, mortality, and long-term follow-up. To reduce anxieties of women and improve the outcome we would recommend to restrict pre-and perinatal care to specially trained staff.

Transvaginal sonography should be incorporated in the complex care of multiple gestations. The obstetrician in charge of management decisions should be capable to perform and interpret the results and follow a protocol designed to reduce poor outcome and to critically evaluate own results. To our opinion, longitudinal measurements by TVS with postural stress should be routine within risk pregnancies. In how far this would also hold true for screening populations cannot yet be concluded from our data.

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Can Regular Exercise Affect Fetoplacental Growth?

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Summary

Maternal exercise during pregnancy can profoundly affect fetoplacental growth. During exercise there is an acute reduction in nutrient delivery to the placental site. In trained women however, the additional training effects of regular exercise on cardiac output, blood volume, blood pressure, placental blood flow at rest and placental functional capacity buffer the acute reduction during exercise and probably increase the absolute 24 hour delivery of nutrient to the placental site. Thus, the effect of maternal exercise on fetal growth and size at birth is dependent on previous maternal physical conditioning, the type, frequency, intensity and duration of her exercise regimen as well as the time point in the pregnancy when the exercise is performed and the quality of maternal nutrient intake. Needless to say this has both preventive and therapeutic clinical implications.

Introduction

The primary maternal factor which regulates fetoplacental growth is substrate delivery to the placental site which can be calculated as the product of the rate of placental blood flow and nutrient level in the maternal compartment, the best studied of which is glucose.(1) Thus, factors which increase placental blood flow and/or maternal glucose levels stimulate fetal growth while those which decrease either or both suppress it. The overall effect is modulated by the placenta using a stimulus-response feedback mechanism involving release of insulin-like growth factors and their binding proteins.(1,2) Examples of this mechanism include hyperglycemia in pregnancy and the effects of processed carbohydrate on maternal blood glucose levels and size at birth.(1,3) Many other lifestyle factors, including recreational exercise, also influence placental blood flow and/or maternal glucose levels.

Sustained bouts of maternal exercise have both short- and long-term effects on placental blood flow and maternal glucose levels. During exercise visceral flows fall dramatically because of the competing needs of exercising muscle and the magnitude of the fall is directly related to exercise intensity.(4,5) During pregnancy, exercise also acutely decreases maternal blood sugar levels and the magnitude and duration of the decrease increases with advancing gestation and is proportional to the duration and intensity of the exercise and prevailing insulin levels.(2,6)

However, repetitive, sustained bouts of maternal exercise enhance many of the maternal physiological adaptations to pregnancy such as blood volume expansion, intervillous space blood volume, increased cardiac output and mid-trimester placental growth and function.(7-11) These long-term adaptations to both regular exercise and pregnancy appear to increase placental blood flow and enhance maternal-fetal transfer both at rest and during exercise especially in late pregnancy.(2,5) Thus, the interaction amongst exercise habits, nutrition, other lifestyle factors, and fetoplacental growth is complex and appears to be largely dependent on their integrative effect on maternal average 24 hour blood glucose and average 24 hour placental blood flow.

There fore we undertook a series of studies designed to quantitate the effects of maternal exercise volume at different time points in pregnancy on fetoplacental growth using either observational or randomized experimental designs.

Materials and Methods

All subjects were enrolled and assessed prior to pregnancy. All were non-smokers, free of coexistent disease and had stable lifestyles. The exercise regimens were monitored carefully in the observational studies(13,14) and, in the randomized ones,(1,8,12) exercise type, intensity and frequency were standardized and the duration of each session was varied. Nutritional information was collected at regular intervals in the all studies and strictly controlled in one.1 To date, over 600 women have been studied.

In every case detailed placental and neonatal morphometrics were obtained at the time of birth and, in most instances, mid-trimester placental volumes were assessed ultrasonically.(9)

Results

The initial observational studies were conducted in well-conditioned women who continued to exercise regularly during pregnancy.13,14 Exercise type, frequency duration and intensity varied widely but the 4 main findings were

consistent. First, fetoplacental growth was not affected by non-weight-bearing or intermittent types of exercise (cycling, swimming, weight lifting). These findings concurred with those previously reported by many other investigators.(2,14) Second, all weight-bearing types of exercise (running, power walking, aerobics, cross-country skiing) stimulated placental growth and anatomic indices of placental functional capacity at term.(9,10) Third, the magnitude of the effect of weight-bearing exercise on size at birth was directly related to the ratio of the subject's overall exercise performance in late pregnancy to her overall exercise performance prior to pregnancy. The greater this ratio the lighter the infant but there was no increase in the incidence of birth weights under 2500 grams. The average birth weight decrement in these offspring was approximately 300 grams. Fourth, the exercise effects on size at birth were primarily due to a reduction in neonatal fat mass not lean body mass and axial growth was unaffected.

The first randomized study examined the effects of beginning a regular weight-bearing exercise regimen for the first time at 8 weeks gestation and continuing it throughout pregnancy.(8) The women randomized to the exercise group exercised 3-5 times a week for 20 minutes at an intensity equal to 55-60% of their maximal aerobic capacity while those randomized to the control group did no regular sustained exercise until the postpartum period. The women who were randomized to the exercise group were delivered of symmetrically larger infants whose birth weights averaged about 250 grams higher than that of the controls. In addition, their mid-trimester placental volumes grew faster (26 versus 21 cc/week), were larger at birth (462 cc versus 421 cc) and had histomorphometric evidence of improved functional capacity.

The second randomized study examined the added effect of dietary carbohydrate in a group of women on the same exercise regimen.1 Briefly, 10 women were randomized at 8 weeks gestation to a diet containing 50-55% of its calories as complex or low-glycemic carbohydrate (fresh vegetables, fruits, nuts, whole grains and dairy) and 10 to a diet containing 50-55% of its calories as processed or high-glycemic carbohydrate (breads, cakes, root vegetables. well-cooked pasta, white rice, snack foods and desserts). These dietary differences altered maternal blood glucose levels and size at bith dramatically. The women who ate the high-glycemic diets were delivered of overgrown infants (4.17 ± 0.12 versus 3.33 ± 0.11 kg) who were longer and fatter with larger head circumferences and much larger placentas (551 ± 42 cc versus 391 ± 22 cc), that grew faster in mid gestation. Maternal weight-gain was also excessive in this group (18.6 ± 1.1 kg).

The third randomized study examined the effects of continuing different patterns of weight-bearing exercise during pregnancy.(12) The women all ate a mixed-glycemic diet and were randomized at 8 weeks gestation to one of 3

exercise regimens: 20 minutes 5 times a week in early pregnancy increasing to 60 minutes 5 times a week in late pregnancy, 60 minutes 5 days a week in early pregnancy decreasing to 20 minutes 5 times a week in late pregnancy, or 40 minutes 5 times a week throughout. Briefly, those who decreased their exercise in late pregnancy had larger offspring and placentas than either of the other two groups and the women who increased their exercise in late pregnancy gained less weight and had the leanest and lightest offspring.

Conclusions

Physical activity and diet can have major effects on fetoplacental growth. The important maternal variable which appears to modulate growth rate through placental mechanisms is average 24 hour nutrient (glucose) delivery to the placental site. During pregnancy, sustained exercise causes a short-term reduction in nutrient delivery but the long-term physiological effects appear to increase it. These opposite effects are also modified by the type of carbohydrate in the maternal diet as well as other lifestyle variables. In women who begin and maintain a moderate exercise regimen during pregnancy the balance between the acute and chronic effects favors increased nutrient delivery which increases fetal growth. This effect is markedly enhanced by a high-glycemic diet and suppressed by a low-glycemic one. Continuing a more rigorous exercise program throughout pregnancy has the same effect on placental growth but suppresses fetal growth especially in late pregnancy. Again, the magnitude of these effects is modified by maternal diet and/or a marked increase or decrease in exercise in late pregnancy.

The magnitude and consistency of these differences suggest that modification of an individual's physical activity and carbohydrate intake either before or during pregnancy may have clical preventive or therapeudic value.

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Does three-dimensional ultrasound contribute significantly to anomaly detection?

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Abstract

Three-dimensional sonography tremendously improved prenatal assessment of fetal morphology. The object of interest is assessed in almost unlimited choice of scanning planes. Different modes of image rendering and storage of the scanned volumes enable close follow-up. Without loosing any information stored volumes can be reassessed in the future. In this article we present and discuss our experience in prenatal diagnosis by the three-dimensional sonography.

Introduction

Tremendous advances in high resolution sonography have increased our knowledge of the normal fetal development and improved prenatal diagnosis of a great number of complex fetal anomalies. Close follow-up by serial sonography expanded our understanding of the natural history of a number of these disorders. Although being very accurate, due to the anatomical reasons, two-dimensional (2-D) sonography is still limited by a rather restricted number of planes in which the object of interest can be depicted. Three-dimensional (3-D) sonography revolutionized ultrasound imaging by the capability of depiction of the unlimited number of planes in which the object of interest can be displayed. Main advantages of three-dimensional ultrasound in perinatal medicine and antenatal diagnosis include scanning in the coronal plane, improved assessment of complex anatomic structures, surface analysis of minor defects, volumetric measuring of organs, "plastic" transparent imaging of fetal skeleton, spatial presentation of blood flow arborization and finally, storage of scanned volumes and images (1-8). An additional progress is achieved owing to the permanent possibility of repeated analysis of previously saved three-

dimensional volumes with additional elimination of redundant structures and artefacts (3,10-13).

Patients and methods

The examined patients were selected from our routine outpatient clinic or send for supervision from other clinics because of a suspected fetal anomaly. The ultrasound devices were Combison 530D and Voluson 530D MT (Kretztechnik, Zipf, Austria) with a 3-5 MHz annular array transabdominal transducer for three-dimensional volume scanning. The Voluson 530D MT was connected to a PC supplied with a 3-D VIEW^{TM} (Volume Imaging Evaluation Workplace, Kretztechnik-Medison) program. This program was integrated in the last version of Voluson 530D MT. It enables precise off-line two and three-dimensional (VOCAL^{TM}) measurements in manual or automatic mode.

Results

Two-hundred-forty-seven patients were send to supervision by 3-D sonography because fetal anomaly was suspected or the examiner did not succeed to clearly visualise fetal anatomy. Seven fetuses had multiple anomalies. For the purpose of this study we took each anomaly separately. Results are presented in Table 1

Discussion

Various studies have already shown that three-dimensional ultrasound can detect or exclude not only major anomalies, but particularly subtle fetal abnormalities. Besides impressive demonstrating of normal fetal structures, 3-D ultrasonography is adding "new window" to the diagnosis of fetal malformations. During the second and third trimester, 3-D sonography makes possible a completely new way of visual perception of unborn baby. Reconstruction and sculpture like images, generated from surface rendering mode, are the most impressive presentations (14-19). Fetal surface abnormalities can be selectively visualized, and the extent of a defect can be determined in all spatial dimensions.

Head and neck malformations

Three-dimensional sonographic evaluation of the neurocranium clearly demonstrate major anomalies, such as anencephaly or hydrocephaly (19,20). Spatial reconstruction of intracranial contents offers ‘‘plastic’’ anatomic and topographic data about ventricle enlargement and consecutive brain tissue damage. It can also define the extent of an encephalocele (17,19). Dysmorphic

appearance of fetus with anencephaly or acrania can be understood much better presenting the fetal head and neck in three-dimensional volume scanning(20).

Three-dimensional surface mode provides clear imaging of all structures previously mentioned, particularly if associated with appropriate amount of the amniotic fluid in front of the fetal face (10,14,18,19,21,22). Merz and colleagues performed 3-D ultrasound examinations in more than two hundred patients with fetal malformations found by conventional two dimensional ultrasound (23). Facial dysmorphias and facial clefts were better seen with 3-D ultrasound. This particularly applies to chromosomal disorders and syndromes associated with subtle facial abnormalities requiring detailed sonographic evaluation (24). Rare morphological anomalies such as single nostril, flat nose, proboscis, cyclopia, hyper or hypotelorism can be easily depicted and diagnosed combining different modalities of 3-D ultrasound imaging techniques (14,23-29).

Lateral head abnormalities such as auricular deformities and low-set ears can also be detected (18-20,30). It is generally agreed that anomalous shape or size of fetal ears is associated with a number of known morphological and chromosomal syndromes. A recognition of a congenital anomaly of fetal ear in utero is generally difficult, possibly due to the complex shape of the ear and the inherent characteristics of conventional 2-D ultrasound. Three-dimensional surface-imaging of fetal ear offers complete analysis of the details related to phenotypic expression of some inherited syndromes (31).

In the neck region, 3-D transvaginal sonography can clearly demonstrate early changes, such as early nuchal translucency (32). Transabdominal scanning can detect later changes: larger cystic hygromas, occipital cephalocele, thyroid tumors etc.(33).

Abdomen and thorax

Three-dimensional surface rendering in fetuses with dorsal cleft anomalies permits an accurate surface analysis that can clearly differentiate level and extent of protrusive lesions (14,19). In fetuses with ventral body clefts 3-D ultrasound offers new capabilities for visualization of the defect and prolapsed organs (14,17,19). Although most of these defects are large and well depicted by 2-D sonograms, the rotating display enables the defect to be viewed from multiple angles and often provides better impression of severity of the anomaly. Even the structural changes of fetal skin surface can be evaluated, emphasizing the possibilities of visual demonstration of congenital ichtyosis (34).

Extremities and skeleton

Surface rendering in 3-D sonography gives clear display of normal and abnormal extremities (14,19,35-37). Using these techniques it is possible to assess.

Clubfoot, reversible or irreversible pathological angulation of the normal anatomical axis and other limb abnormalities are easy to define using available orientation (19). Three-dimensional imaging is very helpful in assessing the precise topographic relationship among three segments of each limb, but also of the wrist, hand and fingers. Significant disproportion and reduction of extremities associated with skeletal dysplasias can be clearly appreciated in the rotating volume display (38). With 3-D ultrasound, fingers are also very well observed. It is thus useful for detecting polydactyly, syndactyly and overlapping fingers. Anomalies of the hands and feet should be looked for in screening for chromosomal defects.

Particular importance is related to the visualisation of malformations and deformationsof fetal skeleton by volume rendering using transparent mode, maximum mode and ''x-ray-like'' imaging (39,40). The most impressive transparent mode reconstruction will result in complete skeletal ''babygram'' (18,19,40).

Cardiovascular system

The heart is poorly displayed by 3-D ultrasound, owing to its motion. However, there are some reports of its use in the fetal cardiovascular system (41-45). It is to be expected that the evolution of fast computing processors will enable real-time 3-D (real 4-D) echocardiography in the near furure.

Fetal tumors

One of the most impressive patterns of 3-D ultrasonography is surface rendering of fetal tumors. Three-dimensional ultrasonography provides accurate and quick detection, associated with instructive visual imaging. Cystic hygroma and sacrococcygeal teratoma are the most frequent fetal tumors easily recognisable by 3-D surface mode (17,19,20).

Parents with malformed fetuses are provided with clear ''photographic'' images of the baby, sonographer can evaluate the extent malformation at the different angles, giving a clear ''plastic'' impression of the shape and severity of the defect to the parents (46).

Placenta and umbilical cord

Three-dimensional color and power Doppler sonography easily depicts spatial position and morphology of the umbilical cord (47). Due to irregular looping of the umbilical chord 2-D sonography depicts only segments of the chord passing the imaging plane.

Instead of the conclusion we will try to summarize advantages and disadvantages of the 3-D sonography.

Main advantages are:

- Capability of displaying unlimited planes of the object of interest (multiplanar mode). This enables accurate measurements and visualization of "hidden" structures.
- Different rendering modalities emphasize investigated structures improving the accuracy of the diagnosis and facilitate understanding of spatial relationships within the investigated area.
- Storage of the acquired volume decreases patients' exposition to the ultrasound energy and enables off-line retrospective analysis without loosing any of the initial information acquired.

Disadvantages of the 3-D sonography:

- Fetal motions produce artifacts that in many cases make the investigation impossible.
- Analysis of the acquired volume is time consuming.
- As in 2-D sonography, oligohydramnios make the examination difficult and, sometimes, inaccurate.
- 3-D sonography requires highly trained personnel and substantial hardware.

Table1: *Suspected diagnosis obtained by two modes of sonography compared to the perinatal outcome*

Suspected anomaly	2-D findings[1]	3-D findings[2]	Outcome[3]
Acrania/anencephaly	3	3	3
Hydrocephalus / ventriculomegaly	54	43	43
Choloprosencephaly	1	1	1
Neural tube defect (spine)	102	23	23
Cleft lip/palate	15	10	11
Cystic hygroma	8	8	8
Diaphragmatic hernia	3	3	3
Gastroschisis	4	4	4
Omphalocoele	3	4	4
Urinary tract anomalies	54	49	49
Dilated bowels	1	0	0
Ovarian cysts	0	1	1
Club-foot	25	15	17
Other limb anomalies	3	3	3
Total	**276**	**167**	**170**

Legend: [1] — initial diagnosis suspected by 2-D sonography at entry in the study; [2] — diagnosis determined after examination by 3-D sonography; [3] — final diagnosis after delivery or termination of pregnancy

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The ethics of first trimester screening.

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Introduction

Some pregnancy screening is meant to treat or prevent serious fetal or maternal disease such as anaemia, rhesus immunisation, or hepatitis. Programmes like this introduce no special ethical issues and will not be discussed further here.

Other screening, in contrast, is both designed to prevent the birth of abnormal babies by abortion and risks causing some normal pregnancies to miscarry. The ethical issues raised by such programmes are the subject of this paper.

I will discuss them in three parts, the ethics of abortion in general, the ethics of selective abortion of abnormal babies, and the ethics of screening for abnormal babies with the aim of aborting them. I will draw a clear distinction between screening as the private choice of the parents, and state funded screening programmes.

The ethics of abortion

Most people's first intuition is that abortion is morally wrong. If killing adult humans is wrong, killing an unborn fetus is particularly wrong, because the child is both innocent and defenceless. Freedom-to-choose arguments seem lightweight in comparison. Philosophers commonly state the case thus:

1. Killing innocent people is wrong.
2. The fetus is a person.
   Therefore — the fetus should not be killed/aborted.
   BUT
3. People should be allowed to do as they like with their own bodies.
4. The mother is a person.

Therefore — the mother should be allowed to empty her uterus and have the abortion.

5. Where one person's right not to be unjustly killed conflicts with another person's right to do as they wish with their own bodies, the right not to be unjustly killed takes precedence.

Therefore — Abortion is wrong.

Personhood arguments

Defenders of the morality of abortion usually criticise point 2. Although the fetus is undoubtedly a member of the species homo sapiens, it is claimed that it is not a person in the sense of an individual who may not be unjustly killed. It is wrong to kill people because they are conscious, and value their lives. The fetus differs in morally important ways. It is not self-aware, and is deprived of nothing by being killed. It therefore fails the test of personhood. Analogies with animals and other beings are used to buttress the argument. If we are justified in killing lower animals to allow us to eat meat, wear leather shoes, or for sport, why should we not kill fetuses for the more serious reasons of preventing the birth of a handicapped child or forcing a woman to bear an unwanted pregnancy? To permit the killing of animals, but to save fetuses, would involve discrimination according to a morally irrelevant criterion, membership of a species. Some people have coined the word "speciesism", analogous to "racism" and "sexism" for this sort of discrimination. Killing higher animals such as gorillas and dolphins would be wrong, if we believed that they were also self-aware and valued their lives.

Alternatively, imagine we one-day meet creatures from outer space. How should we decide in what sense to have them for dinner? Most of us would use behaviour, rather than membership of our species, or appeal to divine revelation, to decide, especially if we remember that the alien may also be deciding whether it is morally permissible to eat us!

This line of reasoning appears to lead to the counterintuitive conclusion that not only may fetuses be killed, but so also may newborn babies and the mentally handicapped, who also fail this test of personhood. Perhaps infanticide is indeed sometimes permissible, although its side effects, such as the offence caused to other persons, mean that it would rarely be permitted in practice. On this view personhood is an arbitrary status, which our society bestows on newborn infants but not on unborn fetuses. We can imagine societies, such as the Spartans, whose members withhold personhood from newborns, or yet others who bestow it on unborn fetuses (these are the societies which do not permit abortion).

Religious arguments

The most coherent defence of the special status of humans comes from religion. Christians, for example, argue that God bestowed special status on humans. At a certain point, perhaps fertilisation, the latest point for twinning, or quickening, the fetus gains a soul, and from then on may not be unjustly killed. Such arguments are supported by reference to religious texts, religious authority or revelation. I do not intend in any way to denigrate them, but would only say here that their main force is as a guide to private choice. Those who wish to follow such teachings should be free to decline abortion for themselves. Those who do not will ignore them, and they provide no basis for public policy in a pluralist secular society.

Women's rights arguments

The other point which defenders of abortion dispute is premise 5. They argue that even if the fetus is granted the status of personhood, the mother should not be forced to carry it for nine months against her will. It may be kind of her to do so, but she should be allowed to escape the burden. We do not expect people to donate even a pint of blood against their will. The American philosopher Judith Jarvis Thompson has put this argument powerfully. She imagined forcing someone to give aid to a paradigm person, in her example a famous violinist. The analogy was with pregnancy resulting from rape, but it can easily be modified to include other cases of unwanted pregnancy.

Imagine that a world famous violinist develops a fatal kidney disease. He will die unless he is connected to the circulation of another person, but the disease is self-limiting, and after nine months he will recover to full health. Unfortunately, he has a rare blood group and no-one with that group is also willing to be connected. A group of music lovers search the world for a suitable person, and find you. You may not agree to their proposal, so they kidnap, anaesthetise and connect you to the violinist's circulation. When you wake up and they explain what has happened. You are outraged and demand to be disconnected. They remind you that you only need to stay connected for nine months and that without you the violinist will die. Must you remain connected?

Thompson's intuition is that while it would be kind of you to stay connected, strong personal reasons, such as career and family needs would justify disconnection. Moreover, disconnection would still be justified if your behaviour had to some extent led to your kidnap. For example, you might have been well aware that the music lovers were searching for a person to kidnap, but nevertheless persisted in going home by a secluded route because you wanted the pleasure of viewing the sunset. By analogy, taking sexual pleasure does not commit you to bearing the unwanted pregnancies, which occasionally result.

This argument is also strongly disputed. Many individuals maintain that their intuitions are different from Thompson's; you should remain connected, and the rape victim should bear the child.

Abortion of an abnormal baby

Neither "women's rights" nor "personhood" arguments carry much force with parents who are considering abortion for fetal abnormality. They want a baby because of its potential to become a healthy human person.

This potentiality argument is often dismissed because it is assumed to commit us to also treating sperms and eggs as potential people, and maximising fertility at all costs. Twenty-eight years ago the philosopher Richard Hare showed that this need not always be the case. His argument was based on the Golden Rule ''do unto others as you would like them to do unto you''.

If we are glad that we were not aborted, we should not abort the fetus. If there were only one potential person to consider, abortion would be wrong. However, if we take potentiality seriously we must also consider all the other pregnancies, which this mother may have if her present pregnancy is ended. The resulting future people may later be glad that the abortion took place. Consider a woman carrying an abnormal fetus who plans a particular family size. There are two potential people to consider, the abnormal fetus and the replacement fetus, which will only exist if the abortion is done. The abnormal fetus would wish that the abortion does not happen. However the replacement fetus will wish the opposite, that the abortion is done. We can resolve the conflict by asking what we would choose if we were forced to live through both potential people's lives. If we chose no abortion, we would have one handicapped life and one non-life. The replacement fetus will not be conceived because its mother will be busy caring for her handicapped child. If we chose the abortion, we would have one non-life (the abortion) and one healthy life (that of the replacement fetus). Surely we would choose that the abortion takes place. Consistency indicates that we should act as we would wish done to ourselves, and do the abortion.

The argument from the Golden Rule is a good one. It appeals to a principle, with which anyone can agree, rather than to intuitions, which often conflict. At a practical level it captures the feelings of parents of a handicapped fetus better than the personhood and women's rights arguments. They love and want to bear their baby, and if they kill it will grieve severely. They will frequently say that they are acting in the child's interest to avoid a life of suffering. If the abnormality is relatively mild, outside observers who consider only the present abnormal child, may find the parents attitude hard to accept, since the child is still likely to have a life of more value than no life at all. Only a few abnormalities can be confidently predicted to be worse than no life at all. However, parents usually

see clearly the choice between the perfect child of their dreams, which they will still probably bear if they undergo the abortion, and the handicapped child they are carrying. If both these potential people are considered it is not difficult to support the abortion even from a fetal perspective.

Screening for fetal abnormality

The ethical issues around screening vary depending on whether the screening is done by private individuals or by the state.

Private screening

Private individuals have to decide what screening tests to undergo, but they have to decide only for themselves. Since there are good reasons to permit individual parents to choose abortion, private screening simply gives them information to help decide.

Even if parents occasionally make choices with which others disagree, there is no discrimination against the handicapped by such screening. Individuals discriminate all the time in ways that it would be intolerable for the state to do. For example, they choose their partners on the basis of skin colour, sexual orientation, height, weight, intelligence, and physical attributes. Similarly, it is acceptable for individuals to discriminate in the children they bear on the basis of physical and mental attributes, but would be unacceptable for the state to do the same. Private testing programmes do not need to make any overall cost/benefit calculation. They are justified solely by their ability to increase parental choice. Parents acting individually decide what abnormality is severe enough to justify screening. Even if individual parents occasionally make bad decisions, they will never all make unjust decisions on, for example, racial grounds or for trivial handicap. There is no need for society as a whole to make any decision about what abnormality is severe enough to justify screening, and therefore no risk of eugenic screening.

Government screening

Government screening is different. Choosing which programmes to offer means making a collective value judgment about handicapped children and pregnancy loss. This risks discriminating against those handicapped groups selected. Insofar as such programmes are justified by the aim of reducing handicapped births they are eugenic.

Screening differs from traditional medicine, in that it does not arise from a patient's request for advice for a specific complaint. This means that people

offering it should be sure that the programme is worthwhile. The disease must be an important health problem, doctors must have a reasonable understanding of the biology, there must be a recognisable early stage, and treatment at this early stage must be of more benefit than treatment started later. Both the tests, and the treatment must be acceptable to people. Finally the gains should outweigh the harms by sufficient margin make the screening more worthwhile than other uses to which the money might have been put. This final decision is difficult to make collectively because people's values differ.

Let us consider first trimester screening for Downs. This requires that a proportion of pregnant women, say the five percent at highest risk, undergo invasive diagnostic testing. In the UK this would involve 30,000 tests per annum and, assuming a one percent procedure related miscarriage rate, the loss of 300 healthy pregnancies. It would also prevent about half the Downs live births in the UK i.e. 300 Downs births.

The correct course of action depends on how the parents feel about having a handicapped child, or undergoing abortion. Some parents feel that having a handicapped child would be terrible, but would not be devastated by abortion, perhaps they have plenty of children already, or the pregnancy was an accident. They would probably choose to undergo screening, and some might even undergo invasive testing at very low risks. Other parents might feel that handicapped children often lead worthwhile lives, and that overcoming handicap can be life enhancing, or the pregnancy might be very precious, perhaps following prolonged infertility, or the parents might oppose abortion for religious reasons. These latter groups would probably decline screening, because their values differ.

The state cannot afford all possible prenatal screening, and even if it could, society would not tolerate paying for it all, so it has to draw a line over what it will fund. In the UK the NHS does not fund screening for toxoplasmosis or sex selection, decisions are being made now about cystic fibrosis and fragile X, and it is unlikely to fund screening for homosexuality, if the putative gene was identified. Such decisions cannot be made on some non-discriminatory calculation of whether the screening did more good than harm overall, since they would require interpersonal utility comparisons that are impossible in a controversial area like abortion.

State providers can only decide arbitrarily, on the basis that some handicaps justify abortion. The short-term side effects of this are unpleasant, with living people with handicaps for which screening is offered feeling justifiably that society does not value them, and parents of such babies feeling social pressure to have an abortion. The long-term risks of such programmes are even more serious. They put politicians on a slippery slope to funding screening for minor abnormalities. The history of eugenics does not inspire confidence that the state

may not one day argue that say homosexuality, short stature, blue eyes or personality disorder should not be screened for. If the state can draw a line at one point now there are few safeguards that it will not draw it at another point in future.

The United Kingdom government has tried to solve the problem by offering a panel of tests but permitting individuals a free choice whether to undergo them. There are two difficulties. Firstly, even offering a test persuades many people to undergo screening, which they would never have chosen for themselves. Secondly, people may assume that if the government offers such a test the government experts have already made the relevant value judgements.

The impossibility of making a non-arbitrary collective decision in these circumstances has been well illustrated by the failure of the UK National Screening Committee (NSC) to give a recommendation whether to implement a national Down's screening programme. This committee was created to evaluate all screening programmes on objective criteria, and has made many clear recommendations both for and against various programmes. However, it has issued mixed messages about Down's screening. Although the committee had issued no written recommendation, and to date still has not, a government minister announced in 2002 that there would be a national Downs screening programme, and claimed NSC support. The NSC has failed to correct the error in public and on different occasions has indicated privately that the decision was ''made by politicians'', ''still awaited'' or that the committee had made a verbal recommendation to the minister, which has not yet been issued in writing. In practice Downs screening is currently the only national screening programme in the UK decided on by politicians rather than by expert committee. The reason is simple. The expert committees cannot decide.

Nevertheless there are positive reasons for government to fund health care, to improve equity or justice and secondly to correct market failure. I now consider whether either of these applies to prenatal screening.

Equity

In general the poor suffer disproportionately from ill health, and in private systems receive disproportionately little health care, particularly expensive interventions. If the state gives them free health care, it redresses some of the inequality. However, prenatal screening for Downs is not a clear benefit like other health care. It is essentially a lottery ticket which might result in the prevention of a handicapped child but which might result in the loss of a normal wanted pregnancy. If poor people do not buy such screening, this means they have other priorities. Giving them free tickets will not correct inequality. It would be better to give them the money the screening would cost. Since the overall

worth of so much screening is disputed the poor may even benefit by getting less.

Market failure

Market failure can arise in insurance-based health care. Private insurance systems have two main problems ''adverse selection'' and ''moral hazard''. Adverse selection is the process whereby sick individuals tend to buy more insurance and use it more, leading insurers to either exclude such customers or charge them more. This makes health care difficult for the sick to obtain, and the defensive efforts by insurers to identify high-risk patients put up health costs without improving outcomes. Moral hazards are the processes whereby insured individuals tend to use more health care as its marginal cost to them is reduced, providers encourage unnecessary treatment if neither they nor the customer is paying, and people take less care of themselves knowing that others will pay the bills. Privately funded screening is affected by neither problem. It is so cheap that it does not need to be insurance-based, so there is little risk of adverse selection, and there is no moral hazard. Moral hazard is created by state provision. For example, a person who valued a screening test below the market rate would not buy private testing, but if it was worth anything at all to them, would accept free state testing.

Public goods are services from which individuals benefit without limiting others' use of them. They are under provided in private markets because it is difficult to find anyone altruistic enough to pay for them. An example would be mosquito control programmes to prevent malaria. Externalities are spillover benefits and harms that do not affect the individual decision-maker. An example of a beneficial externality would be the reduction in transmission of sexually transmitted disease from treatment of asymptomatic infection.

Downs screening is not a public good. There are plenty of incentives for private provision. Similarly, there are no significant beneficial externalities. The benefits affect the individual decision-maker and are therefore weighed in the balance by them. However, there is one harmful externality from state funded prenatal screening for abortion. It causes the living handicapped to feel undervalued, and may develop into a eugenic programme that is unjust to unpopular minorities.

Conclusion

First trimester screening by individuals who wish to prevent the birth of a handicapped child is justified on the grounds that parents should be allowed to decide for themselves whether to carry or abort a pregnancy. There are good

moral arguments to justify abortion and screening simply permits individuals to exercise choice. However government-funded programmes are more difficult. There are no clear moral arguments for them, and they inevitably involve arbitrary collective choices, which discriminate against some handicapped groups.

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Thrombophilia 2003 — overview with a special focus on pregnancy

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The annual incidence of venous thrombosis increases from 1 per 100.000 during childhood to 1 per 100 in older age [1], and is one of the leading causes of morbidity and mortality, particularly in obstetrics and gynecology. In 1847, Virchow postulated three main causes of thrombosis, including alterations of the vessel wall, venous stasis, and changes in the composition of blood (hypercoagulability) [2], the last two predominate in venous thrombosis.

During the past 10 years, knowledge of the etiology of venous thrombosis has advanced with the discovery of specific genetic defects that contribute to venous thrombogenicity. The coagulation abnormalities include, in addition to deficiencies of antithrombin, protein C and protein S, the G1691A mutation in the factor V gene (factor V Leiden), and the G20210A mutation in the prothrombin gene. These disorders underlie about 50 percent of episodes of venous thromboembolism, however, they are also present in at least 10 percent of Western populations [3,4]. Thus, the presence of thrombophilic abnormalities alone does not necessarily lead to a clinical event, since many risk factors are common in the general population (e.g., factor V Leiden up to 8%). As currently considered, venous thromboembolism is a multifactorial disorder in which acquired and genetic risk factors can interact dynamically [1].

Relevant acquired (expositional) and genetic (dispositional) risk determinants for venous thromboembolism are summarized in Table 1. In the majority of these risk factors their expositional or dispositional origin is clearly defined; however, in several abnormalities the origin is unknown or can result from both expositional and dispositional alterations. For example, levels of factor VIII:C and fibrinogen may increase as part of an acute phase reaction, also there is a familial trait for elevated factor VIII:C concentrations, for which no cause has yet been detected within the factor VIII gene [1,5].

Table 1: *Expositional ("Acquired") and Dispositional ("Genetic") Risk Factors for Venous Thromboembolism*

Acquired	Inherited	Combined/Complex
Age	Antithrombin Deficiency	Hyperhomocysteinemia
Previous thrombosis	Protein C Deficiency	Elevated levels of
Immobilization	Protein S Deficiency	— Fibrinogen
Surgery, Trauma	Factor V-Leiden	— Factor VIII:C
Pregnancy and puerperium	Prothrombin G20210A	
Oral contraception	Elevated levels of Factor IX +XI	
Hormonal replacement therapy	Dysfibrinogenemia	
Antiphospholipid syndrome		
Malignant diseases		
Myeloproliferative disorders		

Persons with thrombophilic risk factors typically present with deep-vein thrombosis of the legs, pulmonary embolism, or both. Less common manifestations are superficial venous thrombosis and thrombosis in the cerebral, visceral, and axillary veins. In more than half the cases, venous thrombosis is provoked by pregnancy, surgery, immobilization, use of oral contraceptives or hormone replacement therapy.

Inherited thrombophilia during pregnancy and the puerperium

Pregnancy is an acquired and independent risk determinant for venous thromboembolism leading to thromboembolic events particularly in women with a personal or family history of thrombosis or with additional thrombophilic risk factors. The reported incidence of venous thromboembolism associated with pregnancy varies considerably. When clinical diagnosis is confirmed by objective tests, the incidence of venous thromboembolism associated with pregnancy and the puerperium is approximately 1 per 1000 to 1 per 2000 deliveries [6-9].

To reduce the incidence of venous thromboembolism in pregnancy and improve outcomes, an individual risk stratification based on the probability of thrombosis as a rationale for an individual risk-adapted prophylaxis is required. Within the past 10 years, a significant improvement in risk estimation has been achieved due to the identification of new genetic risk factors of thrombosis.

It is well established that patients with severe deficiencies of antithrombin, protein C, and protein S have a markedly increased risk for venous thromboembolism during pregnancy and the puerperium [1,4]. However, the clinical relevance, particularly the predictive value of mild deficiencies of

antithrombin, protein C and protein S, has been discussed controversially and is still an unsolved problem.

Much of our present knowledge of the risk of venous thromboembolism in pregnant and postpartum women with inhibitor deficiencies is derived from family studies, which are likely to overestimate the risk for unselected women with these defects, particularly for those with mild deficiencies. In these studies, the risk of thromboembolism among antithrombin deficient pregnant women not receiving anticoagulant therapy was judged to be up to 40 % (3 to 40 % in the antenatal period, 0 to 20 % in the puerperium) [10-13]. For pregnant women with abnormalities of the protein C and S system not receiving anticoagulant therapy, the risk of thrombosis during pregnancy ranged from 3 to 10 % for protein C deficiency and from 0 to 6 % for protein S deficiency. In postpartum women, the risk was 7 to 19 % for protein C deficiency and 7 to 22 % for protein S deficiency [10-13]. In contrast to these results obtained from family studies, a much lower risk is found in unselected women [14]. The most likely explanation for this observation is that familial thrombophilia is caused by multigenic effects. Thus, in each of the families, several known and unknown risk factors of thrombosis are present contributing to an overestimation of a single risk factor under study [1].

An increased risk of venous thromboembolism during pregnancy and the puerperium is also associated with factor V Leiden and the G20210A mutation in the prothrombin gene. The risk of venous thromboembolism during pregnancy and the puerperium among heterozygous carriers or the factor V Leiden or the heterozygous prothrombin mutation G20210A increases about 4 to 16-fold [14-20]. Assuming an incidence of 1 thromboembolic event in 1500 pregnancies, the probability of thrombosis in pregnancy among carriers of heterozygous factor V Leiden or heterozygous prothrombin mutation G20210A is approximately 1 thromboembolic event in 400 pregnancies [14-20].

To date, there are only limited data about the risk of pregnancy-associated venous thromboembolism among unselected homozygous carriers of factor V Leiden G1691A. In our study [21], the probability of thrombosis in pregnancy among unselected carriers of homozygous factor V Leiden is approximately 1 thromboembolic event in 70 pregnancies corresponding to a relative risk of 25 for venous thromboembolism. In contrast to these results obtained from unselected patients, a much higher risk is found in women with previous venous thromboembolism and in women with symptomatic relatives [22,23].

Coinheritance of factor V Leiden and the G20210A mutation in the prothrombin gene further increases the risk of thrombosis. The G1691A mutation in the factor V gene (factor V Leiden) and the G20210A mutation in the prothrombin gene are independent risk determinants of venous thromboembolism during pregnancy and the puerperium. We have shown that a

combined defect of G20210A prothrombin gene mutation and factor V Leiden is associated with a disproportionate increase in thromboembolic risk (approximately 1 in 25 pregnancies) compared with the risk of either alone [14].

To date, there is no evidence to support routine screening of all pregnant women for congenital thrombophilia. By contrast, thrombophilia screening is required in women with a personal or family history of venous thromboembolism.

Practice points

Venous thromboembolism in pregnancy is multicausal resulting from the interaction of combined defects. The absolute risk for thrombosis is influenced by the kind of a prior thrombotic event (idiopathic versus transient risk situation), family history of thrombosis (thrombophilic family), and the presence of hereditary and acquired risk factors of thrombosis.

1. Women *without* prior thrombosis
   - — Women who carry a single defect, e.g. heterozygous factor V Leiden or prothrombin G20210A mutation, have a low risk of pregnancy-associated thrombosis (~1 in 400). Heparin prophylaxis is not required.
   - — Women who are carriers of a combined heterozygous defect of factor V Leiden and prothrombin G20210A mutation have a disproportionally higher risk (~1 in 25). We propose a heparin prophylaxis throughout pregnancy plus postpartum for 6 weeks.
   - — Women with deficiencies of protein C and particularly antithrombin who belong to a thrombophilic family have a high risk (probability for thrombosis >10%). Heparin prophylaxis/treatment is recommended.
2. Women *with* prior thrombosis
   - — In women with a single episode of prior thrombosis associated with a transient risk factor, e.g. surgery or trauma, and no additional genetic risk factor, the probability of pregnancy-associated thrombosis appears to be low. Clinical surveillance throughout pregnancy and heparin prophylaxis postpartum is recommended. In contrast, in women who had a thromboembolic event in a previous pregnancy or during oral contraception (without additional risk like immobilization, infection, etc.), we recommend heparin prophylaxis throughout pregnancy and postpartum for 6 weeks.
   - — In women with idiopathic thrombosis or additional hereditary risk factors or a positive family history of thrombosis, heparin prophylaxis is indicated.

Despite the remarkable progress in risk stratification, the precise magnitude of risk is currently unknown in many cases, e.g. deficiency of antithrombin, protein C or protein S without prior thrombosis, and recommendations for prophylaxis are often empiric. Thus, further studies are needed to characterize the risk depending on the degree of deficiency (antithrombin, protein C, protein S) and the family history.

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What is the evidence for the relation of thrombophilia to adverse pregnancy outcome?

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Key words: thrombophilia, pregnancy, factor V, heparin

Inherited and acquired thrombophilia are the main cause of thrombosis in pregnant women. A growing number of reports over the last 4 years have suggested that these disorders are also associated with an increased incidence of vascular pathologies resulting in poor gestational outcome. This review covers recent data concerning thrombophilia and vascular placental pathology, and discusses available therapeutic modalities to prevent placental vascular thrombosis and maximize successful gestational outcome.

Venous thromboembolism

The prevalence of VTE during gestation and puerperium is increased in women with inherited thrombophilic states such as antithrombin III, protein C and protein S deficiencies [1,2]. Pregnancy is an acquired risk factor for VTE, with factor V Leiden and factor II G20210A mutations being the major causes for VTE in pregnant women. However, most carriers will not develop clinical symptoms during gestation [3]. Likewise, factor II G20210A is 3-5 times more prevalent in gestational VTE (10-20%) than in normal Caucasian pregnancy populations (2-5%). Antiphospholipid syndrome can be found in 10-20% of gestational VTE cases.

The risk for first episode of gestational venous thromboembolism (VTE) is 1/200 and 1/500 for factor II G20210A and factor V Leiden mutation, respectively [3]. The risk for gestational VTE in women who had experienced VTE in the past is about 10 to 20-fold higher and is in the range of 5%.

Recurrent fetal loss

Recurrent fetal loss (RFL) is a well-established finding in certain acquired thrombophilic disorders, such as antiphospholipid syndrome [4] and essential thrombocythemia [5].

A case-control study in 60 women with these inherited thrombophilias documented an increased risk for RFL as well [6]. Of 188 pregnancies in women with thrombophilia, 42 (22%) resulted in pregnancy loss compared to 23/202 (11%) in controls: odds ratio (OR) 2.0; 95% confidence interval (CI) 1.2-3.3 [6]. In addition, a high incidence of gestational abnormalities was reported in 15 women with dysfibrinogenemia associated with thrombosis. Of 64 pregnancies, 39% ended by miscarriage and 9% by intrauterine fetal death [7].

A number of recent case-control studies has evaluated the prevalence of factor V Leiden mutation in women with RFL. Despite differences in ethnic Caucasian subpopulations and selection criteria for RFL, all three studies documented significantly increased prevalence of factor V Leiden mutation in women with RFL. Ridker *et al.* [8] studied women with RFL without an extensive etiologic work-up except for ruling out chromosomal abnormalities. They found a 2.3-fold increase in the prevalence of factor V Leiden in women with RFL.

In women with RFL of unknown cause, following exclusion of chromosomal abnormalities, infections, anatomic alterations, and endocrinologic dysfunction, studies by Grandone *et al.* [9] and by our group [10] have suggested that evaluation for factor V Leiden mutation is highly warranted since a significant percentage of women with RFL are found to be carriers of the mutation. Nevertheless, it should be emphasized that other reports did not document an association between factor V Leiden mutation and RFL [11].
The risk for RFL is greater in homozygous carriers than in heterozygous carriers of factor V Leiden [12].

Of interest, activated protein C (APC) -resistance in the absence of factor V Leiden mutation has also been associated with pregnancy loss [13,14]. One potential explanation is the presence of anti-2-glycoprotein-1 antibodies, which induce APC-resistance [15]. A potential explanation for the association between RFL and APC-resistance is that the APC-sensitivity ratio falls progressively throughout normal pregnancy either in correlation with changes in factor VIII, factor V and protein S levels [16], or without such a correlation [17].

The timing of pregnancy loss may be important in terms of pathogenesis and prevention. APC-resistance and factor V Leiden mutation are more common in women with second-trimester pregnancy loss [18], as well as in women with post-embryonic first-trimester losses [19]. Women with thrombophilia have an increased percentage of losses at later stages of gestation. For example, second-trimester losses or intrauterine fetal death accounted for 57 of 158 fetal losses (36%) in 37 women with thrombophilia compared to only 23/135 (17%) in women with RFL without thrombophilia ($p = 0.0004$) [18]. Activated protein C resistance and factor V Leiden mutation are more common in women with second-trimester pregnancy loss [18] and in women with post-embryonic first-trimester losses [19].

In view of the high prevalence of the three common thrombophilic mutations in the general population — factor V Leiden, factor II G20210A and MTHFR C677T — we evaluated their prevalence in women with RFL. At least one was found in 49% of women with RFL of unknown cause compared to 23% in controls (OR=3.2; 95% CI:1.7-6.1, p = 0.0002) [10].

Although this study demonstrated that factor II G20210A and MTHFR C677T as a solitary defect are not associated with an increased risk for RFL, compared to controls [10], it does not rule out the possibility that factor II G20210A mutation is a mild risk factor for certain types of RFL [20].

It is well established that combinations of inherited or acquired thrombophilic states increase the risk for thrombosis [21,22]. Likewise, combinations of thrombophilic states may further increase the risk for RPL. For example, coexistence of factor V Leiden and homozygous hyperhomocysteinemia [23] or a combination of factor V Leiden with familial antiphospholipid syndrome [24] was reported to result in thrombosis and recurrent fetal loss. It is therefore not surprising that the European Prospective Cohort on Thrombophilia (EPCOT) study documented the highest odds ratio for stillbirth (OR=14.3, 95% CI 2.4-86) in patients with combined thrombophilic defects [25]. In our recent study involving 76 women with RFL, 6 (8%) had a combination of thrombophilic polymorphisms compared to 1/106 (0.9%) of controls ($p < 0.02$) [10]. Factor II G20210A and homozygosity for MTHFR C677T both contribute to RPL when presenting in combination with other thrombophilic defects.

Without therapeutic intervention, less than 20% of gestations in women with thrombophilia and RFL result in live birth [10]. This is similar to rates reported in women with the antiphospholipid syndrome who experience RFL. Mechanisms responsible for the association of inherited thrombophilia with RFL have not been elucidated. Pathologic studies of placentae obtained from gestations terminated by fetal loss reveal thrombotic changes and infarcts. These can be observed in the maternal vessels in a large proportion of placentae of women with stillbirth [26].

The location of placental thrombotic changes may involve fetal vessels or maternal vessels. A role for fetoplacental thrombosis has been suggested by studies demonstrating an association between factor V genotype in miscarried fetuses and placental infarction [27]. However, thrombotic changes are commonly found at the maternal side [26].

Other gestational vascular complications

Activation of blood coagulation and endothelial cell stimulation are fundamental findings in preeclampsia [28], clinically characterized by gestational hypertension, edema and proteinuria. Activation of the coagulation and fibrinolytic

systems is more marked in the uteroplacental circulation than in the systemic circulation, and an abnormal pattern of hemostasis has been reported to operate in the uteroplacental circulation in women with preeclampsia [29].

Several recent reports suggest an association between APC-resistance, factor V Leiden mutation and early onset of severe preeclampsia. In one study 14 of 158 women with severe preeclampsia (8.9%) were found to be heterozygous for the factor V Leiden mutation compared with 17 of 403 normotensive gravida controls (4.2%) ($p = 0.03$) [30]. Likewise, in another study, factor V Leiden mutation was documented in 19% of women with preeclampsia compared to 7% of controls [31].

An association of placental vascular complications and hyperhomocysteinemia is increasingly reported. Hyperhomocysteinemia was documented in 26% of women with placental abruption, in 11% of cases with intrauterine fetal death, and in 38% of women delivering babies whose birth weight was below the fifth percentile compared to an estimated 2-3% in the general control population [32]. Likewise, hyperhomocysteinemia was documented in 26 of 84 women (31%) with previous placental infarcts or abruption compared to 4/46 (9%) in controls [33]. In addition, hyperhomocysteinemia was found in 6 of 35 patients (17%) with recurrent abortions [34]. In the Hordaland Homocysteine study, plasma homocysteine levels were evaluated in 5883 women with 14,492 gestations. The study, which is the largest performed to date, reported increased risk for preeclampsia (OR=1.33), stillbirth (OR=2.11), early labor (OR=1.41), and placental abruption (OR=3.03) [35].

APC-resistance and factor V Leiden have recently been associated with placental abruption. Seventeen of 27 women with placental abruption had APC-resistance compared to 5/29 controls (OR-8.2, 95% CI 3.6-12.7) [37]. Factor V Leiden was documented in 8/27 patients (30%) compared to 1/29 controls (3%) [36].

A recent study has evaluated the association of the genetic thrombophilias with gestational vascular complications in 110 women with preeclampsia, intrauterine growth restriction and placental abruption or stillbirth, who were compared to 110 women with normal gestations [37]. One of the three common thrombophilic mutations — factor V Leiden, factor II G20210A or MTHFR C677T — was found in 57/110 women (52%) with gestational vascular complications compared to only 19 controls (17%) (OR=5.2, 95% CI:2.8-9.6). Additional patients had other thrombophilias accounting for a total of 71/110 (64%) compared to only 20/110 (18%) in controls ($p < 0.001$). Patient and control groups differed in parity, with 92/110 patients being in their first pregnancy compared to only 62 controls ($p < 0.001$) [37].

The three inherited thrombophilias were more common in women with severe preeclampsia (OR=5.4, 95% CI: 2.3-12.4), placental abruption (OR=7.2,

95% CI: 2.3-20), intrauterine growth retardation (OR=4.8, 95% CI:2.2-10.3) and stillbirth (OR=3.4, 95% CI: 1.0-11.9). These resulted in earlier delivery, 32 weeks vs. 39 weeks, and decreased birth weight, 1375 vs. 3400 g, in patients with gestational vascular complications compared to controls.

Since up to 65% of vascular gestational abnormalities can be accounted for by genetic thrombophilias [10,37], the implication is to screen for these mutations in all women with vascular gestational abnormalities. Furthermore, this high prevalence of genetic thrombophilias, which is similar to the findings in women with pregnancy-related venous thromboembolism [38], and the findings of thrombotic changes in the placentae of the majority of women with thrombophilia and stillbirth [26], suggest that antithrombotic drugs may have potential therapeutic benefit in women with gestational vascular complications.

Therapeutic regimens

Emerging data on therapy of women with inherited thrombophilia and pregnancy loss are mostly uncontrolled and include small series of patients treated mostly with low molecular weight heparin (LMWH). The potential advantages of LMWH over unfractionated heparin are higher antithrombotic ratio (meaning less bleeding for better antithrombotic effect), longer half-life with a potential need for only one injection per day, smaller injected volume, and less heparin-induced thrombocytopenia. A recent collaborative study has demonstrated the safety of using LMWH during 486 gestations [39]. Successful outcome was reported in 83/93 gestations (89%) in women with recurrent pregnancy loss and in all 28 gestations in women with preeclampsia in a previous pregnancy [39]. Administration of the LMWH enoxaparin, 20 mg/day, to women with primary early RPL and impaired fibrinolytic capacity resulted in normalization of impaired fibrinolysis, conception in 16/20 (80%), and successful live birth in 13/16 (81%) [40].

We have used enoxaparin (Rhone Poulenc, France) to treat 61 pregnancies in 50 women with thrombophilia who presented with RPL throughout gestation and for 4 weeks into the postpartum period. Enoxaparin dosage was 40 mg/day, except for patients with combined thrombophilia or in case of abnormal Doppler velocimetry suggesting decreased placental perfusion, where the dosage was increased to 40 mg twice daily. In the case of previous thrombosis, LMWH therapy was continued for 6 weeks after delivery. Of the 61 pregnancies, 46 (75%) resulted in live births compared to a success rate of only 20% of prior gestations without antithrombotic therapy in these 50 women [41]. These preliminary results are very encouraging. However, the optimal dosage of LMWH is yet unknown and is currently being determined in a prospective randomized trial in order to maximize successful gestational outcome. Livenox is a multicenter prospective

randomized trial comparing two doses of enoxaparin, 40 mg daily and 40 mg twice daily, in women with thrombophilia and recurrent pregnancy loss.

The role of aspirin, if any, in the setting of thrombophilia and vascular gestational abnormalities remains to be confirmed. In patients with antiphospholipid syndrome or in those with combined thrombophilia, aspirin is given along with LMWH. However, whether aspirin has an added benefit to heparin or LMWH alone has not been evaluated. Prospective randomized, dose-finding studies are warranted to assess the potential advantage of LMWH in women with thrombophilia and vascular gestational abnormalities.

Unresolved Issues

Role for fetal genotype?

This is controversial. While there have been reports supporting that fetal thrombophilia is important [27], there are a number of reasons suggesting that this may not be the case. First, most thrombophilic polymorphisms are mild risk factors for gestational vascular complications (GVC) and gestational VTE. Second, thrombotic changes are noted mainly on the maternal side of the uteroplacental unit. Third, LMWH that does not cross the placenta are beneficial. Thus, unless there is a severe thrombophilic defect (i.e., homozygous protein C deficiency), fetal thrombophilic state is probably not a major contributor for GVC or VTE.

Is prediction of complications possible?

Only a fraction of women with thrombophilia experience GVC or gestational VTE. Moreover, the same women can have a normal gestation but in a subsequent pregnancy may experience complications. This may result from involvement of acquired prothrombotic factors operating during certain gestations in certain women. In addition, local hemostasis in the placenta may be crucial for fetal growth and development. *In vitro* and *ex vivo* studies from our laboratory suggest that alteration in placental hemostatic balance is found in women with GVC.

Management of other GVC

The optimal management of women with late pregnancy complications is currently being evaluated in a prospective multicenter randomized placebo-controlled trial, PREGENOX, comparing enoxaparin 40 mg/day and placebo in women with late GVC with or without thrombophilia.

Women with unexplained pregnancy loss

Where current thrombophilia evaluation is negative, the idea is that yet

undiscovered thrombophilia may be implicated, since thrombotic changes can be found in women with GVC without thrombophilia. Following preliminary experience with antithrombotic therapy in these women, a prospective randomized multicenter trial comparing enoxaparin 40 mg/day and aspirin 75 mg/day is about to be launched in Israel.

Future perspectives

Future research is this field will most likely focus on four aspects. First, verification of the potential associations of the various genetic thrombophilias with gestational vascular pathologies is rapidly emerging [42]. Second, currently 30-50% of vascular gestational pathologies cannot be accounted for by thrombophilia. Whether yet unknown novel genetic or acquired thrombophilia will be found to play a role remains to be determined. Third, the pathogenetic mechanisms responsible for placental vascular pathologies in women with thrombophilia have not been fully elucidated. Furthermore, it is not known why some women with thrombophilia express vascular gestational pathologies while others do not. It is possible that this may relate to local factors affecting coagulation, fibrinolysis and vascular tone at the level of placental vessels. Finally, the role of antithrombotic therapeutic modalities deserves prospective clinical trials, several of which are ongoing, to improve outcome for a large population of women who experience poor gestational outcome.

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The value of LMWH treatment in OB/GYN

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Anticoagulation is primarily indicated for treatment or prophylaxis of thromboembolism. Thromboprophylaxis is used in either patients at constant risk or in those who are at increased risk of thrombosis for a limited duration. At present, some inherited and acquired hypercoagulable conditions, termed thrombophilia, are recognized as increasing the thromboembolic risk during pregnancy and puerperium. In addition to DVT and PE, thrombophilia is also associated with vascular pathologies in the uteroplacental unit leading to adverse pregnancy outcome and to a wide range of indications for anticoagulation during pregnancy and puerperium.

Following the recognition of the adverse fetal and neonatal outcome related to warfarin anticoagulation during pregnancy, its use is currently restricted to specific indications when other anticoagulants are judged to be less adequate. Treatment with heparin, although safe for the fetus, may cause maternal side effects and needs close monitoring. During the last two decades a third preparation — low molecular weight heparin (LMWH) — has been introduced. The currently used various regimens of unfractionated heparin and LMWH are summarized in the Table.

Table. *Regimens of unfractionated heparin and LMWH.*

Regimen	Dosage
Mini-dose unfractionated heparin	SC, 5000 U q 12 hrs
Moderate dose unfractionated heparin	SC, q 12 hrs, anti Xa-adjusted dose
Adjusted-dose unfractionated heparin	SC, q 12 hrs, PTT-adjusted dose
Prophylactic LMWH	• SC, dalteparin 5000 U q 24 hrs • SC, enoxaparin 40 mg q 24 hrs
Adjusted-dose LMWH	Weight-adjusted full treatment doses (i.e., enoxaparin 1 mg/kg, q 12 hrs)
Postpartum anticoagulants	Warfarin with a target INR of 2-3

Venous thromboembolism (VTE) in pregnancy occurs approximately 6 times more than in the non-pregnant state. PE occurs in about one sixth of the patients

with untreated DVT and is the most common cause of maternal mortality. The overall risk of DVT during pregnancy (0.05-1.8%) is higher in women with a previous event of DVT, with a recurrence rate of 1:71 cases. Guidelines regarding the management of VTE during pregnancy are regularly updated. Recently, the 6th American College of Chest Physicians (ACCP) Consensus Conference on Antithrombotic Therapy (2001) suggested that LMWHs are as effective and safe as unfractionated heparin for the treatment of acute DVT. The current recommendations for the treatment of VTE during pregnancy are either (a) weight-adjusted dose LMWH throughout pregnancy, or (b) intravenous heparin for at least 5 days followed by PTT-adjusted dose of heparin for the remaining pregnancy. Postpartum anticoagulation therapy should be administered for at least 6 weeks.

Currently, five groups of pregnant patients at increased risk of VTE have been identified by the ACCP:

1. **PriorVTE associated with a transient risk factor** (i.e., with bed-rest, oral contraception, etc.). Management includes close observation during pregnancy and postpartum anticoagulants.
2. **Single episode of idiopathic VTE.** Management includes one of the options: (a) close observation, (b) mini- to moderate dose unfractionated heparin, or (c) prophylactic LMWH. All patients should receive postpartum anticoagulants.
3. **Single episode of idiopathic VTE and thrombophilia.** The same recommendations as in group 2, however, because of the higher risk for VTE, thromboprophylaxis in patients with ATIII deficiency is suggested.
4. **No prior VTE and thrombophilia.** These patients can be offered one of the options: (a) close observation, (b) minidose unfractionated heparin, or (c) prophylactic LMWH. All patients should receive postpartum anticoagulants. Thromboprophylaxis is strongly encouraged in patients with ATIII deficiency.
5. **Multiple episodes of VTE and/or long term anticoagulation.** Management includes one of the options: (a) PTT-adjusted dose of unfractionated heparin, (b) prophylactic LMWH, or (c) weight-adjusted LMWH. These patients should receive long-term anticoagulation postpartum.

To recap, prophylactic treatment should be tailored according to the risk group. Both heparin and LMWH are options, but it appears that LMWH will largely replace unfractionated heparins because of its improved boiavailablity, a longer half-life, ease of administration, no monitoring requirement, and fewer side effects.

Recent studies suggested that inherited thrombophilias are not only associated with increased risk of VTE during pregnancy and puerperium, but also with an increased risk of vascular pathologies in the uteroplacental unit leading to adverse pregnancy outcome, such as first and second trimester miscarriages, intrauterine growth restriction (IUGR), intrauterine fetal death, placental abruption, and preeclampsia. Critical reading of the literature reveals two nuances on these aspects. The data of the European Prospective Cohort On Thrombophilia (EPCOT) study show a certain contribution of thrombophilia to adverse perinatal outcome whereas numerous series show a definitive role of thrombophilia in the pathogenesis of several adverse pregnancy outcomes. There are at present two main controversies related to the validity of evidence associating thrombophilia and gestational thromboembolic phenomena and to the appropriate selection of patients and the timing of prophylactic anti-thrombotic therapy to prevent pregnancy loss and associated pregnancy complications. These conflicting views may be clarified by several ongoing multi-center prospective studies. Data on treatment for women with inherited thrombophilia and pregnancy loss is mostly uncontrolled and based on small series. The optimal dosage of LMWH to prevent thrombophilia-associated adverse pregnancy outcome is also unknown.

The 2001 ACCP Consensus Conference on Antithrombotic Therapy recommends evaluation for inherited thrombophilia as well as for the acquired antiphospholipid antibody (APLA) syndrome in women with recurrent pregnancy loss, prior severe preeclampsia, IUGR, placental abruption, or otherwise unexplained fetal demise. Management of pregnant patients is then subgrouped into five categories.

1. **Pregnant patients with APLA and previous pregnancy complications.** Treatment should consist of low dose aspirin plus either of the following options: (a) mini- to moderate dose unfractionated heparin, or (b) prophylactic LMWH.
2. **Homozygous women for MTHFR.** These should be treated with folic acid before pregnancy or as soon as pregnancy is confirmed.
3. **Women with thrombophilia and previous adverse pregnancy outcome.** One should consider low dose aspirin plus either (a) minidose heparin, or (b) prophylactic LMWH. These patients should receive postpartum anticoagulants.
4. **Women with APLA syndrome and history of VTE.** Long-term anticoagulation (warfarin) should be switched to either adjusted-dose unfractionated or LMW heparins throughout pregnancy and resumption of the long-term anticoagulant postpartum.

5. **Women with APLA syndrome but without history of VTE or pregnancy loss.** Four approaches have been suggested: (a) surveillance only, (b) mindose heparin,(c) prophylactic LMWH, or (d) low dose aspirin.

Persistent peripartum anticoagulation may complicate delivery. No significant difference in hemorrhagic complications was observed between LMWHs, mainly enoxaparin and dalteparin, and unfractionated heparins. Although bleeding complications appear to be very uncommon with LMWH, it was suggested to discontinue therapy 24 hours prior to invasive procedures or before induction of labor.

The incidence of neurological complications from hemorrhage is estimated to be less than 1:150,000 epidurals and less than 1:220,000 spinal blocks, and obviously, traumatic needle or catheter placement may increase the risk of a spinal hematoma. Because the anti-Xa level does not predict the risk of bleeding, the concern about regional anesthesia explains why labor induction or cesarean birth should be a planned elective procedure in women receiving LMWHs. To reduce the risk of anesthesia-related hematoma, regional blockade should start at least 12 hours after the last prophylactic LMWH dose and longer (24 hours) if the patient receives weight-adjusted dose of LMWH. When continuous epidural analgesia is performed, LMWH can be restarted 2 hours after catheter removal.

Finally, among all new therapeutic agents that are currently being developed and investigated in the field of venous and arterial thromobosis, pentasaccharides are the most advanced competitors of LMWHs. Pentasaccharides are a new class of synthetic anti-thrombotics, which selectively inhibit activated coagulation factor X. The use of these drugs in Ob/Gyn has not been studied so far.

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Impairment of Fetal Growth Potential and Neonatal Encephalopathy

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Summary

The objective of this study was to determine the frequency of the growth impairment in neonates with encephalopathy. In a case- control design neonates with encephalopathy (NE) meeting criteria for an acute intrapartum hypoxic event (IHE; n=21) and those who did not meet these criteria (n=20) were compared to controls. The controls were 42 neonates without complications matched 2:1 for gestational age with IHE cases. The percentile of growth potential, a measure of the weight expected in the absence of pathological conditions, was calculated for each fetus. More neonates with encephalopathy with and without IHE were below the 10^{th} percentile of growth potential compared to controls (7/21[33%] and 6/20[30%] vs. 1/42[2.4%]; OR[95%CI] = 20.5[2.2-114.0] and 17.6[1.8-102.5], respectively). These associations remained significant after controlling for gestational age and birthweight. A large proportion of neonates with NE demonstrate signs of antepartum injury reflected in the impairment of their growth. This association was similar in the presence or absence of IHE, indicating that antepartum injury has a causative rather than predisposing character in many cases of IHE.

Introduction

Adverse outcomes of pregnancy are not only extremely devastating, but also extremely rare. Adverse neurological outcomes carry, beside perinatal mortatlity the larges burden for the family, the obstetrician and the society are. They have not been well defined and are difficult to investigate. However, a group of disorders including cerebral palsy, attention deficit disorder, isolated mental retardation and seizure disorders well represents adverse neurological outcomes from the perspective of patients and health care providers.

These long term adverse neurological outcomes are frequently preceded by neonatal encephalopathy manifested by difficulty in initiating and maintaining

respiration, depression of tone and reflexes, subnormal level of consciousness or seizures in the immediate postpartum period. A large body of evidence indicates that neonatal encephalopathy (NE) constitutes a necessary preceding step for the development of permanent neurological injury. If obstetrical complications are not followed by neonatal encephalopathy the risk of permanent neurological injury is not increased.'[1-4]

Current evidence clearly indicates that the vast majority of NEs and cerebral palsies are the result of antepartum events. Only about 8 % of cerebral palsies and 4 — 30 % of neonatal encephalopathies can be attributed to an adverse intrapartum event.[5;6;] Recently, the International Cerebral Palsy Task Force defined criteria to be used for attributing a NE to an intrapartum event.[7] A number of antepartum adverse events which may result in NE have been identified, but many more remain unknown, making ascertainment of their total effect very difficult.[8]

It is likely that antenatal events severe enough to permanently impair fetal neurological development would also impair its growth.[9] ''Growth potential'' is a measure of the optimal weight an individual fetus ought to achieve in the absence of pathological conditions. It is calculated individually for each fetus, reflecting its achieved proportion of individual optimal weight, rather than relation of the fetal weight to the population norms as is traditionally done using birthweight nomograms. Growth potential impairment, therefore, reflects the entire accumulation of genetic and environmental factors that operated upon the individual fetus during the course of pregnancy. Such factors have to be of a significant magnitude to decrease fetal growth from the optimum, 50th percentile of the growth potential, to the individual growth potential below 10th percentile.

The purpose of this study was to determine the frequency of growth impairment in fetuses with NE at or near term attributed to intrapartum or antepartum adverse events, and in gestational age matched normal controls.

Material and Methods

In a retrospective case control design we derived cases from a population of 129 singleton pregnancies delivered from January 1994 to December 1999 and diagnosed with neonatal asphyxia, or hypoxic ischemic encephalopathy (Fig 1).

Neonatal Encephalopathy

From the above group we identified neonates meeting criteria for neonatal encephalopathy based on recommendations of the International Cerebral Palsy Task Force.[7] Patients were included if they demonstrated seizures and or

difficulty in initiating or maintaining respiration within the first week of life, and were delivered at 34 weeks of pregnancy or later.

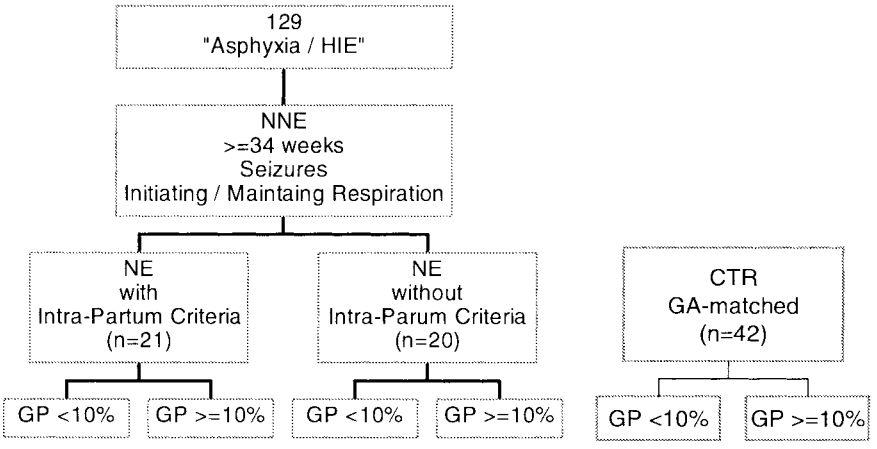

Figure 1. *Study design — Neonates meeting criteria of the International Cerebral Palsy Task Force for neonatal encephalopathy (NNE) were selected from the group of 129 pregnancies with clinical diagnosis of asphyxia or encephalopathy. Two groups of cases: NNE attributed to intrapartum hypoxic event (IP) and not attributed to an intrapartum event (NIP) were compared to control group (CTR) of neonates from uncomplicated pregnancies matched 2:1 for gestational age to IP group The groups were compared for the prevalence of impairment of the individual growth potential (GP).*

Criteria for an Acute Intrapartum Hypoxic Event

From such defined neonatal encephalopathy group, we identified two subgroups of cases. One composed of neonates meeting criteria for an acute intrapartum hypoxic event as a cause for encephalopathy (n = 21) and another group composed of neonates with encephalopathy who did not meet these criteria (n = 20). For controls, we selected a group of 42 neonates without antepartum or perinatal complications matched for gestational age at delivery with cases meeting criteria for intrapartum hypoxic event at the ratio of 2:1. The controls were selected from a randomly identified pool of normal pregnancies without medical and obstetrical complications as previously described.[10]

The criteria used for acute intrapartum hypoxic event were those proposed by the International Cerebral Palsy Task Force.[7] The diagnosis included an umbilical artery pH less then 7.00 and base deficit grater or equal to 12 mmol/l in addition to 3 or more of the following 5 criteria: 1) sentinel hypoxic event such as: ruptured uterus, placental abruption, cord prolapse or amniotic fluid embolism; 2) sudden and sustained deterioration of the fetal heart rate tracing; 3) Apgar score of 0 to 6 for 5 minutes or longer; 4) early evidence of multi-system involvement as evidenced by severe abnormalities of cardiovascular, respiratory,

renal or liver function; 5) early evidence of acute cerebral abnormality on head ultrasound, computed tomography or magnetic resonance imaging.

Growth Potential

For each fetus we calculated the achieved percentile of the individual growth potential. The percentile of the growth potential is a measure of the proportion of the individual optimal weight a fetus ought to achieve in the absence of pathological conditions. It is calculated in three major steps.

First, GROW v.2 software developed by and based on the formula published by Gardosi et al. was used to generate an individual optimal term birthweight for each fetus.[11] The individual optimal term birthweight is based on 6 independent factors (maternal weight at the first prenatal visit, height, parity, ethnicity, fetal gender and gestational age) identified as determining fetal weight from multivariate logistic regression analysis of 40,000 uncomplicated term pregnancies.

In the second step, a curve of the optimal growth as a function of gestational age is extrapolated for each fetus from its calculated optimal birthweight at term. The curve is created by dividing individual term optimal weight into daily fetal weight gains using Hadlock's fetal weight formula transformed into a proportionality equation. The proportionality equation expresses percent of the term weight as a function of gestational age.

Finally, percentiles of the individual optimal weight are calculated for each gestational age by applying the variation in term weights expressed in % of the mean (coefficient of variation) to other gestational ages. A comparison of the actual birthweight with such derived norms for gestational ages provides the percentile achieved of the optimal weight for each fetus, a reflection of its growth potential.

In addition to the individual percentile of growth potential, the population-based percentile birthweight was calculated for each fetus using norms derived from more than 3 million deliveries in the United States in 1991.[12]

The proportions of neonates with growth potential impairment and small for gestational age were compared between the three groups using Chi-square test with Bonferoni correction for multiple comparisons. Multivariate logistic regression and contingency table were used to evaluate the effect of birthweight and growth potential impairments, and gestational age between the groups. P less than 0.05 denoted statistical significance.

Results

The characteristics of the study population in the three groups (I — NE attributable to an intrapartum event, II — NE not attributable to an intrapartum

event and III — controls) are displayed in Table I. There were no significant differences in the factors determining the growth potential with the exception of higher parity in group I and slightly lower median birthweight in group — II.

A larger proportion of patients in groups I and II demonstrated severe impairment of the individual growth potential below 10th percentile. . Conversely, the proportions of patients with the growth potential above 50th percentile were not significantly different (Table II). The odds ratios [95% CI] for the growth potential below 10th percentile in group I (21 [2-114]) and II (18 [2-103]) were not significantly different. After adjusting for gestational age and birthweight using logistic regression the OR (95% CI) were 118 (11-1324) and 35 (3-408), respectively. Comparison of birthweight percentiles using population norms rather than individualized growth potential demonstrated no statistically significant differences between cases and controls (Table III).

Table I. *Variables determining growth potential in cases and controls. Data presented as median [interquartile range] or number.*

	CTR n = 42	**IP n = 21**	**NIP n = 20**	P
Height (cm)	160.0 [157-165]	157.5 [154-162]	162.6 [158-168]	0.17
Weight (kg)	65.5 [54-79]	68.1 [61-79]	67.5 [40-110]	0.25
Parity	1.0 [0-2]	2.0 [1-3]	0 [0-2]	0.005
Ethnicity				
Caucasian	12	4	9	
Hispanic	18	10	5	0.34
African-American	9	7	6	
Other	3	-	-	
Fetal gender				0.07
Male	16	13	13	
Female	26	8	7	
Birthweight (g)	3300 [3065-3576]	3415[2964-3740]	2838[1844-3341]	0.05
GA at delivery (weeks)	39.2 [37.6-40.1]	39.0 [37.8-40.1]	36.7 [34.4-40.]	0.12

Table II. *Proportions of cases and controls in different percentile groups of their individual growth potential. Data presented as number (%) and Odds ratio (95% confidence interval).*

Percentile Growth Potential	**CTR n (%)**	**IP n (%)**	**OR (95% CI)**	**NIP n (%)**	**OR (95% CI)**
<10th	1 (2.4)	7 (33.3)	20.5 (2.2-114.0)	6 (30.0)	17.6 (1.8-102.5)
<50th	18 (42.9)	11 (52.4)	1.5 (0.5-4.8)	11 (55.0)	1.6 (0.5-5.5)
<90th	37 (88.1)	16 (76.2)	0.4 (0.1-2.1)	14 (70.0)	0.3 (0.1-1.4)

Table III. *Proportions of cases and controls in different percentile groups using standard norms. Data presented as number (%) and Odds ratio (95% confidence interval).*

Percentile Birthweight	CTR n (%)	IP n (%)	OR (95% CI)	NIP n (%)	OR (95% CI)
<10th	1 (2.4)	4 (19.0)	9.6 (0.9-67.2)	4 (20.0)	10.3 (0.9-70.7)
<50th	22 (52.4)	11 (52.4)	1.0 (0.3-3.2)	14 (70.0)	2.1 (0.6-7.7)
<90th	39 (92.9)	18 (85.7)	0.5 (0.1-3.3)	18 (90.0)	0.7 (0.1-4.5)

Thirty three percent (7/21) of neonates with encephalopathy due to intrapartum hypoxia had growth potential below 10th percentile. However, none of the patients with neonatal encephalopathy attributable to the intrapartum hypoxic event had birthweight below 10th percentile without impairment of their growth potential (Fig 2). The interaction among growth potential, birthweight percentiles, and gestational age is demonstrated as a 3-D plane. No interaction would result in a straight plane tilted 45°. Displacement of the darkest band representing GP <10 percentile to the right along the birthweight axis demonstrates that a large proportion of these growth potential impaired neonates had normal birthweight percentiles. (Fig 3). Logistic regression analysis demonstrated that the association between growth potential impairment and encephalopathy was independent of birthweight and gestational age (adjusted p=0.002).

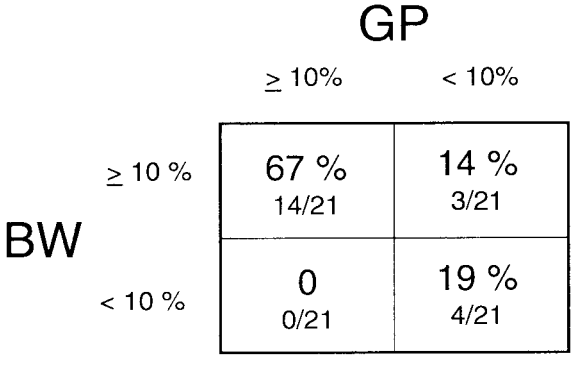

Figure 2. *The contingency table compares the proportion of neonates with encephalopathy attributed to hypoxic intrapartum event according to the presence or absence of impaired growth potential (GP) and/or birthweight (BW). Each cell of the contingency table demonstrates the prevalence of the NNE attributable to the intrapartum event in relation to impairment of the growth potential and or birthweight.*

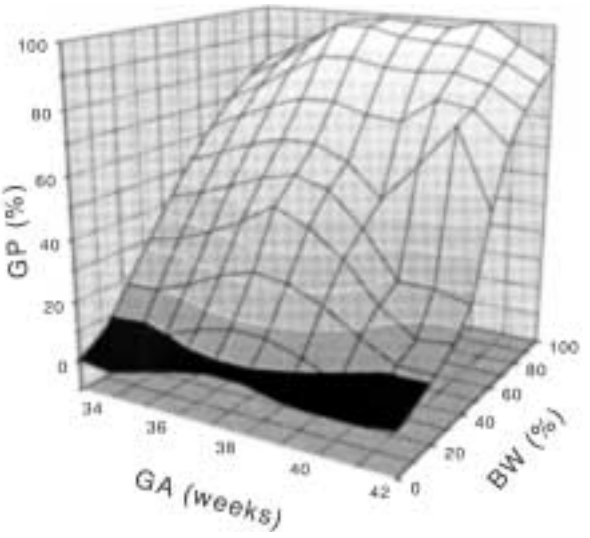

Figure 3. *In the three-dimensional plot, the growth potential (GP) of neonates with encephalopathy due to intrapartum events is plotted relative to gestational age (GA) and birthweight (BW) percentile. Each horizontal line represents cumulative clusters of 10 percentiles growth potential from 0 to 100 %-ile with the darkest area being the group between 0 and 10%-ile. Each horizontal percentile band has a width of 10 percentiles and its shading increases from the darkest 0-10% to the lightest 90-100%-ile-ile. A significant number of the infants with encephalopathy demonstrated growth potential below 10th %-ile, with the birthweight above the 10th %-ile.*

Conclusion

At least one third of the neonates with encephalopathy demonstrate severe (< 10th percentile) impairment of their individually calculated growth potential. The percentile achieved by a given fetus, therefore reflects the degree of impairment compared to its own potential rather than to the norms for a population, as done for birthweight nomograms. Unlike impairment of birthweight, impairment of the growth potential avoids including fetuses who grow normally but are below the 10th percentile of the birthweight for the population. It also avoids missing ones who, although above a given cut off of birthweight impairment, are deficient in their rate of growth. Since optimum growth should occur along the 50th percentile, and lower percentiles reflect impairment of the individual potential, rather than a position in the distribution of the population, the traditional cut-off of 10th percentile may not be appropriate. A higher cutoff may more accurately identify fetuses with growth impairment, and may result in even higher proportion of growth impairment among fetuses with NE.

The growth potential is an indicator of a whole accumulation of genetic and environmental influences that have operated upon the fetus. Therefore, its impairment reflects the sum of pathological conditions that operated ante-

partum and impaired its growth. The pathological conditions that stunt fetal growth are likely to also injure the fetal central nervous system.[9;13] Strong association between NE and impairment of the growth potential confirms prior observations that the majority of NE and cerebral palsies are the results of antepartum factors.[8]

Large cohort studies demonstrated that only between 4 and 30 % of NE can be attributed to the adverse hypoxic intrapartum event.[6;14;] Using criteria of the International Cerebral Palsy Task Force to define this group we demonstrated that at least one third of this small group of NE attributed to intrapartum hypoxic events had severe antepartum injury resulting in profound impairment of their growth. As the cut-off for impairment of the growth potential is likely to be higher then 10th percentile this proportion of NE might be even significantly larger.

Use of population based birthweight norms also demonstrated increased prevalence of fetuses below the 10th percentile in both groups with neonatal encephalopathy. The increase did not achieve statistical significance, likely due to small numbers, which confirms that growth potential better than population birthweight norms identifies the adverse course of pregnancy. The wide confidence intervals of the odds ratios in tables 2 and 3 reflect the small sample sizes. Narrower confidence intervals would require larger number of cases of NNE, which is fortunately a very rare outcome.

Comparison of the prevalence of GP impairment between NE attributed to intrapartum event and ones not associated with such an event and therefore likely to be a result of antepartum insult, indicates that two alternative situations could occur. First the GP impairment is a marker of a causative insult and the prevalence of the GP impairment should be similar in both NE groups. In the second situation GP impairment is a predisposing factor for a causative insult and NE would occur only in a subset of patients in whom a combination of a GP impairment and a causative insult took place. Therefore the incidence of the GP impairment would be lower in the group of NE attributed to an intrapartum event as not all patients with GP impairment, but only the ones in whom it was associated with a causative intrapartum insult would develop encephalopathy. The frequencies and severity of the growth potential impairment in both NE groups were surprisingly similar in our study, indicating that antepartum insult had a direct effect on the occurrence of NE, and that the effect of the intrapartum event was not additive.

While our study design does not allow definite resolution of this issue, observations regarding the impairment of growth in the cases with or without intrapartum events are highly suggestive and encourage further research in this area.

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Ultrasound Biometry and Fetal Weight Assessment

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The fetal weight at term can be estimated by clinical, ultrasound or computer assisted methods using a variety of computational algorithms. Clinical estimation of fetal weight by palpation is generally unreliable, particularly if the woman is obese or if the fetus is macrosomic. Sonographic estimation of fetal weight is widely practiced to detect abnormalities in fetal size that may affect clinical decision-making. However, many of the existing formulae are generally inaccurate at the extremes of fetal weight, and alternative models based on volume estimation have been proposed.

In its latest guidelines, the RCOG recommends the use of either the fetal abdominal circumference (FAC) or the estimated fetal weight (EFW) as a screening parameter for fetal growth restriction. As early as 1983, Chervenak *et al* were advocating the use of estimated fetal weight (EFW) for the detection of FGR. However, in current practice many obstetric ultrasound units rely mainly on the FAC.

Considerable evidence now supports the use of the Gardosi customized growth charts to optimize the screening process for FGR. These charts work best when EFW is used, since the original statistical modeling was based on birth weights. There is additional published data by Chang et al, which suggests EFW to be a marginally superior screening modality than FAC, using FGR defined by neonatal morphometric indices such as ponderal index and skinfold thickness. Our studies using computer modeling technique have shown that plotting the FAC at 2-week intervals is associated with false positive rates for FGR in excess of 16%, whereas plotting EFW instead reduces these rates considerably.

If EFW is going to be the main screening tool, the question arises of what is the optimal weight estimation formula. There is general agreement that equations employing two or more ultrasound parameters are more reliable than those using only one. In Hadlocks' studies, the prediction errors of equations employing three or four ultrasound parameters (BPD, HC, FL and AC) had a standard deviation of around 8%. Slightly better values (about 7%) were reported by Issel et al, by measuring up to 7 ultrasound parameters. However, in

clinical practice outside of tertiary institutions these errors are generally higher and often exceed 10%.

Errors in ultrasound weight estimation may arise from either method error or from observational errors. The former are due to the intrinsic limitation of the formula used, whereas the latter are due to measurement error. We have carried out computer modelling experiments to investigate the tolerance to observational error of commonly used weight estimation formula. We found that Campbell's formula using the FAC only was the least tolerant to observational error, whereas Shepard's formula was most tolerant. This is in agreement with the study of Chien et al, indicating that this formula had one of the best interclass correlation coefficients.

Another important source of bias is the possibility of differential performance of formulae according to gestational age. We have found that, in general, weight estimation formulae tend to overestimate weight at term and underestimate it in the preterm period. This bias was minimal with volumetric formulae.

Why embryo culture to blastocyst stage in human IVF

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Introduction

The goal of in-vitro fertilization (IVF) and embryo culture is to provide high quality embryos capable of continued development and implantation, which will result in viable births. Considerable progress has been made in culturing preimplantation embryos since the initial studies Along with other teams, (Tervit et al. 1972), we began to design and define new, more complex culture media based on the composition of genital tract secretions (Ménézo 1976) During the initial stages of zygote formation and early cleavage divisions, there is a minimal level of transcription only, so that at the time of ovulation, the mature oocyte must contain a storage pool of proteins and/or mRNA transcripts. The cycle during which the zygote genome is activated (Zygotic Gene Activation, ZGA: 4 to 8 cells in human) is the longest cycle of preimplantation development: any delay at this time will result in a decrease in the level of mRNA below critical thresholds. It correspond to a critical point in the dry mass of the embryo (Turner et al 1994). Therefore, for many years embryos were routinely transferred at the so-called ''cleavage stages'', as insufficient knowledge was available to allow prolonged successful culture beyond these stages. Correct timing of blastocyst formation is an initial indication of embryo quality as it shows that the embryos have passed the narrow gap of maternal to zygotic transition. Two solutions have been proposed to satisfy these requirements of the embryo: sequential changes in the composition of the culture media, and providing a dynamic environment with the help of a cocultured feeder layer of cells. Then sequential media replaced this last technology. It is clear that the philosophy is not the same everywhere. It can be used for patients with repeated failures in order to try to understand the problems i.e.the diagnostic tool. But it can be used in good

patients, to reduce the multiple pregnancy rates. We used these 2 approaches for now 15 years:our first ''blastocyst babies'', either ''fresh''of Frozen/thawed are now 14 years of age and more than 700 in total are born. We will explain here why we, as numerous others (Olivennes et al. 1994) do believe in this technology.

Blastocyst transfert and Uterine contractility

Fanchin and coworkers (2001) clearly demonstrated that uterine contractility decrease at the time of blastocyst transfer (hCG +7-8 days) when compared to early stage transfers on Day 2 (hCG +4days). The rythm of contractions is reduced from by one half to 1.5 contraction per minute. This explains why we currently observe that in some cases, patients with high rates of transfers failures for early stage embryos are easily pregnant with the first blastocyst transfer (Ménézo et al. 1992a). This also fits with our observations when comparing ZIFT and Blastocyst transfers(Ménézo and Janny 1995).

A better environment in vivo than in vitro?

A study comparing blastocyst transfers with Zygote Intra Fallopian Transfer (ZIFT) allowed us to compare the efficiency of the selection process. Although tubal transfer (ZIFT) supposedly mimics the ''best ''in vivo conditions, the results did not compare favorably with blastocyst transfer. Since embryos are transferred directly into the Fallopian tubes, the discrepancy cannot be explained by possible expulsion of embryos; selection through in vitro culture is therefore a possible rationale.

Table 1: *Comparison between ZIFT and blastocyst transfers (From Ménézo and Janny 1995)*

	ZIFT	UtB	P
No Transfers	137	217	
No of embryos transferred	356 (Z)	448 (Bl)	
Per transfer	2.6	2.06	0.001
Ongoing Pregnancies	39	61	
Percent per transfer	28.5	27.7	NS
Clinical Gest.Sacs	48	86	
Live birth			
(% of embryo transferred)	13.5 (Z)	19.2 (Bl)	0.038

UtB Uterine transfer of blastocysts,
Z: Zygote, Bl: Blastocyst

Limiting factors

A 45-50 % blastocyst formation rate can be expected in an overall population which includes patients with advanced maternal age, male factor infertility, and repeated failure of implantation. It is now clear that reported rates which exceed this baseline suggest a selected patient population. Prior to a consideration of results, several parameters must be taken into account. Blastocyst development may be affected by:

Maternal factors: maternal age may impair very early blastocyst formation. There is an initial decline around the age of 30, and a further decline after the age of 40. Blastocyst formation, and then expansion, is impaired with advanced maternal age. (Janny and Ménézo 1996). However, once a good quality blastocyst is obtained, within the correct time course, the implantation potential is the same (around 25-30%). In our practice, advanced maternal age is a favourite indication for blastocyst transfer. We have also recently demonstrated (El Mouatassim et al., 2000) that in humans there is a significant regulatory polyadenylation of mRNA during the last phases of oocyte maturation (i.e. between GVBD and MII). As the first cleavage divisions depend upon this stored mRNA and some of these RNAs are important for blastocyst formation, any misregulation of this process leads to inevitable embryo developmental arrest. The effect of maternal factors on developmental arrest is a continuous phenomenon.

Paternal effects: During the early years of IVF, it was commonly believed that all embryos which developed to Day 2 had an equal chance of achieving a pregnancy. Blastocyst technology has destroyed this assumption. Compromised sperm considerably reduces blastocyst formation (Janny and Ménézo 1994). This has been confirmed by ICSI (Shoukir et al. 1999, Ménézo and Barak 2000); in our experience, blastocyst formation for supernumerary embryos decreases from 44% after IVF to 34% after ICSI. Several factors may impair blastocyst formation, epigenetic and/or genetic.

Genetic factors: In the majority of cases, deletions of the Y chromosome have negative effects on sperm count, morphology and/or motility. The direct impact on embryo quality obvious on a large scale. DNA denaturation and fragmentation (Evenson et al.1999) have a strong negative impact, especially after genomic activation, when massive transcription begins. Unrepaired DNA breaks lead to blocks in transcription: this is why many embryos arresting due to paternal factors block more or less at the morula stage (Ménézo and Janny 1997)

Epigenetic factors: Anomalies of the centrosome, defects in ''oocyte activating factor'' and in DNA condensation (Sakkas et al. 1997, Evenson et al; 1999) alters severely preimplantation embryo development. Antisperm antibobies can stop early preimplantation development (Naz 1992)

DNA Fragmentation: It is now obvious that increased sperm DNA fragmentation alters severely the chance of achieving a pregnancy (Evenson et al. 2002). We observed recently that over a certain level of DNA fragmentation, when the DNA repair capacities of the oocyte are overpassed, blastocyst formation is decreased and the ongoing implantation rates of the blastocyst obtained are nil (Menezo and Oger in press). Blastocyst technology does not help in couples where sperm DNA fragmentation is too high.

Cytogenetic problems: in vitro manipulation of gametes increases the risk of cytogenetic anomalies, and cytogenetic anomalies are responsible for the arrest of least one half of the embryos. These anomalies can obviously arise due to maternal and paternal problems (i.e chromosome abnormalities in gametes). Moreover, this process of selection can be used for chromosomal translocation carriers (Ménézo et al. 2001). Ovarian stimulation increases the number of (healthy) oocytes: after fertilization, extended culture increases the selection pressure. Translocation carrier patients who succeed in having embryos that develop to the blastocyst stage have a very good chance of delivering healthy babies. In our activity, 12 babies are born either carrying the translocation of the parents or not. It is obvious that blastocyst transfer is an alternative to PGD for translocation carriers but then if this selection does not work then PGD is required.

Monozygotic Twinning

Monozygotic twinning has been described as a penalty of blastocyst transfer in 5% of ongoing pregnancies, after culture in G2/CCM20 (Vitrolife) and Irvine's Blastocyst Medium and Medicult's Blastassist and M3 (Abusheika et al. 2000 et al. 2000, Peramo et al. 1999, Behr et al. 2000, Schachter et al. 2001, Tarlatzis et al. 2002). It is possible that in certain hypersensitive embryos, there is an over-stimulation of apoptosis in media containing an excessive glucose level: the pro-apoptotic effector Bax was found to be increased in mouse blastocysts exposed to hyperglycemia, through free radical formation (see Pampfer 2000). If a linear polarisation of apoptotic cells occurs, the ICM could split before hatching, thus leading to a monozygotic twinning (Ménezo and Sakkas 2002). Reducing Glucose levels in the culture medium and/or providing a better protection against free radicals within the blastocoele might provide a solution. This was confimed by our recent observations: MZT is not mandatory in Blastocyst technology if culture media and culture conditions are optimum. In our hands, MZT rate remains below O.5% in our blastocyst transfer program (Cassuto et al. 2003)

Blastocyst freezing

Blastocyst freezing has been controversed: It is always said that the probability of freezing is lower for blastocyst, due to the lower number of pre-embryos reaching blastocyst stage.

It is also clear that a strong selection, assessing fragmentation and other anomalies, is also made before freezing early stage embryos.

The results of our freezing programme for the last 4 years, 1995 to 1998 followed co-culture of blastocysts: Following the freeze of 100 blastocysts there were 10 babies born. The extra deliveries, thanks to blastocyst freezing were below 15%. It reaches 28% in 1997 when we were freezing cocultured blastocysts. The use of sequential media for blastocyst culture has led to a reduction in the success rates in our unit. Anyway the extra births related to our freezing program are still over 10%. The results in term of pregnancy rates are then as good as previously described in other teams (Behr et al. 2002) used controlled atmosphere with 5%O2. The current protocols are currently re-evaluated. Vitrification, using cryo loops, is now used in freezing technology. It has been used for blastocyst freezing with some success. However there is still insufficient information to recommend vitrification, whatever the embryonic stage. There is some concern regarding the use of 40% Ethylene Glycol (EG): Catabolism of EG leads to the transitory formation of toxic and teratogenic aldehydes (Klug et al. 2001): a risk which is hard to justify, at the time of high mitotic activity. Moreover the results obtained do not look really better than the ones observed with the slow cooling protocol. In any case, a careful follow-up of the babies born from ethylen Glycol vitrification, is strongly recommended.

Conclusion

The low success rates obtained after culture of human and animal embryos in simple or complex media severely compromises the application of new technologies. New approaches in embryo culture of both mammalian and human embryos have been reported to promote improved quality in embryos.

The empirical approach to comparisons of different culture media formulations was ineffective, until we began to understand some of the interactions which take place in embryo metabolism. Prior to and during culture, it is important to control the interactions of different compounds with each other and with the gas phase. Sequential media are currently effective in human embryo culture, with yields similar to those observed for coculture systems. In order to evaluate new techniques and /or new culture media, *in vitro* grown embryos must be transferred in order to avoid misleading observations based upon morphology alone. In view of early developmental arrests, embryo transfer at early stages is obviously a process which is too blind. The question is no

longer whether or not to use blastocyst technology.Transfer of embryos at the blastocyst stage should not be an exception, but a regular tool. Ultimately this should lead to an improvement in the success of assisted reproductive technologies, with a lesser likelihood of multiple pregnancies.

But one has to be fair. Blastocyst transfer is not a miracle story; the selection effet is important, table 2. It has allowed us to reduce the overall number of embryos transferred down to 1.8 for the last 4 years with no more triplet pregnancies. The percentage of twins is still over 20%. However for old patients and when the quality of sperm is very bad, no one technology can help. In this case, no blastocyst can be obtained in vitro: reproductive physiology has its unavoidable rules.

Table 2: *Effect of culture in sequential media having all the aminoacids in the first and the second phase medium (ISM1/2, Medicult). Regular IVF after Short insemination time.*

Patients		186
Transfers		180 (96%)
Embryos on D2		1499
Blastocyst on D5/6	678	(45.2%)
Blastocysts transferred		330 (1.83 per patient)
Pregnancies		86 (47.8% per patient)
Implantation	100	(30.3% per Bl. transferred)

Mean age of the patients: 33.7 Yrs, mean rate of previous failures 1.6

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ZIFT, GIFT, Co-Culture — where have all they gone and why?

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The allure of any new procedure in assisted reproductive technology (ART) is higher pregnancy rates. However, the advantage must be documented through rigorous trials and is subject to change with rapid advances in medicine today. The promise of higher success did hold true for three such procedures: gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), and co-culture. The current debate associated with GIFT, ZIFT, and co-culture implicates a common root: advances in cell culture techniques in the IVF laboratory. Evaluation of the recent data can aide the physician in sifting through the opinions in reaching a decision based on substance.

With the onset of in-vitro fertilization in the 1980's, the pregnancy rates of IVF-ET were low. Appraisal of the data revealed poor fertilization rates and embryo growth. Using in-vivo systems, GIFT and ZIFT offered the promise of higher pregnancy rates. Although, these techniques were limited to those with at least one patent and functional fallopian tube and those without severe male factor (GIFT precludes intracytoplasmic sperm injection), the anesthesia and surgical risks associated with the procedures were low. Therefore, these procedures were accepted into the management arsenal available to the physician. Initially, pregnancy rates were higher with GIFT and ZIFT versus IVF-ET.

Although some research into GIFT and ZIFT corroborated higher pregnancy rates, randomized controlled trials failed to show an improvement with the use of tubal transfers (1). A meta-analysis of six randomized controlled trials comparing ZIFT to IVF-ET concluded that these two modalities had similar implantation and pregnancy rates with a trend towards increasing rate of ectopic gestation with ZIFT (1). Concurrently, there were substantial improvements in the culturing techniques as well. These advances led to higher fertilization and pregnancy rates. In a review of the published ASRM data of ART

centers in the US from 1985 to 1999, Toner documents the progressively higher IVF-ET pregnancy rates with stagnant GIFT and ZIFT pregnancy rates in the last decade as noted in figure 1 (2). There was a concomitant decline in GIFT and ZIFT cycles (figure 2). The most recent data from the SART registry reports that tubal transfers represent less than three percent of the total IVF cycles (3). A closer look at the cycles from 1999 reveal equivalent delivery rate per retrieval: 63,639 IVF cycles with a delivery rate per retrieval of 29.4%, 945 ZIFT cycles with a delivery rate per retrieval of 29.8%, and 838 GIFT cycles with a delivery rate per retrieval of 27.9%.

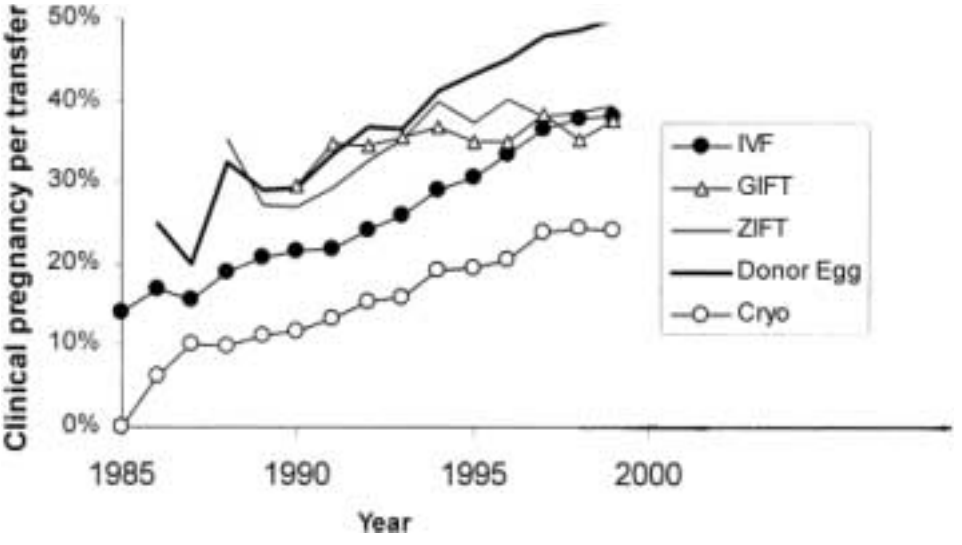

Figure 1: *There is an increase in the clinical pregnancy rate per transfer in ART cycles with equivalent rates among IVF (IVF-ET), GIFT, and ZIFT in the past several years. Reprinted from Fertility and Sterility, V78(5): 943-950, Toner JP: "Progress we can be proud of," 2000, with permission from American Society for Reproductive Medicine.*

Techniques such as serial culture media have significantly improved embryo growth to the blastocyst stage. The technology cascade has resulted in preimplantation genetic diagnosis (PGD) and additional promise of lower rate of higher order multiple pregnancies. The net effect is pregnancy rates of IVF-ET equaling those of GIFT and ZIFT without the associated risks or limitations and with additional options. Accordingly, it is with no great surprise to see the diminishing numbers of cycles using GIFT and ZIFT.

In an effort to find success for the patients with repeat failures, the procedures discussed above have again been heralded as improving pregnancy rates. In 1998, Levran et al proposed the use of ZIFT in patients with repeat IVF-ET failures (4). They conducted a case-controlled trial of 140 patients revealing a

pregnancy rate of 34.2% in the ZIFT group and 17.1% in the control group (another cycle of IVF-ET). Further data is necessary to confirm these preliminary findings.

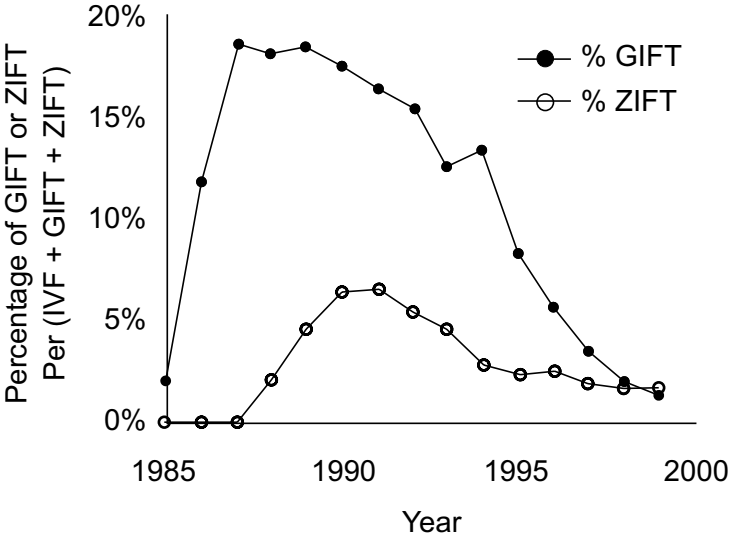

Figure 2: *Rise and fall in the use of GIFT and ZIFT in the US. Reprinted from Fertility and Sterility, V78(5): 943-950, Toner JP: "Progress we can be proud of," 2000, with permission from American Society for Reproductive Medicine.*

As with the use of GIFT and ZIFT, proponents of co-culture hypothesized that the poor success associated with early IVF cycles was due to poor simulation of the in-vivo environment by cell culture in the laboratory. They designed a complementary solution: to reproduce the in-vivo cell-embryo interaction in embryo culture by using feeder cells. Many cell types have been used for the co-culture including cells from the human reproductive tract (granulosa, fallopian, and endometrial cells), bovine reproductive tract, and African green monkey kidney (Vero cells). The improvement in fertilization and embryo development is thought to be secondary to secretory products of or elimination of toxic factors by the feeder cells. Except when autologous grafts are used, there is always the potential for transmission of infection to the embryo. Another disadvantage of this system is the hypothetical risk of imprinting. Use of autologous endometrial co-culture with or without cryopreservation avoids these risks.

The data is conflicting. With human fallopian tube epithelial cell co-culture, Kervancioglu et al reported no change in pregnancy rates (5). However, Seta reports dramatic higher pregnancy rate with the use of autologous endometrial co-culture (6). These two examples represent a significant amount of data supporting and disputing the use of co-culture. The current debate has shifted

the focus from use of co-culture from use in all patients to use for selected indications.

Wiemar et al in a retrospective analysis concluded that the cohorts with the most significant improvement from co-culture are those with prior IVF failures, endocrine abnormalities (PCOS), elevated day three FSH, and embryos subject to cryopreservation (7). In particular, the current trend in co-culture mimics that of tubal transfers: use in IVF-ET failure. In 1994, Sakkas et al concluded that co-culture did not improve pregnancy rates in couples undergoing their initial IVF (<3) cycles using Vero cells (8). Recent studies of autologous endometrial co-culture report its effectiveness in improving embryo quality in patients with repeated IVF-ET failures, but the study designs did not evaluate the effect of co-culture on pregnancy rate (9,10). Simon et al also evaluated patients with repeated IVF failures not utilizing donor oocytes and did not find any difference in the pregnancy rate of blastocyst transfer after co-culture versus day 2 transfer without co-culture in patients not using donor eggs (11). Due to these inconclusive findings, co-culture is not widely employed.

What does the future hold for these once promising technologies? The premise of these procedures regarding the superiority of the in vivo system continues to be valid. Nonetheless, advances in embryo culture have elevated the pregnancy rates to remarkable levels—levels equivalent to those obtainable by use of these technologies. Mystery continues to engulf the embryo's interactions with the reproductive tract, and considerable hurdles still lie in the path of the reproductive specialist. As investigators strive to decrease the rate of IVF failures, the significant rate of higher order multiple pregnancies, and endeavor to improve the status quo, we may success in the inspiration behind these declining procedures.

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Current concepts on role of LH and FSH in follicle development

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It is now over 60 years since Greep and colleagues isolated from sheep pituitaries separate extracts containing either FSH (''Thylakentrin'') or LH (''Metakentrin'') activity (Greep et al 1942). Pituitary and urinary extracts of gonadotrophins containing variable amounts of LH and FSH have been in widespread clinical use for over 40 years for induction of ovulation and ovarian stimulation.

Structure and function of gonadotrophins

FSH and LH are both glycoproteins synthesised by specialised cells of the anterior pituitary. Both proteins are composed of two separate sub-units (and) linked by disulphide bonds. The subunit is common to both LH and FSH (and TSH and hCG); the subunit is hormone specific. Both gonadotrophins are glycosylated, the main oligosaccharides containing sialic and sulfonic acids. These sugar residues impair metabolism and hence prolong their biological action; FSH, which contains up to 30% sugar, has a half life which approaches 24 hours. The anterior pituitary secretes a range of isomers which differ slightly in the amount of glycosylation and hence the electrical charge of the molecules. The relative amounts of isomers which are secreted by the pituitary is sensitive to oestrogen. Post menopausal women secrete relatively more acidic forms which have a prolonged half life.

Both gonadotrophins interact with specific receptors on the surface of granulosa and theca cells to produce their biological effects. FSH classically stimulates growth and differentiation of follicles by interacting with receptors on granulosa cells. Although it stimulates a number of key enzymes involved in steroid synthesis eg aromatase, in biologically pure form it will not stimulate oestrogen synthesis in the absence of androgen precursors (Couzinet et al 1988). LH interacts with receptors on theca cells to stimulate synthesis of steroid hormones including androgens which then are utilized by granulosa cells to make oestrogens (Hillier 1994). This ''two cell — two

gonadotrophin'' model of steroid synthesis by the Graafan follicle was confirmed in women when pure recombinant gonadotrophins became available (Figure I) (Schoot et al 1992).

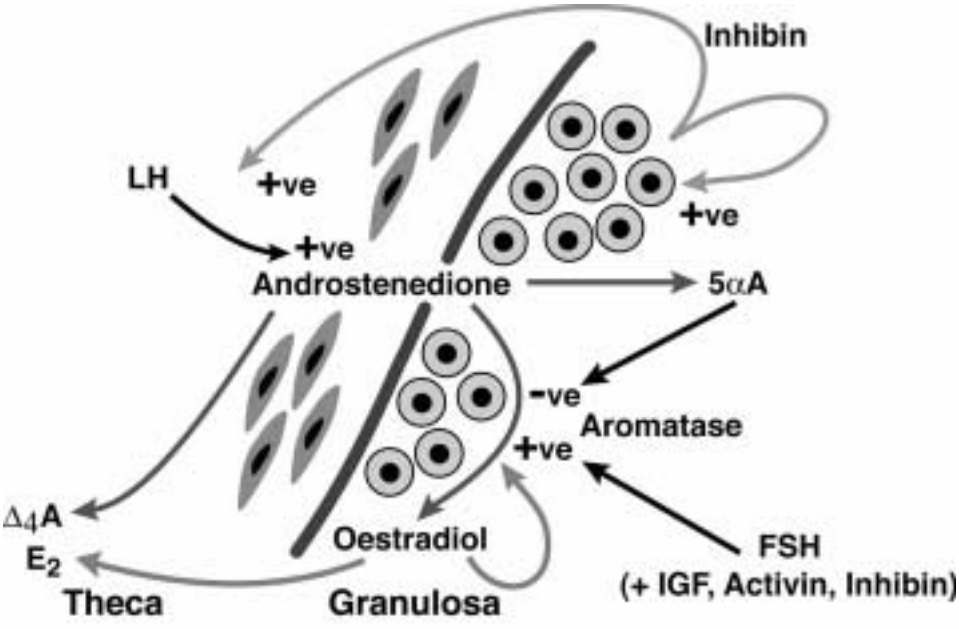

Figure 1.

The structure of both gonadotrophins and their receptors has been defined and synthetic and recombinant FSH and LH available for clinical use (Loumaye et al 1995). However our knowledge of the role of LH and FSH, in the different stages of folliculogenesis is still incomplete, and hence many of our current therapeutic strategies are empirical. In this talk I shall briefly summarize our current knowledge of the process of folliculogenesis and its hormonal control.

Folliculogenesis

Folliculogenesis is the process by which primordial follicles are promoted through a series of developmental stages until a mature oocyte is ovulated (Gougeon 1996). The steps in this process involving initial recruitment from the primordial pool, development of granulosa and theca cell layers, formation of antral cavity, occur in all mammalian species but the time taken varies from around 30 days in rodents to up to 6 months in women (Figure 2).

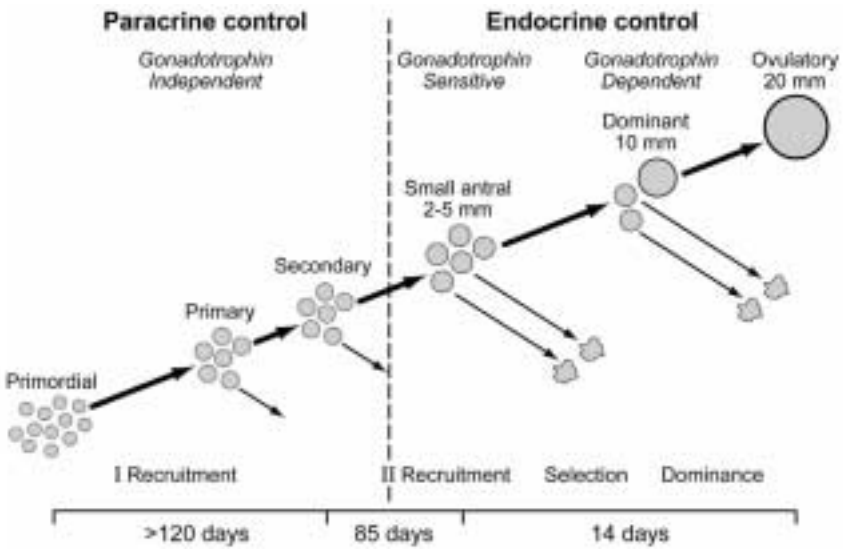

Figure 2.

Initial Recruitment

The factors regulating which follicles are recruited for development from the primordial pool are ill understood (Krarup et al 1969). The most plausible theory involves the production of some inhibitory factors (eg Anti Mullerian Hormone) from growing follicles (Durlinger et al 1999). The proportion of primordial follicles recruited for development increases as the density of growing follicles declines with age. There is no evidence that gonadotrophins influence the rate of recruitment.

Preantral Follicle Development

Once recruited from the primordial pool, the follicle grows acquiring increased number of granulosa cells and a thecal layer. It has been generally considered that because recruitment and preantral development **can** occur after hypophysectomy, that the follicle is unresponsive to gonadotrophins at this stage and that growth is dependant on local paracrine factors. However, mRNA for FSH and LH have been demonstrated in the granulosa and theca cells respectively of preantral follicles in a number of species including women (O'Shaughnessy et al 1996; Oktay et al 1997). Moreover recent research in rodents, sheep and monkeys has demonstrated that gonadotrophins can influence the rate of growth and prevent atresia in preantral follicles (Gulyas et al 1997; McGee et al 1997). The therapeutic implications of this possibility have hardly been explored.

Antral Follicle Development

Once an antrum is formed after about 12 weeks (0.1 — 0.2 mm diameter) the follicle becomes responsive to gonadotrophins (Scaramuzzi et al 1993). Receptors for FSH are confined to granulosa cells while those for LH almost exclusively in theca cells. In the late stages of follicle development functional LH receptors can be demonstrated on the granulosa cells of large follicles (> 8 mm diameter) (Figure III).

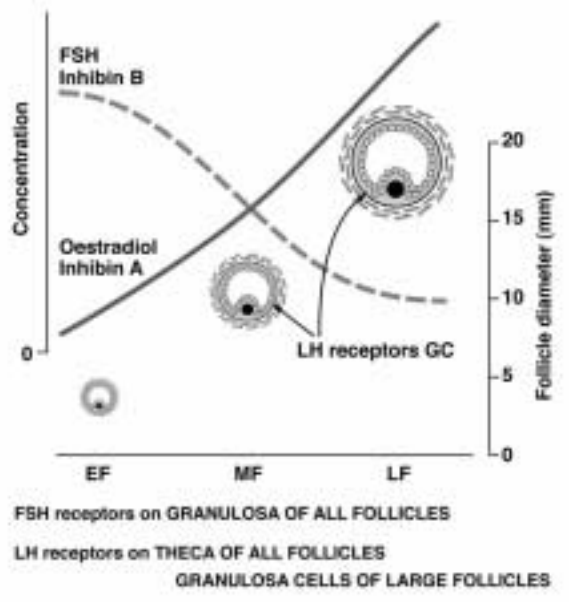

Figure 3.

Gonadotrophin receptors belong to the G-protein 7 transmembrane receptors coupled to adenyl cyclase. Thus they both may generate the same second messenger (cAMP) which accounts for the fact that some functions can be induced by either gonadotrophin eg luteinization (Yong et al 1992).

During the development of the follicle it is necessary to strike a careful balance between proliferation and differentiation. Thus FSH stimulates mitosis of the granulosa cell while at the same time inducing the expression of key markers of differentiation such as aromatase, inhibin. While FSH alone is sufficient for the production of Inhibins A and B, LH is required for the secretion of oestrogen by stimulating adequate supply of androgen precursor from the theca cells (Figure I) (Campbell and Baird 2001). Thus large antral follicles which are stimulated in hypogonadotrophic animals and human by FSH alone secrete

large mounts of inhibin but the addition of LH is necessary for oestradiol production (Couzinet 1988; Campbell et al 1999).

Selection of the Ovulatory Follicle

It is essential in all mammals that the number of follicles selected for ovulation matches the optimum number of offspring (Baird and Campbell 1998). In women the process by which a single follicle is selected for ovulation from the cohort of small antral follicles of similar size (2 — 5 mm) is not fully understood but involved three crucial steps; 1) secondary or cycle recruitment, 2) selection, and 3) dominance.

In the late luteal phase of the cycle as the levels of progesterone, inhibin A and oestradiol fall at the time of regression of the corpus luteum, the concentration of LH and FSH rise (Figure IV).

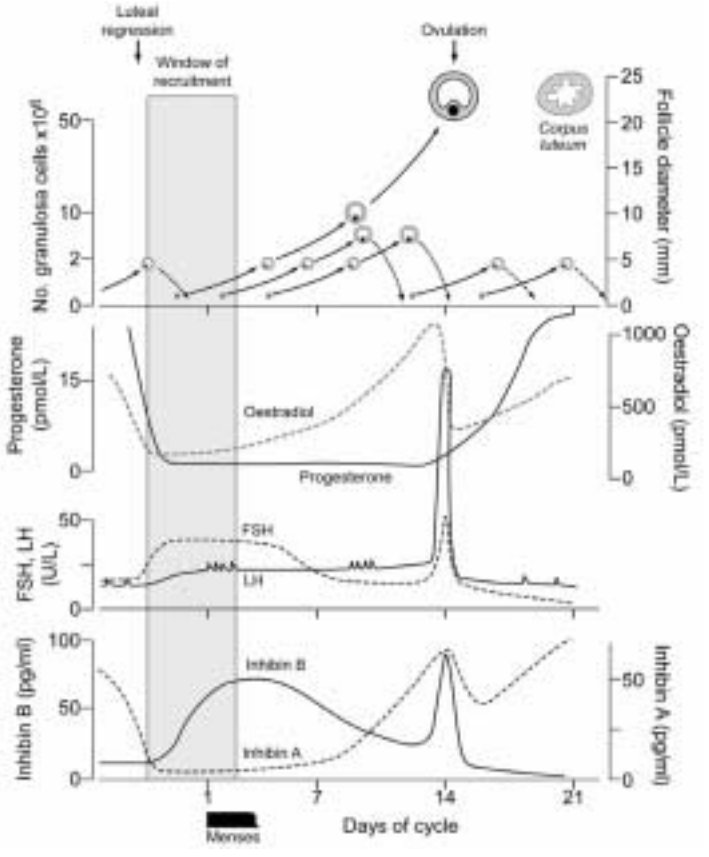

Figure 4.

The FSH rises above the threshold necessary to project the largest healthy small antral follicles through the final stages of follicular development. The maturational process is reflected in the rise in concentration of inhibin B which occurs within 48 hours of the rise of FSH.

The single follicle which is selected is probably by chance at a slightly more advanced stage of development than the rest of the cohort and is able to benefit maximally from the rise in FSH. Once selected it is essential that the dominant follicle suppresses the development of the other antral follicles which have the potential to ovulate. This is achieved by suppression of FSH below the threshold level by the secretion of oestradiol and Inhibin A. In the mid and late follicular phase of the cycle these hormones are derived almost exclusively from the ovulatory follicle which thus has total control of the feedback signals which control the secretion of gonadotrophins.

The dominant follicle maintains its growth in the face of declining levels of FSH by at least two mechanisms.

a) Around the time of selection the granulosa cells of the dominant follicle acquire significant numbers of LH receptors. Thus the granulosa cells can utilize LH as well as FSH for the generation of cyclic adenosinie monophosphate (cAMP). In this way LH may be used as a partial surrogate for FSH (Yong et al 1992).
b) It is also likely that local autocrine and paracrine factors increase the sensitivity of the dominant follicle to FSH (and LH) (Findlay 1993; Hillier 1994). The decrease in IGFBP in the ovulatory follicle increases the availability of IGF (Mazerbourg et al 2000). Inhibin enhances the sensitivity of the theca and granulosa cells to LH and FSH respectively. Thus the dominant follicle becomes increasingly sensitive to gonadotrophins and can survive in an environment which is hostile to the recruitment and from the development of subordinate follicles.

While LH may enhance the survival of the dominant follicle it probably hastens atresia of subordinate follicles which because of relative deficiency of aromatase, accumulate androgens. (Opavsky and Armstrong 1989)

Conclusions

Both FSH and LH play essential roles at virtually all stages of follicle development. The requirements for individual gonadotrophins varies depending on the stage of folliculogenesis, so that normal follicle development is dependent on a carefully controlled sequence of exposure to concentration and ratio of FSH and LH appropriate to that stage of development. Large antral follicles are completely dependent on gonadotrophins; in their absence follicular development ceases. Experiments in hypogonadotrophic sheep have indicated

that in the late follicular phase of the cycle, large antral follicles can transfer their dependence from FSH to LH and continue to develop to ovulation in the presence of subthreshold levels of FSH (Campbell et al 1999). As LH is incapable of recruiting or activating small antral follicles, this may be one of the mechanisms by which the number of ovulatory follicles is restricted. With recombinant FSH and LH now available for clinical testing, studies are underway to devise the optimum ratio of FSH and LH to achieve single ovulation in anovulatory women.

Lessons for Clinical Strategies

- Preantral follicle development may be influenced by FSH (?LH)
- LH as well as FSH required for E_2 synthesis
- FSH alone for inhibin
- Number of follicles recruited related to time of FSH stimulation
- Monovulation may be achieved more easily with LH as well as FSH
- Too much LH may cause premature luteinization and activation of oocyte

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Tibolone in a class of its own! The STEAR concept

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Summary

Tibolone is indicated for the relief of climacteric symptoms and prevention of osteoporosis in postmenopausal women. Tibolone expresses estrogenic effects in a tissue selective manner, resulting in desired estrogenic effects on tissues like the brain, bone and vagina, while avoiding the undesired estrogenic effects on the endometrium and breast. This results in tibolone's unique clinical profile. The effect on climacteric symptoms and on bone is due to the conversion of tibolone into its two 3-OH metabolites. These two metabolites activate the estrogen receptor resulting in estrogenic effect in these tissues. Increased estrogenic activity is however not seen in the endometrium and the breast. This is due to regulation of the sulfatase-sulfotransferase system and/or local metabolism. Inhibition of sulfatase activity and stimulation of sulfotransferase activity leads to a diminished estrogenic activity of both endogenous and exogenous estrogenic metabolites. Furthermore estrogenic stimulation in the endometrium is prevented by the local formation of the -4 isomer, a metabolite with progestagenic and androgenic activity.

In summary it is this tissue selective expression of estrogenic effects that makes it appropriate to consider tibolone as the first representative of a new class of compounds. A drug class should describe and capture the distinguishing properties of the compounds that make up this class.

The proposed name for this new class of compounds is ''Selective Tissue Estrogenic Activity Regulator'' (STEAR).

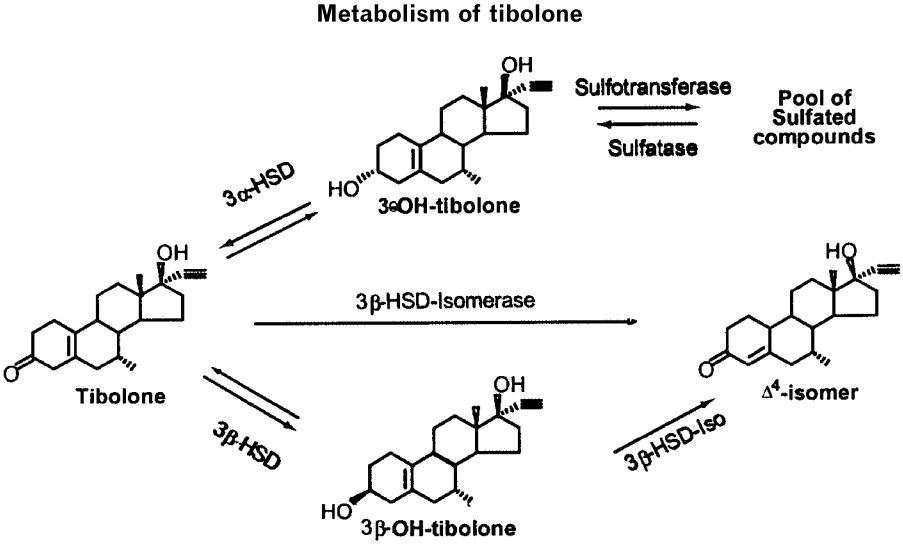
Members of this class should relief hot flushes, prevent bone loss and stimulate vaginal epithelium without stimulating breast and endometrial tissue. Additional positive effects on brain functions such as mood and libido may also be present. The regulation of the estrogenic activity may be accomplished by reducing the formation of biological active estrogenic metabolites or stimulating the formation of biological inactive estrogenic metabolites or further by the local conversion into compounds with progestogenic or androgenic activity. Excluded are compounds that exert their anti-estrogenic effect via a change in the three

dimensional structure of the receptor-ligand complex as has been shown for SERMs. The other reason why SERMs do not belong to this class is that they do not relief hot flushes and have no beneficial effect on the vaginal epithelium. Conventional estrogens like 17-estradiol and conjugated equine estrogens also do not belong to this class because they miss the necessary tissue selectivity. They stimulate the endometrium and hyperplasia can only be prevented if they are combined with a progestogen.

In conclusion, tibolone is the first well-established compound of a new class of compounds called STEAR.

Mechanisms of action

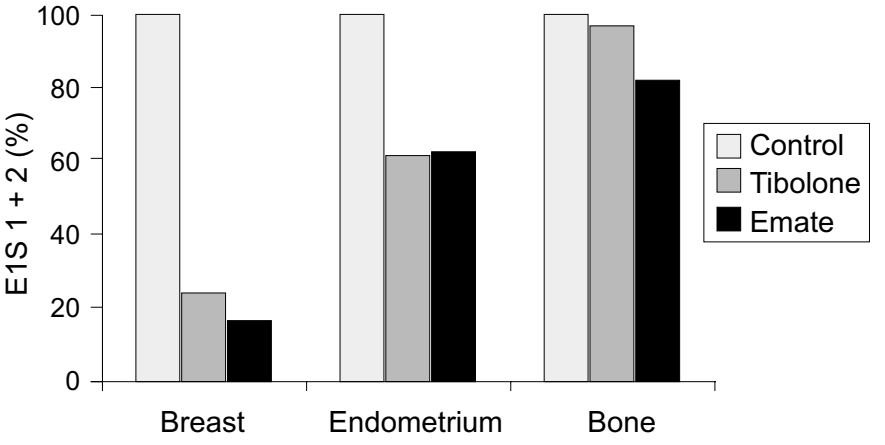
Tibolone itself hardly binds and activates the estrogen receptor (ER). The estrogenic activity results from two 3-OH metabolites that are formed by the enzym 3-hydroxysteroid dehydrogenase (HSD) in the liver and intestine. The 4-isomer of tibolone is formed by the enzyme 3β-HSD/isomerase and has both progestogenic and androgenic properties (1). The proposed metabolism of tibolone is depicted in Figure 1:



Due to the presence of a 3-OH group in the A-ring of the steroid skeleton both metabolites bind and fully activate the ER, with a preference for the ERa (1). A pool of biological inactive sulfated 3-OH-metabolites can be found in the circulation (2). Conjugation occurs in the liver via sulfotransferases and this pool may be reactivated in the various tissues by sulfatases. De Gooyer et al. showed that tibolone and its metabolites inhibit the enzyme sulfatase in a tissue

selective manner. Inhibition occurs in breast and endometrial cells but not in bone cells (3) (see Figure 2).

Selective tissue inhibition of sulfatase activity by tibolone



Gooyer et al Mol Cell Endocrinol, (2001) 183:55-62

The two estrogenic metabolites of tibolone have a lower intrinsic activity than estradiol but they are present in sufficient quantities in the circulation to generate a full estrogenic response (1). Tibolone and its metabolites cannot be metabolized into compounds with an aromatic A-ring, thereby increasing their intrinsic estrogenic activity because they are not a substrate for the enzyme aromatase (4).

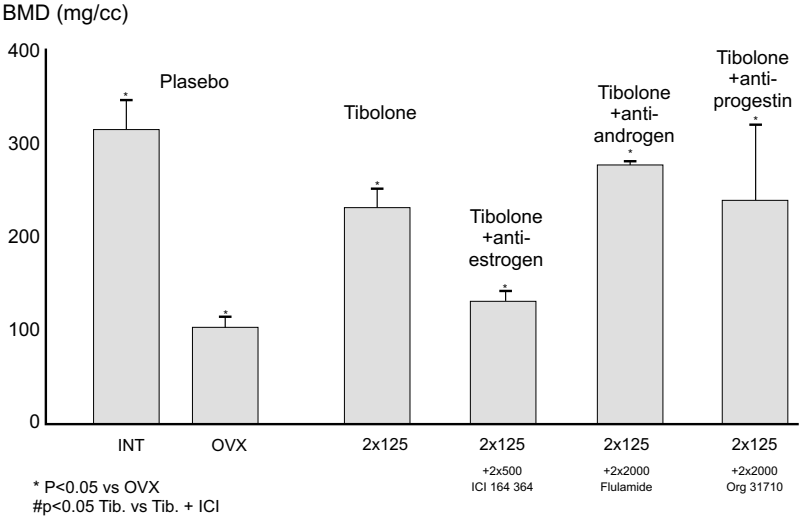
The third metabolite is the so-called 4-isomer of tibolone which exhibits progestogenic activity in the endometrium. This metabolite is locally formed in the endometrium and prevents and counteracts any estrogenic activity (5). The 4-isomer also binds and activates the androgen receptor and its androgenic properties are expressed among others in the liver and brain. The receptor binding and activation profile of tibolone and its active metabolites are shown in Figure 3.

Besides ERs bone cells also have progestogen receptors (PR) and androgen receptors (AR). It was therefore important to determine which hormonal activity is responsible for the positive effects of tibolone on bone. This has been investigated in experiments in which tibolone was combined with anti-hormones for ER, PR and AR and it was shown that only the ER was involved in these effects (see Figure 4). Thus, just like the SERMs, tibolone acts on bone via the ER only (6).

Receptor profile of tibolone and its metabolites in transactivation assays

	ER	PR	AR
Tibolone	+/-	+/-	+/-
3α-OH-tibolone	+	-	-
3β-OH-tibolone	+	-	-
Δ^4-isomer	-	+	+

Effect of tibolone and anti-hormones on bone



Ederveen et al., J Bone Min Res (2001) 16:1651-1657

Preclinical profile of tibolone

Tibolone has been tested in a number of animal models in order to explore its pharmacological effects. Many effects are similar to estrogens with some outstanding exceptions on breast and endometrial tissue. It has been shown for

example that tibolone prevents hot flushes in a similar way as estrogens. Next to that tibolone restores ß-endorphin levels after ovariectomy which can possibly explain the beneficial effects on mood. Tibolone also has an estrogen like effect on sexual behaviour in rodents and reverses vaginal atrophy. It has a positive effect on the cornification of the vagina in rats just like estrogens. In ovariectomized monkeys the improvement of the maturation index of the vaginal epithelium was similar with estrogens and tibolone (7).

In monkeys tibolone does not stimulate the breast tissue (8) and in the DMBA rat model tibolone clearly inhibits the growth of breast tumors (7). Neither tibolone nor its metabolites have any effect on the enzyme aromatase (4). However, they do profoundly inhibit the enzyme sulfatase (3) and stimulate the enzyme sulfotransferase (9). The consequence of the effects of tibolone on these enzymes is that for both the endogenous estrogens and the estrogenic metabolites of tibolone the equilibrium is preferentially towards the sulfated forms.

Tibolone does not stimulate the endometrium in ovariectomized monkeys (10) and investigations with human endometrial fragments have revealed that tibolone is specifically converted to the metabolically stable 4-isomer which has progestogenic activities (5). In addition, tibolone and its metabolites inhibit sulfatase activity in endometrial cells and as a result the sulfated estrogenic metabolites are not activated in endometrial tissue (3).

In the cholesterol-fed rabbit (11) and monkey model (12) no cholesterol accumulation in the vessel wall has been observed with tibolone despite the lowering effect on HDL. Also, the functionality of the vessel wall remains intact under tibolone. Tibolone and its estrogenic metabolites have shown a number of positive direct effects on the vascular system like the lowering of adhesion molecules (13,14).

Clinical profile of Tibolone

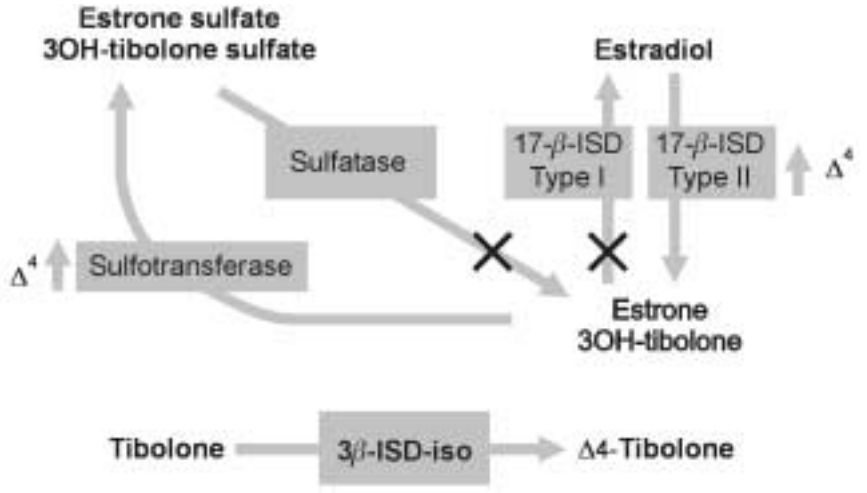
Tibolone exhibits tissue-selective effects via steroid receptor activation, metabolism and enzyme inhibition, leading to the prevention of bone loss (15) and relief of climacteric symptoms in postmenopausal women (16) without stimulating the breast (17) and endometrium (18). The actions of tibolone on bone, hot flushes and the vagina are clearly estrogen-like effects. In contrast no estrogen-like effects have been observed in breast tissue due to the interaction of tibolone with the sulfotransferase-sulfatase system.

In the endometrium, estrogenic effects are not expressed due to the formation of the 4-isomer of tibolone, which has progestogenic properties. See (19) for clinical review.

In addition, the sulfotransferase-sulfatase system is influenced in a similar

way in the endometrium as in the breast (see Figure 5) and thus preventing estrogenic stimulation of the endometrium

Regulation of enzymes in endometrium



The ideal treatment for postmenopausal women would be to relieve climacteric symptoms and prevent bone loss without vaginal bleeding and without compromising the breast and endometrium. Tibolone, SERMs and estrogen (progestogen) treatments all prevent bone loss. In contrast to tibolone, the SERMs raloxifene and tamoxifen do not relieve climacteric symptoms and may even induce them. The negative aspects of estrogen plus progestogen treatment include among others a high incidence of vaginal bleeding, high incidence of breast pain and an increase in mammographic density. These side effects are clearly less during tibolone treatment (17, 20, 21). Next to that tibolone has shown to have beneficial effects on mood and libido (22).

Some effects of tibolone on the cardiovascular system are also different from that of other menopausal treatments in that it induces a different lipid profile (decrease in HDL-cholesterol and triglycerides). This does not, however, appear to lead to a loss of function of HDL (23) or increased risk for arteriosclerosis (24, 25). Similarly, there appears to be no increased risk of VTE with tibolone (25), whilst raloxifene, tamoxifen and estrogen (progestogen) treatments all increase the risk for VTE.

Comparing these clinical profiles (see Figure 6), tibolone appears to differ from the SERMs and estrogen (progestogen) treatment and therefore represents a different class of compounds.

In the past a few names have been suggested for a new class, but either they did not completely cover the clinical profile such as SEEM (Selective Estrogen Enzyme Modulator) (9) or they gave the uniform importance to all three hormonal activities of tibolone as in SPEAR (Selective Progesterone, Estrogen and Androgen Regulator) (26).

Tissue effects in postmenopausal women

	Tibolone	Estrogen	Estrogen+ Progestogen	SERMS
Bone (BMD)	+	+	+	+
Brain (hot flushes)	+	+	+	—
Endometrium	=	—	=	=/—
Vagina	+	+	+	=
VTE	+/=	—	—	—
CAD	=/?	—	—	=
Breast	=/+	=	—	+

+ beneficial effect = no change — unwanted effect

A new class should cover the main properties of a compound. For tibolone, these are the effects on climacteric symptoms and prevention of bone loss, both of which are clearly linked to the estrogenic activity of the compound. Since estrogenic activity is not seen in tissues like the endometrium and breast, one can define the compound as a tissue selective estrogenic activity regulator.

We therefore propose Selective Tissue Estrogenic Activity Regulator (STEAR) as the name for this new class of compounds. Tibolone is the first well-established compound in this class.

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The evolving clinical story of tibolone

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Tibolone: the extensive preclinical data and clinical experience

Almost 20 years ago a new compound, a steroid, (7 alpha,17 alpha)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-yn-3-one (code name Org OD 14 or Tibolone), was found to possess concomitant weak estrogenic, androgenic and progestational activities. A number of experimental studies demonstrated that the drug suppress blood FSH and LH in without affecting PRL levels. The effects demonstrated using various endocrinological tests, inhibition of ovulation, prevention of bone loss following ovariectomy and restoration of sex drive, corresponded to this hormonal profile. Studies of the metabolites of Tibolone in rats suggested that these are involved in the complex endocrinological properties displayed by the compound. Tibolone induced a significant dose-related increase in plasma and pituitary lobe beta-EP concentrations in ovariectomized rats as compared with the results on placebo treatment (4).

The attention on this compound was extremely vivid, and a series of clinical studies have tested Tibolone. Administration of 2.5 mg/day Tibolone suppressed gonadotropins in both post-menopausal women and fertile women. At the beginning Tibolone, administered orally in a daily dose of 2.5 mg for 21 days, was shown to inhibits ovulation in young normal healthy female volunteers, aged 20-34 years and with established ovulatory cycles. The most promising results were obtained however in postmenopausal subjects. In postmenopausal women Tibolone induce an increase in plasma beta-EP and beta-LPH levels (4). Tibolone has a significantly better effect than placebo on hot flushes, sweating, sleeplessness, mood and libido, as well as on fatigability, irritability and psychic instability (5-13).

Tibolone and the concept of the tissue selective stimulator

In post-menopausal women Tibolone induced virtually no endometrial proliferation, only occasional, very slight proliferation being seen. Even after years of therapy no endometrial hyperplasia is observed. The rate of amenorrhoea

between six months and six years treatment is 90% in the tibolone treated subjects (14-27). Tibolone caused significantly fewer bleeding or spotting episodes, which were reflected by low drop-out rates due to bleeding than usual continuous combined regimens. The lack of a stimulatory effet of tibolone on the endometrium and on the myometrium, parallels a moderate and clinically relevant stimuli on the lower urogenital tract (28-30). Tibolone induces a significant increase in the karyopyknotic index and maturation value. There is a quite rapid improvement in reported vaginal symptomatology in women suffering from vaginal dryness and dyspareunia with a significant symptomatic improvement in sexual enjoyment and libido. A weak stimulatory effect on the vaginal mucosa was apparent. This null effect of Tibolone on endometrium, prompted pilot studies on patients suffering from endometriosis: Tibolone has been shown to be a safe hormonal treatment for post-menopausal women with residual endometriosis. In premenopausal women suffering form severe fibromatosis, the administration of Tibolone in association with GnRH-a reduces vasomotor symptoms and prevents bone loss, without compromising the therapeutic efficacy of GnRH-a alone. On the other hand, treating menopausal symptoms with Tibolone does not affect preexisting asymptomatic uterine fibroids. Thus Tibolone has different clinical effects at different target tissues/ organs: estrogenic on the brain, vagina, and the bone, and antiestrogenic on the endometrium. The term Tissue Specificity was then created to define the specific actions of Tibolone ion the woman's body.

The safety of a long term tibolone clinical experience

In addition to the improvement in the symptoms of vaginal dryness, dyspareunia, sexual enjoyment and libido occurs. The beneficial effect of Tibolone administration on sexual life has been attributed to the mild androgenic action of this compound. However, the incidence of side effects is very low: in particular, there are no significant changes in body weight, hair distribution of blood pressure. Extensive safety studies of up to 5-8 yr duration, including liver function tests and metabolic studies, indicated no untoward effects. Biochemical studies revealed no alteration in live enzymes, bilirubin, CBG, or cortisol, while SHBG levels were slightly suppressed. Tibolone does not negatively influence glucose metabolism and may indeed improve both the peripheral tissue sensitivity to insulin and the lipid profile (31). There was a certain drop in HDL-cholesterol with a tendency to normalise even the long-term and a simultaneous decrease in VLDL and triglycerides which should minimise the risk of cardiovascular pathology. No damaging interference with blood clotting was noted. Tibolone increased fibrinolysis parameters without significantly altering coagulation parameters. Thus Tibolone changes hemos-

tasis parameters toward a more fibrinolytic profile, which may diminish the risk of venous thrombosis (32). The cardiovascular actions of Tibolone have been extensively investigated and a possible protective action on the vascular apparatus has been recently reported (32). Tibolone and its estrogenic metabolites exert direct actions on the vascular wall, decreasing the expression of endothelial-leukocyte adhesion molecules, thus producing potentially important direct anti-atherogenic effects (33).

Tibolone, the bone and the lowering dosage

Tibolone was found to have a powerful suppressive effect on skeletal metabolism in both postmenopausal women when compared with control subjects (34-49). Tibolone reduce bone resorption in early post-menopausal women and the degree of suppression was similar to that found with estrogen therapy. Tibolone induces a significant increase in trabecular (lumbar spine) and cortical (femoral neck) bone mass in postmenopausal osteoporotic women compared to placebo, suggesting its potential to treat postmenopausal osteoporosis. In addition, Tibolone in women suffering from established osteoporosis, was found to be a bone-active compound with not only anti-resorbing effects but also anabolic activity. This was fully supported by changes in biochemical markers of bone resorption and bone formation. Thus, Tibolone increases bone mass in the spine and prevents bone loss in the forearm in early as well as in late postmenopausal women. The attention of scientific community on the effects of Tibolone on bone extended the evaluation of this drug administered at different (lower) dosages. Tibolone at two doses (1.25 and 2.5 mg/day) had similar effects, indicating that even lower doses may be efficacious. Tibolone warrants consideration not only for the long-term prevention of bone loss but also for curative treatment of post-menopausal osteoporosis (47). Recently, the use of Tibolone has been proposed as a real cost-effective treatment for either climacteric symptoms and the prevention of fractures in women with low bone mass (48). This paper related to the costs of tibolone at the standard dose of 2.5 mg/day. Data produced in our Department (49) are in line with these observations and reinforce the contention that Tibolone can offer a positive cost/benefits ratio since the lower dose, at proportionally lower costs, can exert similar bone sparing effects.

Tibolone, the breast and the ultimate indications

Extensive preclinical data have been also accumulated on the effects of Tibolone on breast tissue (50-58). Human breast cancer tissue contains all the enzymes (estrone sulfatase, 17 beta-hydroxysteroid dehydrogenase, aromatase) involved

in the last steps of estradiol biosynthesis. This tissue also contains sulfotransferase for the formation of the biologically inactive estrogen sulfates. In the past years, it has been demonstrated that tibolone and its metabolites as well as various progestins are potent inhibitors of sulfatase and 17 beta-hydroxysteroid dehydrogenase activities. It was also shown that tibolone can stimulate the sulfotransferase activity for the local production of estrogen sulfates. All these data, in addition to numerous agents which can block the aromatase action, lead to the new concept of Selective Estrogen Enzyme Modulators (SEEM) which can largely apply to breast cancer tissue. The inhibitory effect of Tibolone and of its metabolites on the enzymes involved in the biosynthesis of E2 in human breast cancer cells, points to a potential beneficial effect of Tibolone which may be of relevance in its application for the treatment of climacteric complaints. In addition, the exploration of Tibolone in trials with breast cancer patients, showing an inhibitory effect on sulfatase and 17 beta-hydroxysteroid dehydrogenase, or a stimulatory effect on sulfotransferase, will provide a new option in the treatment of postmenopausal women treated for breast cancer. The lack of a clinically relevant breast stimuli is supported also by the mammografic studies showing no increase in breast density in women treated with Tibolone (59,60). Thus, Tibolone has advantages in screening for breast cancer with mammography, which is often impaired by conventional HRT due to increased density of breast tissue. Conventional HRT induces and increase in mammographic breast density in almost 2/3 of the patients, while Tibolone can only slightly increase mammographic density in a small proportion of women. A large clinical trial on Tibolone administration in breast cancer patients will hopefully provide a definitive answer on the ultimate effect of this tissue selective modulator on breast cancer.

Tibolone today

Tibolone is an effective and well-tolerated therapy supported by a wealth of preclinical and clinical experience. Tissue-Specific activities which has the capacity to exert estrogenic or progestogenic/androgenic effects, depending on the tissue substrate. These Tissue-Specific properties of Tibolone permit it to be active in specific parts of the body as an estrogen, providing effective relief of climacteric symptoms and preventing osteoporotic bone loss. However, no estrogenic unwanted effects on endometrium and breast tissues have been reported. For this Tissue-Specific hormonal activity, we can conclude that Tibolone is a secure preparation for the treatment of menopausal women. The results of ongoing clinical trials will give us the definitive information on the effects of chronic Tibolone administration and breast safety.

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Is there a preferred protocol for ART?

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Controlled ovarian hyperstimulation (COH) has undergone endless changes since the very beginning in both stimulation protocols and physician's attitude. We have witnessed a pendular phenomenon. In 1978, Edwards and Steptoe achieved the first IVF pregnancy from a natural cycle: a single oocyte was obtained, and to do so, the cycle required continuous, exquisite monitoring of follicular growth and steroid production, a perfect coordination between physicians and embryologist to perform the retrieval at any time, and even so, success rates were very low. Then, during the 80s and 90s, multiple follicular development expanded due to gonadotropin production and administration. The introduction of the GnRH agonists that allowed a better control of the cycle reducing premature LH surge as well as cycle programming. However, these more aggressive protocols brought a higher complication rate, namely ovarian hyperstimulation (OHSS) and high order multiple pregnancies. Today, the pendulum is moving again in the other direction. COH is oriented to a milder stimulation, as we do not need too many oocytes to transfer 2 good quality embryos due to the impressive IVF lab improvements, reducing this way both complications. Some people even question today whether ovarian stimulation is required at all to do IVF.

Generally speaking, ovarian hyperstimulation may affect several critical areas in human reproduction: a higher incidence of OHSS, a higher morbi-mortality in newborns from high order pregnancies, a lower endometrial receptivity in hyperstimulated women, a social problem with frozen embryos, and last but not least, a higher cost for the patient in the IVF cycle. Considering that a natural cycle still has very low success rates and the COH is needed, the first question would be how much we should stimulate the ovaries in order to achive a reasonable success rate. We reviewed the pregnancy rate obtained at our institution according to the number of oocytes retrieved, and when only 1 to 5 oocytes were obtained, pregnancy rate was around 25%; when 6 or more oocytes were retrieved, pregnancy rates raised to 45 to 50%. Obviously, cycle cancellation rate decreased accordingly, from

almost 70% when 1 oocyte was obtained to less than 20% from 6 oocytes onwards. These data are a clear reflection of the idea that a good embryo selection influences IVF outcome. Recent work from different groups has shown through multiple linear regression analysis that a good ovarian response will yield a better embryo score, increasing pregnancy rates. Devreker et al (1) recently showed that when 2 embryos were transferred, embryo score and pregnancy rates were significantly better if more than 6 oocytes were obtained. Probably, this is due to the fact that a lower ovarian response is close to poor oocyte quality. So, if a low oocyte yield is observed with current COH protocols, we will not be able to select the best embryos to transfer but we will transfer whatever we have, and obviously these patients have a poorer prognosis than the rest.

Then, if the final goal is to transfer 1 blastocyst or 2 day-3 embryos, we would probably need 4 day-2 embryos, which require 5-6 fertilized eggs or 6 to 8 good quality oocytes. How should we stimulate a patient if we only need 6 to 8 good oocytes? New, friendlier approaches have been described.

— **"Classical" COH with lower gonadotropin doses**: a simple minded, easy principle is to reduce both the starting dose to recruit less oocytes and also the total dose. We showed that using a step-down, low dose approach the incidence of ovarian hypestimulation was significantly reduced and endometrial receptivity improved in a group of high responder patients (2).

— **Recover drugs from the past: the new clomiphene citrate (CC) protocols**. Although extensively used in the past, CC was forgotten in ART protocols after the introduction of the potent gonadotropins and GnRH agonists the blocked the pituitary gland. Another reason not to use CC was the antiestrogenic effects observed on the endometrium. With the new GnRH antagonists, that do not need a previous down-regulation, and the lower number of oocytes required to do a good embryo transfer based on the recent improvements developed in the IVF labs and embryo culture media, CC is being reintroduced in combination with gonadotropins to balance its antiestrogenic actions. In a recent report, Williams et al (3) reported their experience in 55 good prognosis women who were offered a mild stimulation protocol to reduce the costs of medication in the cycle. They received 100 mg CC from cycle day 3 to day 7, continued with 150 IU of rFSH and GnRH antagonist was introduced when the leading follicle reached 14mm. When compared to patients undergoing a long protocol, they observed a similar length of stimulation, with obviously a lower number of FSH ampoules and a lower number of oocytes retrieved but a similar number of embryos transferred and a comparable pregnancy and implanta-

tion rates. It is interesting to note that 8/55 cycles were cancelled in the CC groups opposed to 1/55 in the long protocol group. Similar results have been obtained by other groups (4).

— **New drugs: GnRH antagonists**. These new drugs have been introduced in our routine protocols, but we are still trying to elucidate how can we obtained the most benefit from them compared to the widely used agonists. The needless down-regulation offers the possibility to rely much more in the physiology of the ovarian cycle, recruiting a hypothetical better oocyte cohort which did not undergo the direct effects of the GnRH agonists. Using this approach, Hohman et al (5) recently performed a very interesting study, randomizing patients into three groups: long protocol, antagonist protocol starting rFSH on cycle day 2, and a similar protocol but starting rFSH on cycle day 5. The rationale under this protocol was to wait for the ovary to select the best oocytes available in the cycle. Although cancellation rate was higher in this last group, a significantly higher number of patients reached embryo transfer when compared to the other two groups, probably due to a better oocyte quality. Pregnancy rate per embryo transfer as well as per started cycle was similar among groups. This ''late start rFSH'' group showed also a significantly better embryo score and a higher number of embryos score as 1. Thus, a higher number of patients may reach the oocyte retrieval stage with the traditional protocols, although their embryos may be arrester prior to embryo transfer. A more physiologic approach offers the possibility to select the best available oocytes for a good embryo development.

— **The mildest approach: natural cycle**. The first IVF baby born was, as we all know, from a natural cycle. There are substantial benefits from such an approach: natural, physiologic oocyte selection and endometrial development, no complications derived from COH will appear, oocyte retrieval may be performed without anaesthesia, reducing the cost for the patient and improving patient tolerability, as well as it allows month after month repetition if pregnancy does not occur (6). However, the major drawbacks from the natural cycle are the lower success rates (around 7% per cycle) and a very high cancellation rate (over 30%) due to many different reasons as abnormal folliculogenesis, premature ovulation, unsuccessful oocyte retrieval, fertilization failure or poor embryo quality (6). It may be an option in young, low responder patients in whom IVF failed and before offering them oocyte donation. We have performed 62 natural cycles in 25 young patients who did not produce more than 2 eggs in prior IVF attempts. Although 66% of the cycles were cancelled, in those who reached embryo transfer, a respectable 19% pregnancy rate was obtained.

New developments and improvements in IVF labs together with the new and old drugs in the market are changing our attitude towards ovarian stimulation in ART. Friendlier ovarian stimulation may influence the duration of the stimulation, improve patient tolerability and compliance, and reduce the secondary effects and the costs for the patients. However, we still have to precisely define what it is considered a ''good'' response to a mild ovarian stimulation protocol.

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Why LH is important if pure FSH works

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Abstract

Recent evidence suggests that an appreciable proportion (up to 50%) of normogonadotrophic women subjected to long protocol GnRH-a/FSH treatment acquire endogenous LH levels that are so low that the outcome of ART is compromised. Controversy exists regarding the level at which (the lower LH threshold) ovarian stimulation with gonadotrophins containing LH activity is required in order to optimise ART outcome. Based on a retrospective analysis of data from two previously published studies from our group, it is proposed that serum LH levels below 1.2 IU/l at the start of stimulation constitute a clinical significant threshold that requires addition of exogenous LH to the stimulation protocol. However, further research is needed to defined the optimal timing and daily dosage of exogenous LH during COH.

Introduction

It is undisputed that both LH and FSH are required for normal ovarian function. It is also clear that FSH is the principal regulator of antral follicular growth. However, the precise gonadotrophin requirements of the developing ovarian follicle are not yet established, in spite of intense research and clinical use of gonadotrophin preparations with various content of FSH and LH over the years, Especially, the importance of increased or decreased levels of circulating LH during ovarian stimulation in ART has been intensely debated during recent years.

Deteriorating effects of increased basal LH levels on reproductive outcome - both in women achieving pregnancy spontaneously and in those undergoing ART treatment for infertility - was reported in the 1980's (1,2). These reports boosted the development of protocols for controlled ovarian stimulation (COH) that aimed at minimizing levels of circulating LH. To this end two measures were introduced in COH for ART treatment: i) use of gonadotrophin releasing hormone agonists (GnRH-a), and recently also GnRH antagonists, to down-regulate the pituitary output of gonadotrophins, and ii) administration of

gonadotrophin preparations containing negligible amounts of LH activity (u-FSH and u-FSH-HP) or no LH activity at all, such as the recombinant FSH (rFSH). This has resulted in protocols, which effectively reduce circulating LH during COH to levels considerably lower than observed during the follicular phase of the normal menstrual cycle. Superiority of the so-called long protocol, i.e. initiation of GnRH agonist in the mid-luteal phase, over other protocols (short and ultra-short) with regard to reproductive outcome after IVF is well documented (3). Moreover, since this protocol allows for programming of COH and avoidance of oocyte aspirations in weekends, the long protocol has gained tremendous popularity in the fertility clinics through out the world. Thus, the long protocol is used in more than 80% of IVF cycles in Europe.

Obviously, the combination of long protocol GnRH-a down-regulation and rFSH results in the lowest possible levels of circulating LH during COH, and in many cases levels (< 1.2 IU/l) that are comparable to those found in WHO group I women with hypogonadotropic hypogonadism. These patients are known to require stimulation with both FSH and LH (HMG or recombinant LH) to achieve pregnancy and delivery (4,5). Nevertheless, large prospective studies have documented good results by use of the long protocol GnRH-a/FSH in normogonadotrophic women, suggesting that LH levels much lower than during the follicular phase of the natural menstrual cycle may be sufficient to secure a satisfactory outcome of ART (6,7). Against this notion are the results of a number of recent studies demonstrating that an appreciable proportion of normogonadotrophic women subjected to long protocol GnRH-a/FSH treatment acquire endogenous LH levels that are so low that the outcome of ART is compromised (8,9). The exact lower threshold level of endogenous LH that separates a good or bad outcome of ART in normogonadotrophic women during therapy with GnRH-a/rFSH, however, is poorly defined and remains a controversial issue in the current scientific debate. In addition, controversy exists regarding the importance of low LH levels at the start of stimulation or during the mid- or the late follicular phase of COH.

Defining a lower LH threshold in GnRH-a/FSH treated normogonadotrophic women

A number of recent retrospective studies have addressed this issue, but the results are conflicting and difficult to compare due to differences in the definition of severely depressed LH levels (9,10,11,12). Since mid-follicular LH levels <3.0, <2.0, <0.7 and < 0.5 IU/L have all been used as threshold to assign women to the ''low'' LH group, and since preparations and mode of administration of GnRH-a differ, it is not surprising that the proportion of women in the low LH group varies widely among the studies from 7% (12) to 49% (9). In keeping with the two-cell, two-gonadotrophin concept most studies find

significantly reduced levels of E2 in the low LH group compared to the normal LH group, while other outcome parameters related to the LH threshold varied among the studies. Some studies reported significantly decreased fertilization rates, reduced in vitro embryo development, decreased rates of implantation (10,11) and increased occurrence of early pregnancy loss in the low LH groups (9), while one study was unable to confirm this (12). Not withstanding these discrepancies, the above findings indicate that at least some normogonadotrophic women subjected to GnRH-a down-regulation might improve their chances for a positive outcome of ART by being treated with gonadotrophins containing LH activity (HMG or rLH).

Comparing use of recombinant FSH and HMG in GnRH-a down-regulated ART cycles

Considering that recombinant FSH has been available for clinical use for the last decade (and HMG for more than 40 years), surprisingly few clinical trials directly comparing the effects of rFSH and HMG in ART cycles have been published so far. A recent meta-analysis of available (N=4) truly randomised trials comparing rFSH and HMG in GnRH-a down-regulated ART cycles showed no significant difference regarding ongoing pregnancy/delivery rates, although the clinical pregnancy rate was of borderline significance ($p=0.05$) in favour of HMG (13). While these results do not give a clear guidance as to the right choice of gonadotrophin preparation for COH in long protocol GnRH-a down-regulated normogonadotrophic women, they do not rule out that individualization of COH on the basis of LH levels could lead to significant improvements of reproductive outcome. Thus, in one of the studies included in the meta-analysis, we could demonstrate a significant correlation between the stimulation protocol, mid-follicular serum LH levels and reproductive outcome of ART (14). More recent results by others support this notion (15,16).

Supplementation of the stimulation protocol with gonadotrophins containing LH activity

Although the above findings give strong evidence to suggest a benefit of supplementation with LH to some normogonadotrophic women subjected to GnRH-a down-regulation, we are still left with the following questions: a) at what stage of the cycle (early-, mid or late follicular phase) and at what level of circulating LH should supplementation with exogenous LH be started, b) what dosage of LH should be chosen and c) can overdosing with LH occur, i.e. is there an LH ceiling level that if surpassed will affect the outcome negatively?

In order to address some of these questions we analysed the combined the results of two published studies from our clinic: a retrospective study including 200 couples (9) and a prospective study including 379 couples (14). Assuming

that a serum LH below 1.2 IU/l represents a hypogonadotropic state in analogy with WHO group I women requiring LH supplementation throughout COH, we analysed the reproductive outcome in relation to treatment with FSH or HMG in the women with serum LH above and below 1.2 IU/l at the start of gonadotrophin stimulation.

Material and methods

All women who underwent embryo transfer in the two studies were included, totalling 507 normogonadotropic women, who were treated for infertility with IVF or ICSI. All women were subjected to a standardized hormonal treatment regime, including pituitary down-regulation with Buserelin from cycle day 21 for fourteen days. After down-regulation was ascertained by ultrasound and se-E < 200 pmol, all women were treated with rFSH or hMG 225 IU/day for 7 days. Hereafter, the dose of gonadotrophins was individualized according to ovarian response. Ovulation induction with hCG (10.000 IU) was given when at least 4 follicles with diameter of 17 mm was seen by ultrasound. Oocytes were retrieved 36 hours later by ultrasound-guided aspiration. After fertilization embryos were cultured for 3 days, and a maximum of 2 embryos transferred back to the uterus.

Results: Of the 507 women 283 (56%) had se-LH on stimulation day 1 above 1.2 IU/L, and the remaining 224 (44%) below that level. In the former group (LH > 1.2), 174 and 109 were treated with rFSH and hMG, respectively. In this group of women pregnancy and delivery rates were similar (rFSH: 42% and 33%; hMG: 47% and 36%, respectively), while the implantation rate was higher in the hMG group than in the rFSH group (31% vs. 26%; $p = 0.05$). In the group of women with LH < 1.2, 165 and 59 were treated with rFSH and hMG, respectively. Pregnancy and delivery rates were significantly higher in the hMG group: 58% and 49%, compared to the rFSH group: 38% and 27%, respectively ($p < 0.01$ and < 0.005). The implantation rate in the hMG group was 37%, which is significantly higher than the 22% found in the rFSH group ($p < 0.005$).

Conclusions

An appreciable proportion of normogonadotrophic women subjected to long-protocol GnRH-a down-regulation experience so low levels of circulating LH that the reproductive outcome may be compromised. A serum LH level of 1.2 IU/L at the start of ovarian stimulation seems to constitute a clinically significant threshold. Patients with serum LH below 1.2 IU/L on stimulation day 1 will benefit from co-administration of LH during ovarian stimulation for ART.

Since doubts still exist regarding the right dosage of exogenous LH and

whether overdosing with LH can occur, further prospective trials are required to define the optimal stimulation protocol in relation to serum LH levels.

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Ovarian surgery for PCO. Why is it not more popular?

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While the polycystic ovarian syndrome (PCOS) appears at an incidence of 20% in the infertile population, it appears at a prevalence rate of 3-7% in the general population. It is characterised by an increased body mass index, an increased LH/FSH ratio, hyperandrogenemia and hyperinsulinemia. While the ovarian androgens are increased (testosterone), the serum sex hormone binding globulins (SHBG) are decreased. This is followed by an increase of the serum-free testosterone concentrations. As high levels of androgens lead to chronic anovulation and massive disturbances in the menstrual cycle, these patients typically have elevated LH levels, increased LH/FSH ratios and elevated insulin resistance. As the hyperandrogenism is closely tied to the insulin resistance, the decline in insulin levels leads to a decreased androgen production. The insulin resistance seems to be most important in the development of PCOS. A lowering of the androgen level does not lead to an improvement in insulin resistance. Hyperandrogenism leads to abnormal folliculo-oogenesis and endometrium development. There is a risk for the development of glucose intolerance type II diabetes mellitus and gestational diabetes mellitus (Dahlgren et al. 1992a,b, Franks et al. 1999, Legro et al. 1991). **Mechanism of insulin resistance development**

Although it is not yet known whether patients with PCOS have an increased severity of cardiovascular morbidity and mortality, these patients certainly have an increased prevalence of cardiovascular risk, such as diabetes mellitus, dyslipidemia, obesity and hypertension (Legro et al. 2001). While observing PCOS patients, doctors need to pay attention to the potential long term medical outcome of the disease, endometrial hyperplasia and carcinoma. There is also evidence of a positive correlation between the serum insulin levels and the plasminogen activator inhibin (Sampson et al. 1996).

Treatment options

The most common treatment option is still ovarian drilling.

Homburg 2002 and Nestler 2002 have reviewed the evidence for using insulin sensitising agents to treat infertility. These papers reviewed the evidence for the role of metformin alone or in combination with chlomiphine citrate in increasing the incidence of ovulation induction and menstrual cyclisity.

The question still remains unanswered in some reviews (Homburg 2002, Nestler 2002) whether the insulin to glucose ratios are useful in predicting which patients will benefit from the insulin sensitising drug or whether all PCOS patients will benefit. It is not clear whether glucoseratios in slim PCOS patients are different from those in obese women in terms of their benefit for metformin. Should metformin be used in all patients undergoing gonadotrophin stimulation for ovulation induction or IVF? Does metformin reduce the risk of miscarriage? Is there an increase in cardiovascular disease among women with PCOS and, if so, does metformin reduce the risk of diabetes and cardiovascular disease in these patients?

It remains clear that simple laparoscopy with ovarian biopsy and ovarian drilling is still an invasive procedure and therefore questioned by many endocrinologists. The technique is simple, consisting of a laparoscopic drilling of approx. 20 holes in each ovary, releasing the follicular fluid and ensuring that the entry hole is large enough for further fluid production to exit.

Our experience

In 54 cases of polycystic ovarian disease an increased LH/FSH ratio occurred in 35 patients. Of these 35 patients, seven were obese and 26 had an increased insulin resistance test. Ovarian drilling was meticulously performed with the monopolar hook or a branch of the monpolar forceps. Both instruments were introduced to the depth of the follicle, leaving a gap at the follicular surface of at least 1 mm.

Description of technique

Ovarian biopsy and ovarian drilling

While the ovary is held by atraumatic forceps, the biopsy forceps are inserted through a 5 mm port and a biopsy is taken from the depth of the right ovary. The wound is coagulated with the bipolar forceps. Twenty holes are drilled into the unruptured follicles and the fluid is released. This is followed by careful rinsing with Ringer's lactate and inspection of the ovary (figure 1). Within the last year we had no case of pelvic irritation in the postoperative phase.

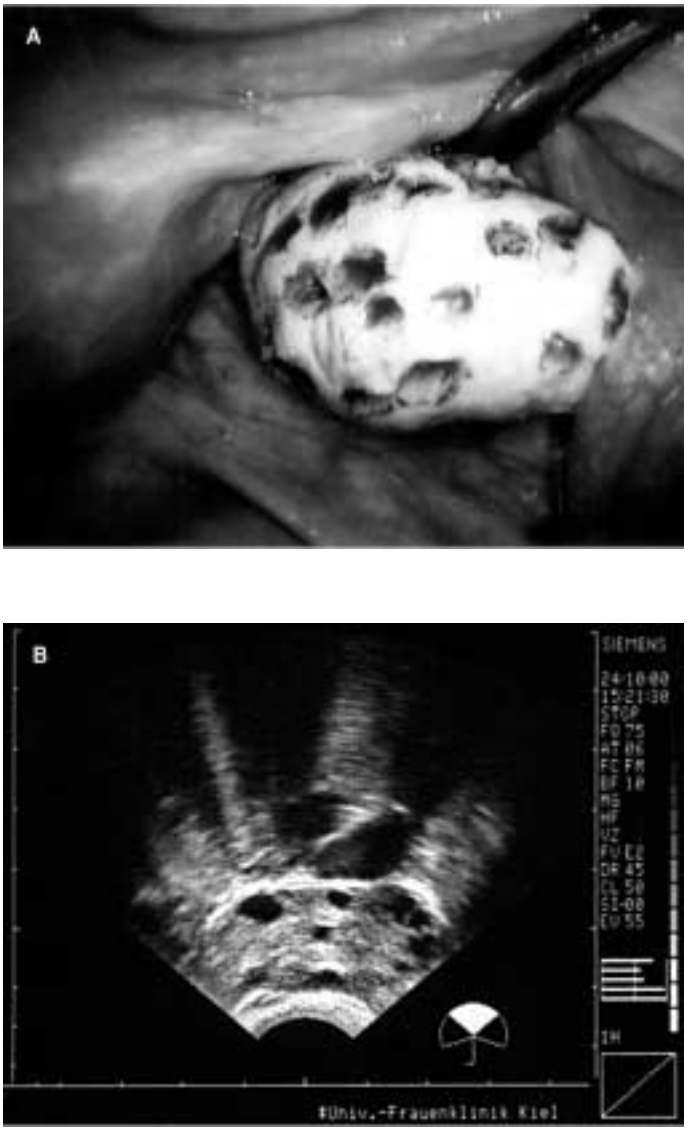

Figure 1: *Original ultrasound scan and pelviscopic site of polycystic ovarian syndrome*
A) laparoscopic site after puncture of the ovar
B) vaginal ultrasonic scan of the ovary

Why is ovarian surgery for PCOS not more popular?

It requires laparoscopy, anaesthesia and surgical skill. Clomid and Gn-Rh agonist or antagonist treatment are still in the experimental phase.

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Can we meet expectations of the chronically ill patient? — A multidisciplinary approach to endometriosis

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Summary

There is no blue print treatment of choice for endometriosis, nor a reliable diagnostic procedure to predict treatment outcome. In fact, the evidence supporting the use of many medical therapies for endometriosis may not be as robust as we might wish to believe [1]. Subsequently treatment failures within this field are common.

"Let's see what happens" is potentially the most damaging phrase to patient confidence. However, by listening to the patient, communicating in a positive language, setting realistic expectations, and involving her in decisions about her treatment, outcomes may improve.

Assessment of therapeutic effectiveness should not rely exclusively on clinical data, but should include patient based outcome measures. Thus, an open mind and willingness to explore a combination of therapies becomes essential.

Introduction

To borrow from Sir Winston Churchill, endometriosis is "...a riddle wrapped in a mystery inside an enigma". There is still no agreement about its origin nor a definitive cure for it. And, if endometriosis is a mystery to physicians, imagine how frustrating it is to those who suffer from it?

A woman with endometriosis must deal with a disease that in many cases will interfere with her life, her ability to function sexually, threaten her

fertility, and impedes her from having a satisfying work life and participate in the normal activities of living, playing, and carrying on meaningful personal relationships.

Furthermore, women with endometriosis must struggle with the taboos that surround it (menstruation, female sexuality, and to a lesser extent infertility). The disease is particularly hard to cope with because it is not visible, and it is hard to speak about in society.

In short, from the woman's point of view, endometriosis can be a nightmare of misinformation, myths, taboos, lack of diagnosis, and problematic hit-and-miss treatments overlaid on a painful, chronic, stubborn disease.

The pressures of treating patients that do not get better are huge, and a challenge both for the women themselves, but also for the treating physician.

Background: the stigma of chronic pain

The most widespread misconception about chronic pain is that it results from a psychological disturbance. However, three studies (one for chronic pelvic pain and two specifically for endometriosis) show that psychological profiles return to normal or near normal after patients are successfully cured of their pain — or in the endometriosis studies, after the endometriosis is removed [2, 3, 4].

The late Arnold Kresch has said that the psychologising of endometriosis represents gynaecology's failure to diagnose. Dr Kresch was trained as both a gynaecologist and a psychiatrist. Because he was a psychiatrist, hundreds of women with chronic pelvic pain were referred to his practise as "mental cases". In 850 laparoscopies performed on patients referred for pain of six months or longer, Dr Kresch found histologically proven endometriosis or adhesions in 92% [5]. If women are told symptoms are in their head, they will find it much harder then to cope — especially when they know in their own bodies that their pain is real.

This has been further substantiated in a recent study by Jon Stone [6], which confirmed that many diagnostic labels that are used for symptoms unexplained by disease have the potential to offend patients. Although "medically unexplained" is scientifically neutral, it showed to have surprisingly negative connotations for patients. Not surprisingly, a diagnosis of "symptoms are all in the mind" and "hysterical weakness" had the highest offence scores, and their continued use is hard to justify.

In reality, what happens when pain occurs and is not adequately controlled by treatment, is that a pattern often emerges, where it soon becomes difficult to tell which feeling leads to which, or where one emotion stops and another begins (see figure 1).

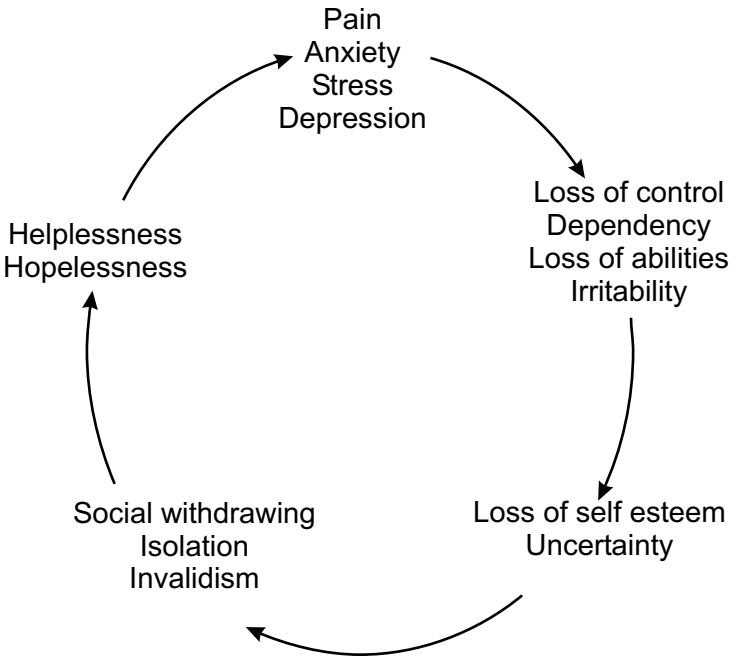

Figure 1: *The pain cycle*

Ray Garry's study from 2000 also suggests that if pain has been with an individual for a very long time, then even after the disease has been removed and the pain has disappeared, she might not necessarily feel that her quality of life has improved [7]. The pain may have caused the woman to feel that she has lost control, because she has become so used to the pain controlling what she could do. So even when the source of the physical pain is under control, emotions can compound pain and these can continue to affect an individual.

Communication becomes key: set realistic expectations!

The way in which the diagnostic news is delivered affects how it is received, and the role of the communicator becomes very important. To quote David Barlow: "communication and listening are vital in the management of endometriosis".

Disbelief is often the first emotion to strike, followed rapidly by fear. Fear of the unknown. The woman will have lots of questions: Will she be able to have children? Will the disease lead to hysterectomy? Will the pain ever end? Will endometriosis lead to cancer? Will the disease recur? What will happen if she does not get treatment? Will it get worse? Will she pass it to her daughter? She may fear further tests and treatments, and experience anxiety when having to

face unfamiliar medical tests or therapeutic procedures. And then comes the worry about possible side effects.

Reality is that most fears are due to a lack of information — and the way in which this information is communicated.

"Let's see what happens" is potentially the most damaging phrase to patient confidence, and doctors sharing verbal uncertainty can unnerve patients [8]. The worst 10 words or behaviours have shown to be:

1. "Let's see what happens"
2. "I don't know"
3. Asking a nurse for advice
4. "I think this might be..."
5. "I haven't come across this before"
6. "I'm not sure about this"
7. "I need time to find out more"
8. Using a book to find out about a condition
9. Using a computer to find out about a condition
10. Asking another physician for advice.

The physician *has* to explain what is happening, *must* provide information, and *needs* to take the time to answer questions.

The resentment and frustration, which many with chronic diseases develop over time, more often than not, result from a mismatch between clinical management and the patient's expectations — a mismatch that leaves the patient unprepared for the possibility of recurrence after treatment.

For example, if a patient has been promised a 100% cure, and yet she finds herself only 80% symptom free, the outcome is not successful. Conversely, if it has been communicated to her that any given treatment may only give 75% relief, and indeed it does — expectations have been met! (see figure 2)

Expectation	Outcome	Successful?
100% symptom free	80%	No
80% symptom free	75%	Maybe
75% symptom free	80%	Yes

Figure 2 *Lone Hummelshoj, 2002*

The multi-disciplinary approach

Because physicians see patients for short amounts of time, and these visits often are about specific aspects of the disease, the true chronic aspect and full scope of endometriosis may not always be apparent.

With no blue print in place for the treatment of endometriosis, and the knowledge that different treatments may work for differently for individuals, a team of medics more often than not need to get involved. This therapeutic network may consist of gynaecologists, surgeons (from a number of disciplines), nurses, physiotherapists, counsellors, psychologists, and nutritionists, who can all play an important role in providing a holistic and individualised treatment plan for a woman or girl with endometriosis.

It is imperative that patients have the full support of their physician to ensure that science — not untested myths — determine treatment options. However, given the sometimes chronic and stubborn nature of the disease, there may be times when it can be beneficial to explore complementary therapies beyond the medical mainstream. These include homeopathy, osteopathy, herbs, traditional Chinese medicine, and others, and these may work well alongside traditional medical management [9].

Highly personal issues mean that the decisions involved in treatments are very individual. Yet despite no overwhelming medical evidence to support particular treatments over others at this time, some clinicians do not present a full range of options to patients to allow them to be involved in their treatment decisions. Long-term, individual, holistic treatment plans needs to be worked out between the woman *and* the physician. If a woman has been part of the decision making process herself, her treatment outcome may also improve.

Extending the therapeutic network

For some women, all they need is to talk to others with the disease — to share a mutual experience, coping techniques, and effective treatment methods. The Internet, such as EndoZone.org, and patient support groups may play an important role in providing women and girls with emotional support, and these organisations can, and do, collaborate closely with physicians in providing guidance and information about the disease — as an extended therapeutic network.

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Should we treat endometriosis in teenagers?

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Introduction

Endometriosis remains a disorder which is difficult to diagnose and to treat. It is often a progressive disease reduces fertility and always threatens to recur. It is for this reason that management is primarily dictated by the patients hopes for permanent pain relieve and/or future pregnancies. We know the frequency of endometriosis (fig. 1.)in special groups — for example 1,5 — 8 % in teenagers — but the true incidence in the female population is unknown and estimated with 8 % — 12 %, and the disease accounts for 10 % to 20 % of all female infertility.

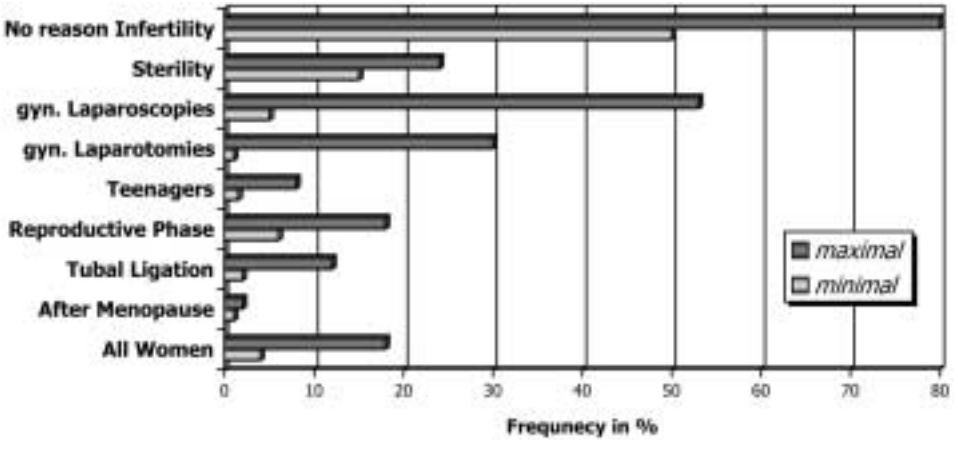

Fig. 1: *Frequency of endometriosis in special groups*

Beside endoscopic surgery medical treatment designed to suppress ovarian function or exclude estrogen activity most likely offers the best chance for clinical remission of endometriosis. Follow-up studies have shown, that endometriosis seems to be a chronic disease with a high recurrence rate of symptoms after medical and / or surgical treatment and morphological

examinations lead to the concept, that only permanent deprivation of estrogens can cure the disease (Schweppe et al. 1981).

Special diagnostic Problems

Endometriosis has a wide range of symptoms mostly depending of the location and activity of the foci; in contrast to the general patients with endometriosis teenager presented mainly — in more than 2/3 of the cases — with dysmenorrhea and lower abdominal pain (fig. 2). Teenagers with clinical suspected endometriosis have the disease confirmed by laparoscopy in only half of the cases and confirmed by histological verification in only, that means: the false positive rate is more than twice as high as compared to the women in the 3rd decade. In consequence: the diagnostic and therapeutic laparoscopy has to be indicated very strongly in teenagers.

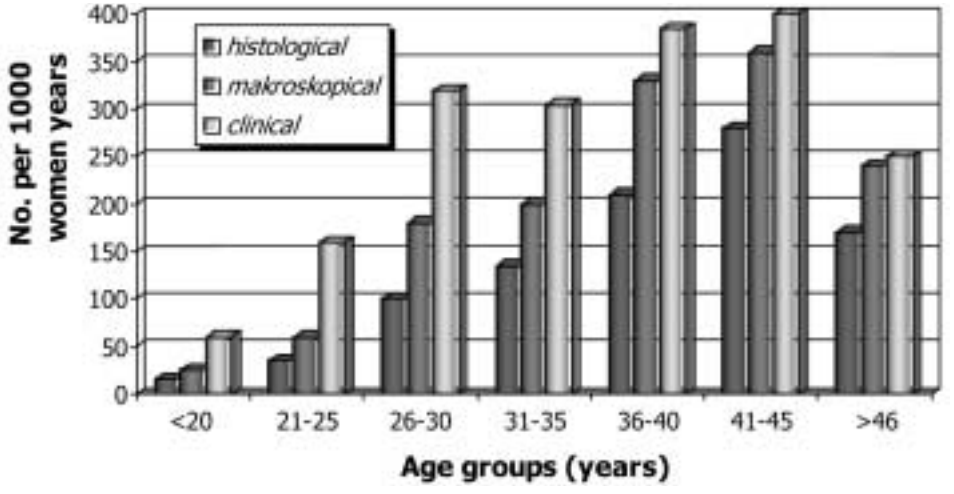

Fig. 2: *Symptoms and gynaecological findings in patients with endometriosis: teenagers in comparison to women > 20 years of age.*

Waller and Shaw reported (1993) about 130 patients medicated with different GnRH-agonists, who had been followed up for a minimum of 5 years. The cumulative recurrence rate after 60 months was as high as 53,4% with a higher risk for progressed disease. For mild endometriosis they found a 36,9% recurrence rate, while for severe endometriosis a 74,4% recurrence rate was reported. A study of the German Endometriosis Foundation (1992) reported during a follow-up period of 36 months in 476 patients recurrence rates between 32% and 50%. There were no statistical significant differences in the recurrence rates between the different treatment groups. Similar results were published by

Nieto et al. (1996) who observed a 67% recurrence rate of symptoms after intranasal application of buserelin for 6 months. Regidor et al. (1997) reported recurrences in 28 of 42 patients (mean follow-up 82 months) but associated with an improvement on quality of life due to less severe subjective symptoms. These data were confirmed in a study with additional laparoscopy to confirm the recurrent endometriosis histologically (Martschausky et al. 1996). The recurrence rate was stage related and as high as 90% in stage IV and only 28% in stage I rAFS, which demonstrates the need for an early diagnosis and early treatment of endometriosis (fig. 3) — especially in young patients.

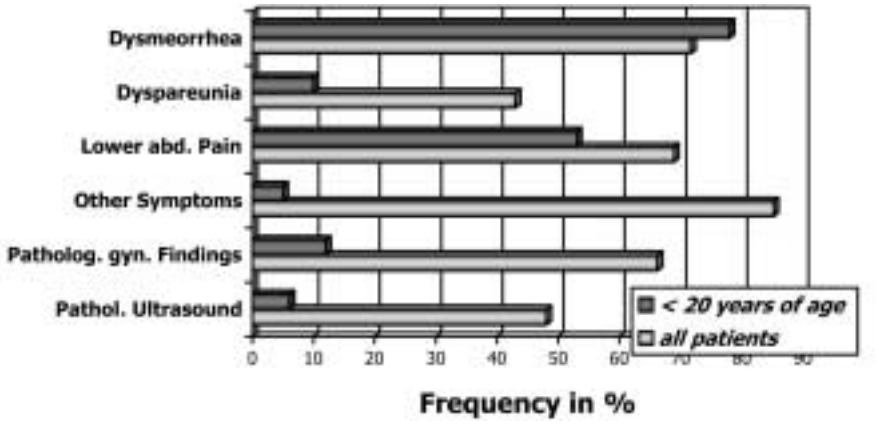

Fig. 3: *Recurrence rates of endometriosis in relation to the stage of the disease.*

These disappointing data are in agreement with the recurrence rates after progestin medication or laparoscopic surgery and emphasise the point, that our strategies in the past, which were based on eradicating the implants, nodules or cysts surgically or medically, are insufficient. We need individualised concepts, which take into account from the first therapeutic activity, that repeated treatment or long term medication will be necessary especially in very young patients — but at the same time we have to avoid overtreatment.

Therapeutic strategies

The treatment of external endometriosis in young patients is governed by the patients symptoms and complains; next in importance are the reproductive expectations for the future. The treatment has to be tailored to the individual situation and the stage of the disease, because no method of treatment will certainly cure all patients. One of the main reasons for an individual treatment is the individual character of the disease. Endometriosis has a broad variation and

there are different types of disease. This is true for the macroscopy, histology and biochemistry of this enigmatic disease. These biochemical, morphological and macroscopical characteristics will decide whether or not endometriosis can be modulated by hormones, i.e. whether hormonal therapeutic regimens will be effective (Schweppe, 1984) If endometriosis can not be eradicated but only suppressed by drugs alone there is the question about the length of treatment. Most paper recommend a period of medication between 3 to 9 months but there is very little research done on this problem especially in teenagers. The main reasons for limit of duration of treatment are symptomatic and/or metabolic side effects, costs and the physicians desire to give as few drugs as possible. The best solution seems to use drugs, which give sufficient pain relief, which have few side effects and are cheep in price too — oral contraceptives are. But is there progression of endometriosis during the intake of combination pills ? Are oral contraceptives effective in moderate and severe stages also ?

The place of GnRH-Analogues

As early as 20 years ago Meldrun (1982) introduced the GnRH-Analogues as a possible new approach to the treatment of endometriosis. The beneficial clinical results were loss of symptoms caused by endometriosis such as dysmenorrhea, lower abdominal pain, and even loss of bowel bleeding or disappearance of hydronephrosis if these organs are involved in severe cases. However, the follow up studies published during the last years observed a significant rate of recurrences indicating that the medical treatment by GnRH-Agonists alone provided only temporary suppression of the disease, such as other hormonal approaches to endometriosis, so that either pregnancy or continuation of treatment had to be envisaged.

Between 1988 and 1995 ten randomised comparative or double blind placebo controlled studies have been published, which have compared the efficacy of different GnRH-Analogues against the danazol regimen (Cirkel et al. 1995; Henzl et al. 1988; Kiesel et al. 1989; Lemay, 1988; Matta et al. 1986; The NEEG 1992; Rock et al. 1993; Shaw 1992; Sondheimer et al. 1990; Wheeler et al 1992). The effect of the differing medical treatments has been estimated on the basis of subjective symptoms, clinical findings by gynaecological examinations and objective findings by second look pelviscopies using the r-AFS-score or the additive diameter of implants to compare the drugs. Tolerability of the medication and side effects were carefully evaluated and compared. Shaw (1995) has summarised the results as follows: Because the subjective side effects of GnRH agonist therapy are better tolerable as other medical options against endometriosis and there are no metabolic disturbances (liver enzymes, lipid metabolism, clotting system ea.). These drugs

are the primary treatment of choice. There are no data in the literature if this is true for teenagers also.

Some studies have followed the patients for a period of at least 12 months after treatment. An increase in pain scores were usually associated with the return of dysmenorrhea, however to a lesser degree than in pre-treatment. Prolonged relief of dyspareunia and pelvic pain was also observed.

With respect to the extent of regression, the location and type of endometriosis seems to react different to medical treatment. Whereas peritoneal endometriosis and superficial ovarian implants react well to ovarian suppression (Schweppe, 1984), deep infiltrating endometriotic nodules in the bladder, rectum or the recto-vaginal septum don't cause symptoms during therapy, but the are not significantly reduced in size and early recurrences are characteristic. Ovarian endometriomas larger than 3 cm in diameter reduce in volume but regrow rapidly and surgical treatment is necessary. Donnez et al. (1990) found with 3 months preoperative medical therapy a regression of more than 25% of the volume, which was achieved in 30% of the danazol treated patients and in 82% of the GnRH-analogues treated patients. This paper stressed the indication for presurgical treatment with GnRH-agonists for endometriomas specifically.

Repeated therapy with GnRH-Analogues is limited by the most important side effect — the bone loss, which is even more important in patients, which have not reached there peak bone mass yet. Therefore, several add-back trials have been investigated to solve this problem (Schweppe, 1996). Because we have learned from add-back studies for the GnRH agonist treatment of uterine myoma, that the therapeutic efficacy can be reduced by ovarian steroids, the question if add-back therapy for the treatment of endometriosis should be deferred was answered by Kiesel, who presented the results of a prospective placebo controlled study (1996) with medrogestone 10mg daily for all 6 months to the last 3 months of goserelin treatment in 123 women, which was performed in four German centres. The data demonstrates that the addition of either immediate or deferred add-back medication, such as medrogestone, does not reduce the efficacy of GnRH-Analogues treatment judged by r-AFS score. The frequency and severity of hot flashes were reduced, but the bone loss was only insignificantly reduced by the progestin. Therefore, starting estrogens and progestins together with the GnRH-analogues treatment is the add-back medication of choice. These data confirm the so called estrogen threshold theory.

Management Strategies of Endometriosis in Teenagers

A summary of our current therapeutic strategies considers the following points:

The group of teenagers presenting with secondary dysmenorrhea and a normal pelvis by gynaecological examination and vaginal sonographie should be treated for their symptoms by NSAR and / or with an oral contraceptive (monophasic, low dose). If these medications are ineffective — especially if a more potent pill is used for 3-6 months — an indication for diagnostic laparoscopy is given.

In stage I and II the diagnostic laparoscopy can be extended to thermocoagulation, vaporisation, ultracison and adhesiolysis and resection of implants and residual fibrosis. Thereafter, follow-up is directed to recurrence, which then would ask for an additional hormonal regimen — that is postsurgical medical treatment with delay.

In stages III and IV, a 3 to 6 months medication of a GnRH-analogues — preferable depot preparations — should follow endoscopic surgery if histological examination shows an active disease. The question remains, whether preoperative use of a hormonal regimen would be beneficial. Besides the advantages with respect to reduction in extent of surgery, reduced vascularity and less inflammation of pelvic organs noted at the time of revaluation may also be helpful in preventing the development of postoperative adhesions. In fact, in some cases, filmy adhesions noted at initial laparoscopy were not visible at revaluation. The reason for this is unclear.

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Should laser vaporization and electrocoagulation of endometriosis be banned?

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The title seems to ask a pointless question. Why should two forms of treatment which are used around the world be banned? The answers are straightforward:

1. Neither has been validated as being effective in eradication or reduction of endometriosis

Validation of efficacy in eradication or reduction of endometriosis requires a systematic examination of disease extent in patients both before as well as after surgical treatment. In other words, at least two surgeries are required to validate the efficacy of a method of surgery (or medical therapy for that matter). At the first surgery, disease extent is measured, then the surgical modality is applied. At the second surgery, disease extent is measured and compared with disease extent noted at the first surgery. Both laser vaporization and electrocoagulation have been in use for over a quarter of a century, yet we still do not know how effectively either eradicates endometriosis. It is outrageous for those who promote these treatments to ignore the question of efficacy. The published studies have measured only the response of symptoms, not the response of the disease.

2. Both violate common surgical sense

Endometriosis can be a very invasive disease. In some cases, fibrotic disease can be located up to 3 cm beneath the visible surface, with impressive nodular spherical volumes to match. Endometriosis can invade the bowel, bladder, and ureter, and can be associated with fibrosis which can envelope tubular structures in the pelvis including arteries, veins, and ureters. During surgical treatment, it is necessary to separate healthy tissue from the diseased tissue. It is inconceivable that a surgeon can expect to completely and safely treat such a

disease by shining a light at it or spraying electrons at its surface. This violates simple common sense.

3. Both convert all manifestations of endometriosis into superficial disease in the eye of the surgeon

This is a corollary of #2. The surgeon who uses laser vaporization or electrocoagulation to treat the disease may be aware of its invasive potential but doesnt want to be accused of incompletely treating deep disease. Therefore, many will fool themselves into imagining that the disease they are treating is fairly superficial and is being destroyed by shining a light at it or by spraying electrons at it.

4. Electrocoagulation has never been described in the literature so no one can reproduce it

For a surgeon to use electrocoagulation in a reproducible manner, he would have to know several things, including: 1. The type of electrosurgical generator used; 2. Power settings; 3. Type of active electrode; 4. Manner of use of the electrode; 5. Visual endpoint marking completion of treatment. No article on electrocoagulation contains all of these details. A surgeon must either cobble together a list of specifications or, more commonly, just use electrocoagulation in a hopeful manner. Although laser vaporization has been described in more technical detail, the technique of complete treatment of all deep disease has still not been published.

5. Both rely too heavily on opinions of the surgeon

a. What is being treated?
   Neither shining a light at nor spraying electrons at endometriosis produces a pathology report. The surgeon has no idea what is being treated. In some instances, cancer, or carbon or scarring from previous treatments rather than endometriosis is being treated.
b. How complete was the treatment?
   Surgeons using these techniques have no objective means of ensuring that all endometriosis of any depth of invasion has been eradicated. While occasionally a surgeon might accurately judge that extremely superficial disease has been adequately treated with photons or electrons, the question becomes more problematic with more invasive disease in parenchymal structures such as the uterosacral ligaments or muscularis of the bowel or bladder.

6. Both contribute support to an incorrect theory of origin, namely reflux menstruation

Reflux menstruation is not the origin of endometriosis. If it were, it would not be called a theory. Reflux menstruation cannot be the origin of endometriosis for several reasons:

a. Endometriosis is not identical to endometrium.
It has been taught incorrectly for many generations that endometriosis is identical to endometrium because of reflux menstruation leading to pathologic autografts. Modern studies have illustrated the dozens of profound differences between endometriosis and endometrium.(1)

b. Endometriosis is curable by conservative surgery.
It has been taught incorrectly for many generations that endometriosis is a chronic, enigmatic, incurable disease. It is said to be ''incurable'' because of reflux menstruation: even if a surgeon really does destroy all disease, it will just come back because the pelvis will be re-seeded with refluxed endometrium during the next menstrual flow. Yet, it has been known for over half a century that endometriosis can be cured by conservative excision without the need for medical therapy.(2) The minimum cure rate following one conservative surgery is 41%. Following two conservative surgeries, it is 67% (Redwine, unpublished data.) If endometriosis is present after aggressive excision, it will be present in much smaller amounts. (3). Robust photomicrographic evidence of initial attachment and secondary proliferation and invasion of the peritoneum by endometrium does not exist. It is estimated that 10% of women have endometriosis which will be symptomatic during their reproductive years. In the industrialized world, it seems likely that there are probably at least 25,000,000 women with endometriosis at any one time. These women will have monthly menses for at least 10 to 20 years. If we take 17 years as a conservative estimate of a womans menstrual lifespan, then such a woman will have about 200 menstrual cycles between the age of 13 and 30. If a typical flow lasts four days and on each day of flow 10 endometrial cells are refluxed into the pelvic cavity, then each woman will have 8,000 endometrial cells refluxed into her pelvis in her menstrual lifetime. If this is true, then during the menstrual lifetime of our cohort of 25,000,000 women with endometriosis, there will be $8 \times 10^3 \times 2.5 \times 10^7$ or 2×10^{11} instances of endometrial cells being refluxed into the pelvis in those women. If the rate of attachment of refluxed cells during any one menses is only 1%, then 2×10^9 instances of attachment will have occurred during the menstrual career of these women, or 1.2×10^8 instances of attachment annually. The actual numbers would be higher if previous generations

of women are considered or if some women have more than 17 years of menstrual flows or if some women have flows lasting longer than 4 days or if some women have heavier flows. These calculations may seem to be on the low conservative side to some. In any event, if conservative estimates are that several hundred billion possible instances of attachment of refluxed endometrium will occur in this cohort over their menstrual careers, and if over one hundred million instances of attachment occur annually, isn't it strange that no photomicrographs exist to confirm this supposedly ubiquitous and common phenomenon? The lack of photomicrographic proof of the initial two steps (attachment and proliferation/invasion) of the theory of reflux menstruation is the strongest evidence that this is not the origin of endometriosis. The theory of reflux menstruation as the origin of endometriosis and ineffective surgical treatments such as laser vaporization and electrocoagulation share a symbiotic relationship. Neither can exist without the other. So long as the theory of reflux menstruation exists to explain all treatment failures, surgeons dont have to consider whether their treatments are effective since they can blame all failures on the theory of origin. So long as ineffective surgical treatments exist, the persistent disease which is left behind can be called ''recurrent'' disease because of this theory and surgery will be considered ineffective or futile. As a result, many surgeons will prescribe medical therapy, which we know does not eradicate the disease.

7. Both aggravate the rising cost of health care around the world

It is axiomatic that ineffective treatments applied repeatedly are more expensive than effective treatments applied once or twice, especially when one of the treatments requires an expensive machine.

8. Both reduce a surgeon's skills

Shining a light at endometriosis or spraying electrons at it are easier than taking the time to remove the disease from the pelvis by careful dissection. As a result, surgical skills will erode since less skill is required for thermal ablative techniques. Removal of the disease, on the other hand, requires meticulous pelvic dissection, knowledge of anatomy, courage, and mental as well as physical stamina.

9. Laser can cause pain by leaving behind carbon which can cause a painful foreign body giant cell reaction

Carbon left behind by laser vaporization is more problematic than just the question of being mistaken for recurrent disease. Carbon can stimulate a foreign body giant cell reaction which can be its own cause of pain and lymphadenopathy. Further attempts at treatment by laser can aggravate the problem.

10. "Everybody does it"

Don't forget what your parents told you: ''Just because everybody does it doesnt make it right.'' With a disease which seems so mysterious such as endometriosis over the last 80 years, if everybody thinks alike and treats alike, then it is likely that everyone is wrong. Otherwise if everybody was right, it would be obvious by now. Progress is not made without change.

11. Both contribute to the belittlement and medical oppresson of women

How can we as a profession justify treatments with so many flaws provided to so many women for so many times over so many years? We cannot hide behind the notion that ''endometriosis is a mysterious, enigmatic, chronic, highly recurrent disease which is difficult to treat.'' We cannot continue to ignore the ill effects of these treatments on women. We must face the fact that these treatments are a large part of the problem in so many ways. Until they are abandoned, our patients will not be freed from our prejudices and from our mistakes. Most who read the twelve conditions above will immediately understand all of them, will abandon laser vaporization or electrocoagulation of endometriosis at once, and adopt excision which is the recognized treatment of choice for endometriosis. Welcome to a better way for your patients.

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NovaSure Impedance Controlled Endometrial Ablation System. Technology Overview & Clinical Results

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Summary

The NovaSure Impedance Controlled Endometrial Ablation System was developed to treat women suffering from menorrhagia due to benign causes. This technology allows for a customized, controlled, contoured endometrial ablation, without the need for hysteroscopic visualization and endometrial pre-treatment of any kind. Average treatment time is 90 seconds. The safety features employed (Perforation Detection System and Device Position Feedback) combined with a highest reported rate of effectiveness and patient satisfaction qualifies this system for consideration as the treatment modality of choice in patients requiring endometrial ablation.

Introduction

Hysterectomy is currently the leading treatment method for patients symptomatic for menorrhagia. Over 600,000 hysterectomies are performed every year in the US alone. This aggressive method of treatment was found to be efficacious, but is associated with a number of well-known and analyzed serious drawbacks. Hysterectomy has a relatively high morbidity and mortality rate[1], and direct and indirect costs associated with the procedure are also found to be quite significant[2].

Ablation of the endometrial lining of the uterus as an alternative to hysterectomy was found to be a less invasive and aggressive method. A large number of clinical trials, as well as retrospective analysis of clinical and financial data has shown that endometrial ablation allows for a lower morbidity and mortality rate and significantly lower procedure costs[3]. Endometrial ablation is increasingly being adopted by the gynecological community

worldwide[4]. The risks associated with the hysteroscopic approach are well known. Among these are uterine wall perforation, intravasation of fluid distention media, hyponatremia, encephalopathy, and death[5-8]. Technical challenges, requirement for a very well developed hand-eye-foot coordination[9], potential risks and other drawbacks of this treatment modality do not allow for a successful adoption of this procedure by the vast majority of gynecologists.

This particular fact and an alarming rate of hysterectomies performed triggered the development of other, less challenging methods of endometrial ablation. Treatment modalities are as different as the technologies employed. Each of the newer (Second Generation) treatment methods has a number of advantages over the ''Gold Standard'' Resection-Rollerball ablation, most importantly ease of use. Most of them are found to be quite effective. Randomized controlled studies have been and continuously are conducted to evaluate safety and effectiveness of each treatment modality[10-13].

Nevertheless, each of these technologies has several drawbacks associated with them (need for endometrial pre-treatment, long treatment and procedure time, e.t.c.). The NovaSure System (Novacept, Palo Alto, USA) successfully addresses many of the drawbacks of other global ablation devices.

Description of the novasure system

The NovaSure System (Picture 1) consists of a single use, 3-Dimensional bipolar ablation device and Radio Frequency Controller that enables a controlled endometrial ablation in an average of 90 seconds without the need for concomitant hysteroscopic visualization.

Endometrial ''pre-treatment'' of any kind (mechanical, hormonal, or cycle timing) is not required when using NovaSure[14]. The technology is easily employed in the actively bleeding patient.

Novasure disposable ablation device

The NovaSure disposable ablation device consists of a conformable, bipolar, gold-plated, porous, fabric mesh, mounted on an expandable metal frame. Integral to the hand-held device is the Intrauterine Measuring System (IMD) used to determine uterine cavity width (cornu-to-cornu distance). The unique geometry of the electrode allows for a controlled depth of ablation. It is characterized by a more shallow depth of miometrial penetration (2 mm) at the cornu and lower uterine segment, and a deeper (5 mm) ablation in the mid-body of the uterus[15]. The NovaSure device can treat uteri with sounding lengths up to 12 cm.

Figure 1. *NovaSure System*

Novasure controller

The NovaSure Controller contains a constant power output generator with a maximum power delivery of 180 watts. Measurement of uterine cavity length (determined during sounding and cervical dilation), and width (measured by the device at the time of device deployment), are key-entered into the controller, which automatically calculates the unique power output required to assure an optimal, confluent endo-myometrial ablation. Throughout the 90-second procedure, the depth of ablation is continuously controlled by monitoring tissue impedance (resistance). Vaporization of the endometrial layer is a low impedance process owing to a high concentration of conductive liquid (saline) present in the endometrial tissue. As a result, the endometrial tissue is not slowly ablated, but vaporized instead. The vaporization front is continuously moving deeper and closer to the edge of the myometrium. Once the ablation process reaches the myometrial layer, the content of the saline becomes significantly lower. Tissue impedance (resistance) rises rapidly during myometrial tissue desiccation process and reaches 50 Ohms, which is equivalent to the impedance of the ablated superficial myometrium. This signals the NovaSure generator to automatically terminate the ablation process. This approach allows for an effective ablation independently of the endometrial layer thickness. An

important component, unique to the NovaSure, is a vacuum pump, contained within the RF Controller. This pump provides continuous suction during the procedure, thus allowing for the removal of steam, blood and other by-products of ablation from the cavity. Use of constant vacuum assures intimate contact between the ablation electrode and the endometrium[15].

Novasure safety features

A Cavity Integrity Assessment System (Perforation Detection System) is another integral part of the NovaSure System. This automatic safety feature assists the physician in the timely detection of a uterine perforation, and prevents energy delivery in such cases. The Cavity Integrity Assessment System utilizes the same technology employed by conventional hysteroflators[16], in which there is an inverse relationship between flow rate and pressure. CO_2 is delivered into the uterine cavity at a safe flow rate and pressure. The goal is to generate and maintain an intrauterine pressure of 50 mm Hg for a period of 4 seconds. The pressure of 50 mm Hg was chosen in order to avoid false positive results due to leakage of CO_2 through the Fallopian tubes (cracking pressure of the Fallopian tube is 75-80 mm Hg). Once the controller determines that this pressure is maintained, thus confirming uterine wall integrity, it signals the generator to proceed with the ablation process.

Another very helpful safety feature is the Device Position Feedback. This system was designed and implemented in order to allow the physician to track and control the process of opening of the device electrode in the uterine cavity. It will prevent RF energy delivery in the event of inadvertent placement of the device into a false passage.

Clinical results

A large multicenter, international randomized clinical trial was conducted in support of the PMA application (FDA trial) and included 265 pre-menopausal women with excessive menstrual bleeding due to benign causes[13]. In this clinical trial the NovaSure system (175 patients) was compared to the Loop Resection followed by a Rollerball Ablation (90 patients).

Two-year results demonstrated that the two treatment modalities were effective in reducing excessive menstrual blood loss. 98% of the NovaSure patients and 92% of the Rollerball patients reported normal bleeding or less (PBLAC scores < 100) at 2 year post-treatment. In-patients with 24-month follow-up, 47% of the NovaSure patients and 35% of the Rollerball patients experienced amenorrhea. The mean procedure time (from device insertion to removal) was 4.2 minutes in the NovaSure Group and 24.2 minutes in the

Rollerball Group. Treatment time, defined as the time during which RF energy was delivered, averaged 84 seconds in the NovaSure Group. 73% of the NovaSure patients had the procedure performed under Local and/or IV sedation compared to 18% in the Rollerball group. Intra-operative adverse events occurred less frequently in the NovaSure Group (0.6%) than in the Rollerball Group (6.7%). There was a 13% occurrence of postoperative adverse events in the NovaSure Group and 25.3% occurrence in the Rollerball Group. There was a significant decrease in premenstrual symptoms and dysmenorrhea in both treatment groups at 24 months following the procedure. Based on the results obtained in this clinical trial the FDA found that NovaSure system is both safe and effective.

Gallinat *et al*. conducted a clinical trial on 107 pre-menopausal women with excessive bleeding[17]. At 12-months following the NovaSure ablation 58% of patients reported amenorrhea and 96.1% of patients reported reduction to normal bleeding or less. Busund *et al.* conducted a multi-centre study in which 58% of patients reported complete cessation of bleeding (amenorrhea) at 12-month follow-up. Abbott *et al.* reported the results of a randomized clinical trial comparing NovaSure with Cavaterm balloon. Amenorrhea and satisfaction rates reported in the NovaSure arm at 12-month follow-up were 43% and 92% respectively, compared to 11% and 83% in the Cavaterm group. A randomized clinical trial comparing the NovaSure with the ThermaChoice balloon system was conducted by Bongers *et al.* One-year follow-up results were reported with NovaSure patients yielding 55% and 94% amenorrhea and success rates respectively, compared to 8% and 77% in the ThermaChoice group. Fullop *et al.* reported on the 3-year long-term follow-up for the patients (n=75) treated using the NovaSure system[18]. Amenorrhea rate in this study was 90% with the success of 100%. Intra- and post-operative pain was assessed by Laberge *et al.* in a clinical study comparing the NovaSure with the ThermaChoice balloon [(19)]. Clinical results demonstrated that use of NovaSure system is associated with a statistically significantly lower intra- and postoperative pain level when compared with the use of the ThermaChoice system.

Conclusions

Considering the fact that NovaSure system allows for a short treatment (~90 seconds) and procedure time without the need of endometrial pre-treatment of any kind, associated with the highest reported amenorrhea, success and patient satisfaction rate achieved in a randomized clinical trial, it should be considered a treatment modality of choice and standard of care in treatment of patients suffering from menorrhagia due to benign causes.

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Five-Year Post-Procedure Follow-up of Patients Participating in a Randomized Trial of Uterine Balloon Therapy vs. Rollerball Ablation for the Treatment of Menorrhagia

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Objective

The objective of this investigation was to collect five-year post-procedure follow-up information from women participating in a randomized trial comparing uterine balloon therapy (GYNECARE THERMACHOICE Uterine Balloon Therapy System) to rollerball ablation for the treatment of menorrhagia. The purpose of this follow-up was to report the long-term outcomes of ablation treatment, and in particular, uterine balloon therapy.

Background

A randomized trial to compare uterine balloon therapy (UBT) and rollerball ablations was conducted at 14 centers during 1996 and 1997. Women participating in this trial had menorrhagia (menstrual blood loss in excess of 80mL/menstrual cycle), were premenopausal, had no evidence of cervical or uterine malignancy, and no uterine anatomic abnormalities.

Treatment success and safety demonstrated in 1 year post-procedure results lead to U.S. market approval of uterine balloon therapy. [1]Subsequent two and three year post-procedure surveillance of this patient cohort indicated continued treatment success in most patients.[2,3]

Methods

Twelve (12) of the original 14 centers in the randomized trial participated. Reviewing Institutional Review Boards either accepted notification or approved the patient follow-up contact at 5 years post-procedure.

Participation in the randomized trial received an introductory letter from

their physicians explaining the purpose of the study. Following this communication, patients were contacted by telephone at 5 years post-procedure (+3 months). A questionnaire regarding menstrual status, dysmenorrhea, pelvic pain, satisfaction, and additional gynecologic treatments/conditions was administered.

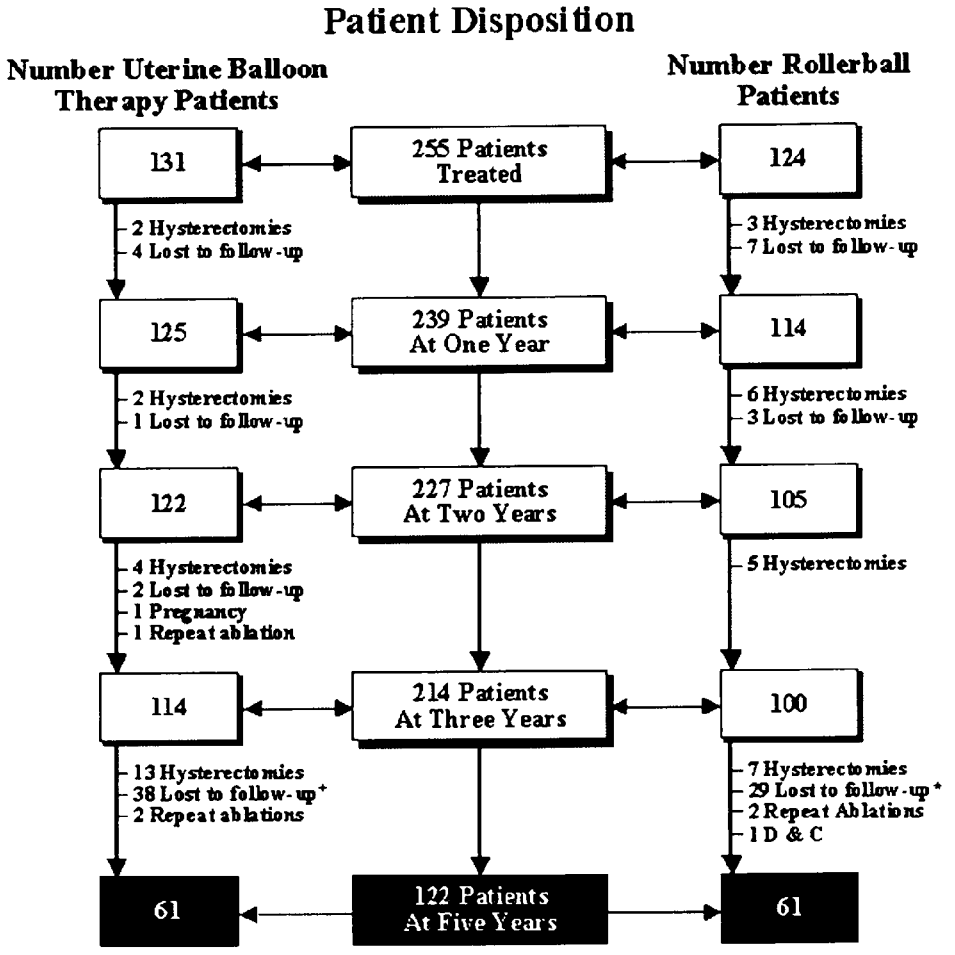

Figure 1. + *Includes 11 patients from 2 non-participating sites;* * *includes 9 patients from non-participating sites.*

Results

Among the 214 women available for follow-up at the last contact (3 years post-op), 147 completed the 5-year questionnaire (69% response rate). Of these, 25 patients reported either hysterectomy, repeat ablation, or D & C, leaving 122

(61 UBT; 61 rollerball) eligible for analysis of bleeding patterns and pelvic pain. The overall follow-up rate at 5 years was 58% (147/255). The patient disposition for this trial is outlined in Figure 1.

Between years 3 and 5, 20 new hysterectomies (13 UBT; 7 rollerball) 4 repeat ablations (2 UBT; 2 rollerball), and 1 D & C (rollerball) were reported.

In total, by year 5:

- UBT group: 21 hysterectomies and 3 repeat ablations
- Rollerball group: 21 hysterectomies, 2 repeat ablations, and 1 D & C.

The reasons for hysterectomy are outlined in Table 1. Among the 35 (17 UBT; 18 rollerball) patients reporting bleeding, pelvic pain, or both as reason for hysterectomy, 11 (7 UBT; 4 rollerball) were known to have fibroids at the time of hysterectomy. Two additional patients (1 in each group) reported fibroids as the reason the hysterectomy.

Table 1. *Reasons for Hysterectomy*

	Uterine Balloon Therapy N=21	Rollerball N=21
Bleeding	9	7
Pelvic Pain	3	10
Bleeding and Pelvic Pain	5	1
Fibroids	1	1
Ovarian Cyst	1	–
Mood swings, depression	–	1
Uterine prolapse	2	–
Endometrial hyperplasia found on histology of pre-treatment D&C tissue at the time of study ablation procedure	–	1

The 122 women completing a questionnaire at 5-years and without additional uterine intervention were eligible for analysis of bleeding patterns (Table 2), dysmenorrhea (Table 3), and pelvic pain (Table 4). The following statements also apply to this 5-year population.

At the time of the ablation procedures, the mean age of study participants was approximately 40 (+5) years. At the five year contact, the mean age was 46 (+5) years.

57 of 61 (93%) UBT patients and 61 (100%) rollerball patients reported satisfaction with the procedure.

Success of the procedure is defined as having normal menstrual bleeding or less and not undergoing hysterectomy, repeat ablation, or D & C. As such, the success rate for the procedure is calculated as follows:

$$\text{Success rate} = \frac{\text{\# women with normal bleeding or less at 5 yrs and without hysterectomy, repeat ablation, or D\&C (successes)}}{\text{\# successes at 5 years + all known treatment failures to date (excessive bleeding at yr 5 or hysterectomy, repeat ablation, or D\&C)}}$$

The success rates at 5 years were 68% (58/85) for the UBT group and 69% (59/85) for the rollerball group.

Table 2. *Menstrual Status*

	Uterine Balloon Therapy N=21	Rollerball N=21
None	14 (23%)	20 (33%)
Spotting	6 (10%)	7 (11%)
Light	23 (38%)	15 (25%)
Normal	15 (25%)	17 (28%)
Excessive	3 (5%)	2 (3%)

* 58 of 61 (95%) UBT patients and 59 of 61 (97%) rollerball patients included in this analysis reported nrmal bleeding or less.

Table 3. *Dysmenorrhea*

	Uterine Balloon Therapy N=21	Rollerball N=21
None	32 (52%)	43 (52%)
Mild	13 (21%)	16 (27%)
Moderate	13 (21%)	8 (13%)
Severe	3 (5%)	4 (0%)

Table 4. *Pelvic Pain/Cramping (other than dysmenorrhea)*

	Uterine Balloon Therapy N=21	Rollerball N=21
None	46 (75%)	45 (74%)
Mild	7 (11%)	13 (21%)
Moderate	5 (8%)	3 (5%)
Severe	3 (5%)	0 (0%)

Discussion

The menstrual status results are influenced by age as this cohort moves closer to menopause. To explore this potential confounder, we analyzed the change in menstrual status from year 3 to year 5 and selected age 50 as a possible marker for menopause. All but 3 of the 14 patients newly reporting amenorrhea at year 5 were under age 50.

Among the 25 patients with hysterectomy, repeat ablation, or D & C reported between years 3 and 5, 22 (88%) reported satisfaction with the study ablation procedure.

These results indicate that nearly 7 of 10 women undergoing an ablation procedure will remain treatment successes five years after the procedure. It is important to consider that the success rate is affected by 1) the lost to follow-up group and 2) the fact that women who experienced eventual hysterectomy were considered treatment failures despite the fact that the reason for the operation could be construed as unrelated to treatment failure (e.g., fibroid development, uterine prolapse).

There were no reports of endometrial cancer among study participants.

Conclusion

Uterine balloon therapy continues to be an effective treatment for menorrhagia and clinical outcomes are similar to rollerball ablation at 5 years post-procedure. Endometrial ablation is a viable treatment for menorrhagia and a practical alternative to hysterectomy in this at-risk group of women.

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Can we reliably predict or diagnose preterm labour and how useful is fetal fibronectin?

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The prediction of preterm labour is not only difficult, as it often occurs in women with few risk factors, but is also problematic to diagnose. Maternal perception of uterine activity is a poor guide to the risk of delivery, clinical examination is unreliable and there is no single test to guide management. To date, long-term medical prophylaxis has not been demonstrated to improve the outcome in high-risk individuals. Although cervical cerclage is likely to be effective in women with an appropriate history, it is inappropriate for the majority without risk factors. In women with preterm uterine contractions, cervical length measurement by ultrasound is reported to predict preterm labour before 34 and 37 weeks gestation(1). Fetal fibronectin is also helpful in the detection of those women with labour symptoms who will deliver, particularly in the 7-10 days following identification(2). These investigations have not however been universally introduced into clinical practice. Since preterm labour is multi-factorial, it is not surprising that there is no single method of diagnosis and/or treatment. Prevention and treatment should thus be directed towards the cause, such as systemic maternal conditions or bacterial vaginosis, where appropriate. In women with uncomplicated preterm labour who are likely to progress to delivery, short-term tocolysis is appropriate to instigate measures likely to improve neonatal prognosis, such as the administration of corticosteroids.

1. Crane et al 1997.
2. Honest et al 2002

Extending the opportunity of tocolysis beyond 48 hours; particularly at more extreme gestational ages.

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Introduction

The World Health Organisation defines preterm birth as a birth before a gestational age of 37 completed weeks[1]. Preterm birth is the single most important cause of perinatal mortality in Europe and North America[2]. The annual number of preterm births worldwide is estimated at approximately 13 million[3]. In North-America and Europe together there are each year approximately 800.000 preterm births, and it is estimated that 200.000 of these preterm births are potentially preventable[4]. About two third of preterm births results from spontaneous preterm labor or preterm premature rupture of the membranes, the rest is due to medical interventions for maternal or fetal indications[5]. In developed countries the incidence of preterm birth is estimated at 5-10 % of all births[4,6]. The incidence of preterm births has been stable over the last four decades despite remarkable improvements in perinatal care facilities, the widespread use of tocolytic agents, and the introduction of steroids to enhance fetal lung maturation. Preterm birth is responsible for more than 80% of all neonatal deaths not due to congenital anomalies, and 50% of childhood neurological disabilities[7]. The costs of preterm birth and its associated neonatal intensive care unit admission include immediate and long-term costs. The weekly costs of approximately $10,000 per baby, gives an estimated total costs in the United States of more than $5 billion[8]. The mean costs to graduate a sick infant from admission to discharge from the newborn intensive care unit is between $20,000 and $100,000 per infant, up to $140,000 for those weighting less than 1000 gm[8]. Infants with severe handicaps can have long-term care costs estimated to be more than $100,000, and lifetime custodial care as much as $450,000[8]. The exact causes and aetiologies of preterm birth are not known. It is therefore difficult to influence the incidence rate. Causes can be multifactorial. Management strategies to prevent preterm birth are mainly focused on

identification of those women who are at high risk for preterm delivery. Eventually when preterm labor ensues sometimes tocolytic agents are required.

Perinatal outcome after preterm birth

The last decades there has been a significant improvement in the reduction of neonatal mortality and morbidity for preterm infants mostly due to better perinatal surveillance, and improvement in neonatal intensive care facilities. The gestational age of viability is around 24 weeks. Recent studies suggest that the limit of aggressive intrapartum management should be 24 weeks[9,10]. Viability requires the availability of effective clinical interventions known to support the extremely preterm infant and a favourable follow-up of these infants[11]. The analysis of perinatal outcome should not only be based on mortality rates but also on morbidity and long term prognosis. For the infant undergoing aggressive neonatal management, a fair balance should be maintained between clinical goods as intact survival versus impaired survival or mortality. The last years the 50% survival rate has shifted from 26 weeks to 24 weeks[12,13]. Lumley[6] showed that the incidence of preterm births under 28 weeks is approximately 10%, responsible for 57% of the perinatal mortality preterm. The incidence of preterm births under 26 weeks is approximately 7%, responsible for 46% of the perinatal mortality preterm[6]. Recently within the Epicure study[14,15] the outcome was investigated of infants born between 20 and less than 25 completed gestational weeks. The percentage of singleton infants who survived and were discharged from the NICU increased from 23% at 23 weeks, to 38% at 24 weeks, and 54% at 25 weeks. Long term follow-up of these children born at less than 25 completed weeks, at the age of 30 months, showed that the incidence of disability was 48%, 23% of these infants had even a severe disability[14,15].

Management of preterm labor

When preterm labor ensues admission in a hospital is required. The advantages and disadvantages of medical treatment of preterm labor should be weighted against each other. Factors that influence this decision are the gestational age and the estimated fetal lung maturation. Delay of delivery i.e. prolongation of pregnancy can be an advantage because of the estimated lower neonatal morbidity and mortality at higher gestational age. This can be established by administration of corticosteroids to promote fetal lung maturity and transfer of the patient to a tertiary referral hospital with NICU facilities. Crowley et al.[16] demonstrated that the use of corticosteroids administered antenatally gives a significant reduction in perinatal morbidity and mortality.

Tocolytic agents

Use of betamimetic agents is associated with a delay of delivery for 24-48 hours, the time that is gained can be used to administer the patient steroids and maternal transfer to a tertiary centre with NICU facilities. Fetal contraindications for the use of tocolytic agents have to be excluded such as: fetal distress, intrauterine infection, congenital anomalies, abruptio placentae, and severe intrauterine growth retardation. Maternal contraindications for the use of tocolytic agents are: preeclampsia, hyperthyroidism and cardiovascular diseases. It is estimated that from the women with preterm labor only 10-30 % are eligible for administration of tocolytic agents[17].

Betamimetics; the most commonly used tocolytic agents are far from ideal. Betamimetics are only effective for a maximum of 48 hours, and by itself their use is not associated with an improvement in perinatal outcome[18,19]. Furthermore use of betamimetics is associated with a lot of substantial side effects[20-22]. The use of betamimetics for maintenance therapy has been investigated in several placebo controlled trials, but these studies have not shown any beneficial effect with respect to delay of delivery or improved perinatal outcome.[23-25]

Calcium channel blockers; and especially nifedipine, have been used in obstetrics since the early 1980s and have shown that nifedipine is an effective smooth muscle relaxant with in animal experiments a low teratogenicity and toxicity, and when used in pregnant humans associated with a lower incidence of maternal and fetal side effects compared with betamimetics[21,26-28]. A recent Cochrane systematic review demonstrated that calcium channel blockers (CCB's) (mainly nifedipine) are at least equally effective as tocolytic agent as any other tocolytic (mainly betamimetics)[29]. CCB's are associated with a significant lower perinatal morbidity and a lower incidence of maternal side effects[29]. Calcium antagonists have not been licensed as a tocolytic agent.

Oxytocin antagonists: Oxytocin receptor antagonists like atosiban block oxytocin receptors in the myometrium and myoepithelial cells of the mammary gland. Blockage of the oxytocin receptor by oxytocin receptor antagonists will result in a lower intracellular calcium, resulting in myometrial relaxation. The efficacy of atosiban has been investigated in several studies. In placebo controlled trials atosiban has a similar tocolytic efficacy with less maternal side effects[30]. When atosiban is compared with betamimetics there is a similar tocolytic efficacy and perinatal outcome with less maternal side effects[31,32]. A maintenance trial of atosiban versus placebo has shown that atosiban can prolong uterine quiescence after successful treatment of an acute episode of preterm labor with atosiban[33].

Tocolysis for the future: which one to choose

Early preterm birth accounts, as stated in the introduction, for more than 80 percent of perinatal morbidity and mortality not related to structural anomalies. Particularly in the period 24-28 weeks of gestation much is to be gained from postponement of delivery even if for several days. At early gestational ages each day delay of delivery increases neonatal survival with 3%[34].

Currently licensed tocolytics atosiban and ritodrine are registered for only a limited time span. Atosiban in this regard has a better action/safety profile then ritodrine.

Calcium antagonists, in particular nifedipine, are associated in recent meta-analyses with a lower neonatal morbidity and less maternal side effects than betamimetics[29].

Calcium antagonists have been applied orally and for a prolonged period of time. It is not clear if the more favourable outcome is due to the use itself of calcium antagonists or the prolonged treatment schedules. Calcium antagonists have the disadvantage not being licensed as a tocolytic drug, which may qualify them as less attractive for the first 36-48 hours. When not applied at the start of tocolytic treatment, their use can be considered for maintenance.

A randomised controlled trial is planned in which atosiban is prescribed for the first 48 hours after which tocolytic treatment is stopped or will be followed up by a switch to maintenance therapy with nifedipine, particularly at very early gestational ages.

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Can we effectively arrest premature labour in women with multiple gestations or other high-risk factors?

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The practice of suppressing preterm labor by pharmacologic intervention has been used for over 40 years.

A number of different pharmacologic alternatives have been used in the clinical setting since the 1980s, although most were used off-licence. The tocolytic agents available include the more selective β_2-agonists, calcium channel blockers, magnesium sulfate, and prostaglandin synthetase inhibitors (1).

Historically, β_2 adrenergic agonists have been the most widely used agents in clinical practice, but at high cost in term of adverse effects. For this reason, and the fact that the delay in delivery achieved by their use did not transitate into objective perinatal benefit (as measured by mortality or morbidity), their use has been removed from USA FDA and much scientific and clinical research has focused on the development of new tocolytic agents (2).

Atosiban ([1-deamino-2-D-Tyr(Oet)-4-Thr-8-Orn]-oxytocin) is an oxytocin receptor antagonist that was developed for the treatment of preterm labor. Atosiban is a competitive antagonist of oxytocin that inhibits oxytocin-induced uterine contractions in bot in vivo and in vitro animal models. Atosiban has also been shown to diminish uterine contractions in women with threatened preterm birth without causing significant maternal, fetal or neonatal adverse effects (3).

Tocolysis is mainly used for the short-term suppression of uterine contractility to allow corticosteroid administration, patient transfer to high-level perinatal centres, as well as improving neonatal survival. This is particularly true for women with multiple pregnancies (MP) and preterm premature rupture of the membranes (PPROM) (<32 weeks) in which prolongation of tocolytic therapy may be needed (4).

This study compared the clinical effectiveness, safety profile and metabolic effects of atosiban with that of ritodrine administered either as standard

protocols (i.e. 48 hrs to allow the administration of drugs enhancing fetal lung maturity), or for a longer period (>48hrs to 7 days consecutively) in different clinical settings of threatened preterm labor.

Methods and patients

Atosiban (Tractocyle, Ferring Pharmaceuticals) was given as a single intravenous bolus dose (6.75 mg in 0,9 mL isotonic sodium chloride solution), followed by an intravenous infusion of 300 μg/min in 5% dextrose for the first 3 hours and then 100 μg/min in 5% dextrose for as long as 45 hours.

Ritodrine (Miolene or Yutopar, Bristol-Myers Squibb Pharmaceutical) was given as an intravenous infusion in 5% saline solution at 0.10 to 0.35 mg/min. by increments every 10 minutes, as required, or until contractions ceased.

Inclusion criteria (all of these criteria had to be present): Preterm labor, as defined — Regular uterine contractions >30 s in duration at a rate of >4 contractions/30 min confirmed by external tocography — Cervical dilatation of 0-3 cm (nulliparous women) or 1-3 cm (primiparous or multiparous women) — Cervical effacement >50% — Gestational age between 23 and 33 completed weeks confirmed by ultrasonography performed before 20 weeks' gestation or by reliable menstrual dates or by both of these.

Forty-three women were enrolled in the study evaluating the use of atosiban vs ritodrine. They were admitted consecutively and randomly allocated to one or two other treatment short schedule: 21 patients were evaluated in the atosiban group, 22 in the ritodrine group. Their characteristics were not significantly different (table 1).

Table 1: *Characteristics of the study population*

	Atosiban (n=21)	Ritodrine (n=22)
Gestational age (mean, range)	28 (24-31)	27,8 (25-32)
Parity <1	16	14
>/ 1	5	8

Moreover, the patients affected by threatened preterm labor associated to PPROM or multiple pregnancy were treated for longer period with atosiban (same schedule except the 100 μg/min treatment was prolonged for more than 2 days, mean 198 hrs).

Tocolytic effectiveness was assessed in terms of the total mumber of women in the intent-to-treat population who had not been delivered at 48 hours and 7 days after starting treatment. Safety was assessed by maternal side effects, with particular emphasis on cardiovascular adverse events (pulmonary edema, chest

pain, myocardial ischemia, dyspnea, palpitation, tachycardia, hypotension and syncope) and neonatal morbidity.

For all women blood samples were collected before, during (24 hrs) and after completion (48 hrs and 7 days) of study drug infusion for measurement of various laboratory parameters. Specifically, blood samples for free T3, free T4, TSH, electrolytes (Na+, K+, Cl-, Ca+, P-, Mg-), glycemia, cholesterol, creatinine, uric acid, triglycerides, γGT, AST, ALT, alkaline phosphatase, bilirubin were assayed by standard laboratory techniques (Central Lab., University Hospital, Perugia,).

Maternal and fetal tachycardia were defined as heart rates of >120 and 170 beats/min respectively.

Electrocardiogram was recorded before, after 24 hrs, 48 hrs and 7 days (where appropriate) administration of drugs.

Results

Tables 2 and 3 summarize the results of the study. No statistically significant difference was detected on the tocolytic effectiveness between the two *regimen* protocols.

However, important differences were detected in metabolic effects (table 2) among all parameters evaluated: *K+* levels and glucose levels were respectively significantly lowered and increased by ritodrine infusion with no modification during atosiban treatment. Apart from tachycardia, electrocardiographic findings were somewhat altered during ritodrine infusion (changes in ST waveforms in 12 cases with no modification during atosiban treatment).

Table 2: *Metabolic effects of different tocolytic regimens*

		Atosiban (21)	Ritodrine (22)	Range normal values
K+ (mEq/l)	0h	3.9 ± 0.2	4.0 ± 0.2	(3.6-5.5)
	24h	3.7 ± 0.2	2.9 ± 0.4	
	48h	3.9 ± 0.3	3.3 ± 0.4	
Na+ (mEq/l)	0h	142 ± 5	140 ± 5	(135-155)
	24h	140 ± 6	137 ± 6	
	48h	141 ± 5	142 ± 6	
Glycaemia (mg/dl)	0h	74 ± 11	72 ± 12	(60-110)
	24h	96 ± 12	117 ± 21	
	48h	82 ± 10	107 ± 14	

No significant variations for AST, ALT, γGT, FT3, FT4, TSH, cholesterol, creatinine.

Maternal morbidity was also significantly increased in the ritrodine group compared to atosiban (table 3).

Table 3: *Tocolytic efficacy and maternal side effects during different drug treatment*

	Atosiban 1 (n=21)	Atosiban 2 (n=10)	Ritodrine (n=22)
Suppression of labour > 48h	19	10	18
Maternal tachycardia (>120 bpm)	1	1	21
Trembling/shivers	1	1	16
Nausea/vomiting	6	2	10
Giddiness	4	1	8
Suspension of treatment	0	0	2

Atosiban 1: standard protocol (<48 hrs)
Atosiban 2: prolonged protocol (> 48 hrs)

Prolongation of treatment with atosiban (table 4) has not been followed by particular changes in maternal metabolism neither in side effects of the mother (table 2) or of the fetus compared to ritodrine.

Table 4: *Treatment duration and prolongation of pregnancy with atosiban in different clinical settings*

Cases (nr)	Treatment duration (hrs, mean)	Prolongation of pregnancy (days) mean — (range)
PPROM* (5)	168	12 (8-22)
MP** (5)	108	26(6-36)

* Preterm premature rupture of membranes (< 33 weeks)
** Multiple pregnancy (1 triplet, 4 twins)

A remarkable recent case just underlines the usefulness of atosiban: triplet pregnancy in primigravida (after ART) at 22 weeks + 5 arrived at full cervical dilation with one fetus in breech presentation. The delayed delivery of the remaining twins was attempted successfully with atosiban promptly administered after the delivery of the first fetus (470 g, Apgar 1-7 died at 12 hrs) and with no maternal adverse events. Pregnancy continued to 26 weeks for the two remaining twins. At this time one newborn (female) is surviving well (> 700 g) with no apparent problems.

Discussion and conclusion

Literature evidence and our experience with atosiban (5) found short term (at least 48 hours) tocolysis in 85% of women, with much fewer maternal adverse events (especially pulmonary, myocardial, metabolic) than the beta-agonists.

This treatment con be safely prolonged up to 7 days when needed (PPROM, multiple pregnancy, restarting of uterine contractions).

The improved safety and tolerability of atosiban, and high efficacy, represent a clinical advantage over existing tocolytics, especially in high-risk women.

Cost-effectiveness calculated by length of hospital stay and the need for further intervention, was another advantage for atosiban. We believe that there is no longer a role for traditional tocolytics for women with MP and PPROM.

Atosiban, indeed, appears to have a clinical effectiveness similar, if not even better, than to that of ritrodine in the treatment of preterm labor but is much better tolerated.

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Long-term safety with oxytocin antagonists

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The oxytocin antagonist, atosiban, represents a new generation of agents specifically designed for the management of preterm labour (PTL). During the development programme, two randomised, double-blind, placebo-controlled trials, PTL-096 and PTL-098, were conducted to evaluate the efficacy and safety of atosiban (1,2). Child assessments were made at 6, 12, and 24 months post delivery. The main outcomes evaluated were infant weight, length and head circumference, neurological examination, the Bayley II assessment of mental and motor development, and serious adverse events. For 288 atosiban-exposed infants and 295 placebo-exposed infants in the PTL-096 trial, follow up at 6, 12, and 24 months was conducted in 73%, 66% and 55%, respectively. In the PTL-098 trial, 291 infants exposed to atosiban IV and SC and 272 exposed to atosiban IV and placebo SC, follow up at 6, 12, and 24 months was conducted in 81%, 75% and 55%, respectively. Atosiban-exposed infants did not differ from placebo-exposed infants with respect to any of the physical or psychometric variables studied. Any long-term adverse events that were observed were attributable to the effects of prematurity, not drug therapy. Stratification of the data by tocolytic/gestational age and length of exposure will allow more specific safety claims to be made regarding atosiban, with long-term safety shown at early and late gestational ages. The 2-year follow-up results for infants enrolled from these two studies provide the most detailed, long-term assessment of childhood development after tocolytic therapy currently available.

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Severe Preeclampsia at the limit of viability: Is there a role for expectant management ?

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Severe preeclampsia at any gestational age can rapidly progress to a life threatening syndrome and an obstetrical emergency. Hence, traditionally women with evidence for severe preeclampsia were expeditiously delivered regardless of gestational age. Clinical studies from the eighties and nineties indicated that expectant management for women with severe disease should be considered since a median prolongation of pregnancy by one to two weeks may significantly improve neonatal outcome with favorable maternal outcome. It should be emphasized that these studies, some of them prospective randomized controlled trials (1,2), recruited patients with severe disease defined mostly on the basis of severe hypertension or proteinuric criteria. Patients with hepatic involvement, renal dysfunction beside proteinuria, severe thrombocytopenia or with evidence of hemolysis were not included and were delivered without delay or shortly following corticosteroids administration. Moreover, the reported studies had, eventually, limited power to rule out increase risk for maternal mortality or catastrophic complications of severe preeclampsia such as intracranial hemorrhage, liver rupture, acute renal tubular necrosis etc. Based on these studies and on clinical experience, most tertiary centers adopted guidelines for considering expectant management remote from term (before 32 gestational weeks) for patients with controlled hypertension, platelet count > 100,000, no evidence of oliguria following routine fluid intake, pulmonary edema or compromised renal function, and without epigastric pain, elevated AST/ALT (> 2x upper limit of norma), severe persistent headache or visual disturbances (3). This policy is limited for centers with availability of intensive care facilities, real time laboratory facilities, blood bank services, as well as equipment and personal trained for frequent maternal and fetal monitoring.

I addition to the acceptance of expectant management of the so-called moderate type cases of preeclampsia, a dramatic improvement in neonatal care occurred during the last two decades. In most tertiary centers in North America and Europe, gestational age of 23 weeks is considered to be the limit of viability

at the present. The clinical data regarding conservative management of severe preeclampsia remote from term at the limit of viability (22 to 25 weeks) are limited. In a randomized prospective observational study, Sibai and colleagues (4) evaluated the results of a protocol for expectant management of patients with severe preeclampsia developing at the second trimester (before 27 weeks of gestation) at the University of Tennessee, Memphis between 1985 and 1989. Women who required immediate delivery for maternal or fetal indications such as HELLP syndrome, pulmonary edema, fetal distress and oligohydramnios were excluded from this report. Fifteen out of 25 patients who were less than 24 weeks gestation and were offered termination or expectant management elected to continue the pregnancy. Among patients at 24-27 weeks, 54 elected expectant management whereas 30 women were delivered 24 hours following receiving the last doses of steroids because of the patient desire or physician advice. In patients > 24 completed weeks while developing the severe preeclamptic state, the perinatal mortality rate was significantly lower in the expectantly managed group (24%) than in the aggressively managed group (64%). Women who were < 24 weeks and elected an expectant management had a mean pregnancy prolongation of 19.4 days, but yet a mean gestational age at delivery of 24.8 weeks. The perinatal mortality rate at this group was 93%.

From these data as well as from unpublished clinical experience of experts across the world, it is clear that patients who are managed expectantly at these gestational weeks should expect potential prolongation of pregnancy that is similar to that achieved at later weeks (median of one to two weeks). Moreover, the maternal risk of developing catastrophic life threatening events is low while being under intensive clinical and laboratory monitoring. This median prolongation is associated with highly significant neonatal improvement outcome, since at this age group (24-25 weeks) each day of pregnancy prolongation is associated with 2-3% on average increase in survival. Hence, when gestational age is clearly at the viable range at the time of facing the development of the severe disease (> 24 completed weeks), expectant management should strongly be considered. When gestational age at that time is less than completed 23 weeks, termination of pregnancy should strongly recommended since there is a high probability that the expected prolongation will leads us to gestational age with yet very limited survival and high neurological significant morbidity. Termination at less than 23 completed weeks is also not associated in the majority of cases with survival or with significant ethical conflicts between the parents and medical staff. In borderline cases where the severe disease develops between complete 23 to complete 24 weeks, the decision should be tailored according to factors such as specific results of this neonatal center, the level of disease severity, and the ethical and regulatory environment of that specific society regarding issues such as late fetocide,

parents rights to refuse neonatal resuscitation etc. In any case of expectant management of severe preeclampsia, informed consent must be obtained. The risk and potential benefits of such management versus aggressive treatment regarding both the mother and the fetus must be explained. It must be made clear that even though the infant may survive, it may not be neurologically intact, compromising its quality of life. Nevertheless, such a policy of pregnancy prolongation on behalf of the fetus at the limit of viability is accepted by most centers for additional obstetric situations associated with maternal risk as well as limitation in predicting the length of prolongation (preterm PROM, premature labor, bleeding placenta previa, etc.).

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Is there a preferred drug to stop uterine contractions?

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Tocolytic drugs have been shown to be highly effective at reducing the incidence of preterm delivery within 1-7 days of treatment[1]. However, some investigators question their use based on the failure of randomised controlled trials to demonstrate a significant benefit on neonatal outcome. The neonatal effects of short-term prolongation of gestation with tocolytic agents have not been adequately evaluated in placebo-controlled trials, all of which have been characterised by low steroid usage, no in- utero transfer, enrolment at late gestations, and lack of power[2]. Although, some beneficial effects are suggested by (i) Bayesian trends towards reduced neonatal morbidity and mortality, and improved long-term outcome in the — agonist trials and (ii) randomised controlled trials showing that calcium antagonists improve neonatal outcome and delay delivery more effectively than — agonists[3].

The choice of drug is determined both by tocolytic efficacy, and by the materno-fetal side- effect profile. To date, Oonly three drugs have been shown to prolong gestation in placebo- controlled trials: the — agonists, indomethacin, and atosiban[1]. Calcium antagonists, such as nifedipine, have not been evaluated in placebo-o controlled trials. Furthermore, despite its popularity in North America, there is no controlled evidence to demonstrate that magnesium sulphate functions as a tocolytic agent. There is also insufficient evidence from randomised controlled trials to support use of nitric oxide donors as tocolytics[4].

The β-agonistsagonists, especially ritodrine, have been extensively used in the past but are no longer recommendedused for preterm labour, because of but adverse maternal side effects. s and some fatalities have occurred limiting its widespread use. Adverse effects from tocolytic agents largely result from their non-specificity for uterine muscle. Stimulation of — sympathoagonist receptors causes the increased frequency of adverse events, such as maternal palpitations (48% vs 5% placebo), tremor (39% vs 4%), hyperglycaemia (39% vs 6%), headache (23% vs 6%), nausea (20% vs 12%), dyspnoea (14% vs 1%), vomiting (13% vs 8%)

and chest pain (10% vs 1%) in women onadministered — agonists[1]. Pulmonary oedema occurs rarely (1 in 266 women based on three cases in 797 treated women[1,5]), but has resulted in some maternal deaths. Such side effects are not only potentially life threatening, but also mask signs of intrauterine infection, rendering the tocolytic inefficient and preventing the optimal dose being achieved. In addition, alternative tocolytics are required in aroundapproximately 15% of women.

Cyclo-oxygenase (COX) inhibitors appear to be effective tocolytiicstocolytics, but theritheir use has been limited by concenrconcern about serious fetal side effects, particularly oliguria and ductal constriction., For example, in a large case-control study, indomethacin was associated with increased neonatal necrotising enterocolitis (29% vs 8%), intraventricular haemorrhage (28% vs 9%) and patent ductus arteriosus (62% vs 44%)[6]. To date, there is littellittle evidence that these side effectts are reduced by use of COX-2 selective inhibitors[7].There is as yet littel

Thus, the choice of tocolytic agent in the year 2003 is between the calcium antagonist nifedipine and atosiban, both of which have been shown to ahvehave fewer matenralmaternal side effects than ritodrine.

Although the tocolytic efficacy of calcium antagoonistsantagonists hasve never been validated in randomised controlled trials against placebo, The calcium-antagonist, nifedipine,nifedipine has a greater chance of delivery delay and a lower risk of neonatal complications, tl siide and less maternal side effects orsuch as neonatal respiratory distress syndrome and jaundice, compared towith — agonist therapy. However, the data are based on poorly designed trials:. Indeed, Tsatsaris[1] criticised a nifedipine meta-analysis[2] citing lack of ITT analysis and blinding, inadequate power analyses and poor/no randomisation leading to bias. ; all were unblinded, most lacked an intention-to-treat analysis, some used calcium antagonists as second- line therapy, and others included non-contracting women with ruptured membranes[8,9]. Although the studies suggest a safer maternal side effect profile thancompared with β-agonists (odddsodds ratio 0.4), there are still concerns over serious side effects, including maternal and fetal hypotension and the rare interaction with magnesium leading to respiratory arrestpulmonary oedem[a]a[3,4]. In addition, animal studiiesstudies showuggest that calcium anatagonistantagonists fetal risks of impair uteroplacental perfusion[10,11], although to date, this has not been found in clinical studies. While nNifedipiine has the advantage of oral administration and low cost, it has not undergone rRobusrobusttt t information aboutits short- and long-term safety studies, which would be is required for licensing as a tocolytic agent. For the same reason, the optimal dose and frequency of administration remianremain unclearIndeed, nifedipine is not even indicated for preterm labour, and is actually contraindicated in early

pregnancy with special warnings for later stages. The main advantages are oral administration and low cost..

Unlike nifedipine, atosiban, an oxytocin antagonist, was specifically designed, evaluated and licensed for the management preterm labour. Pooled analysis of atosiban versus β-agonists from three parallel multinational, double-blind randomised-controlled trials representing the largest tocolytic study to date, documentsIt has a comparable effect on delivery delay to the beta-agonists, but with ten- fold fewer maternal side effects. Indeed, the only matenralmaternal or fetal side effect of atosiban is nausea in the mother (11% vs 5% placebo), although this results in the discontinuation of therpaytherapy in <1% of cases. A pooled analysis of atosiban and beta-agonists from three (multinational, double-blind) randomised-controlled trials represents the largest tocolytic study to date, statistically powered to demonstrate real differences[7]. Serious maternal side effects, such as pulmonary oedema and myocardial ischaemia, occurred only with β-agonist but not atosiban therapy. This resulted in a much lower rate Two-year infant folowfollow up neonatal safety of over 300 infants exposed tofor atosiban in from two large placebo- controlled trialstrials revealedfound no untowardexpected safety or developmental concerns findings compared with placebo[12]. The favourable safety profile of atosiban and equivalent effectiveness represents an advance over current tocolytic agents, and accordingly, it is currently the licensed drug of choice for the short termshort-term inhibition of threatened preterm labour.

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Do mothers of triplets over age 40 represent a new obstetric entity?

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The literature provides no consensus on the definition of advanced maternal age. Depending upon the author and year of publication, readers are confronted with >35 years, > 40 years, > 45 years, and, occasionally, menopausal. This lack of clarity is complicated by the occasionally perceived need, to be ''politically correct''. Thus, clinicians are confronted with a choice between the elderly gravida, the mature gravida, the elderly dangerous gravida, women in their ''reproductive twilight'', or obstetrically senescent patients.

The reasons for delayed childbearing are numerous and vary from culture to culture. Among the most important are the following: 1) the post-war (WWII) baby-boom; 2) the emergence of career oriented lifestyles in the developed and developing worlds; 3) access to a variety of methods of fertility control; 4) late and or second marriages; and, finally 5) socio-economic concerns related to childbearing and childrearing.

Prior to addressing the question posed by the title of this presentation, it is reasonable to ask what are some of the major problems that may be associated with pregnancy over aged 40. Aside from medical complications of pregnancy which may be increased and reflect an accumulation of minor and major disease processes, two additional problems need attention. Each is significant in its' own right and, when combined, strongly effect the worldwide trends of pregnancy outcome in older women. The first is generally underemphasized in contemporary textbooks of either obstetrics or gynecology, and relates to the fact that older women either have impaired or reduced fecundity or simply are unable to get pregnant when they want to. In terms of inability to get pregnant, it has been shown that if women marry after their late 30's, one third to one half remain involuntarily childless (Stein, 1985). It has also been determined that each additional year of schooling provided to young

girls in societies where early childbearing had been the norm delays first birth by nine months. Indeed, in some countries health officials freely admit that the greatest impetus to their national family planning programs was teaching young girls to read.

The causes of age related reductions in fertility or, if you will, fecundity, are well known and include poor ovarian factor (in terms of follicular response and/or oocyte quality), endometrial changes (whereby the number of conceptions declines and the number of spontaneous abortions increases), and other considerations such as the accumulation of deleterious effects from leiomyomas, P.I.D., and endometriosis, either individually or collectively (Keith and Oleszczuk, 1999). Regardless of whether these factors operate one by one or in an additive manner, studies of the marital fertility rate per 1000 females consistently show a peak at or about age 24 and a gradual but continual decline until age 45-49. This consistency is trans-national and trans-cultural and has been shown in as diverse populations as American Hutterites (a small closely intermarried religious community), diverse populations in 17th and 18th century Europe, and women residing in the Third World (Stein, 1985). As steep as is this decline, however, natural conceptions do occur in a very small percentage of women between the ages of 45 and 54 in all groups examined.

A second problem of the older woman contemplating pregnancy is that she is at risk of having a multiple gestation, regardless of whether the pregnancy is spontaneous or induced. Indeed, advancing maternal age was recognized as a risk for multiple gestations long before physicians had any concept of how to induce ovulation and secondarily cause these eggs to become fertilized. The truth of this statement is born out by the changes of the numbers of live births per 1000 women in the United States between 1990 and 1998 in the respective cohorts of women aged 35 to 39 and age 40. For the former, the rate was 31.7% in 1990, and 37.4% in 1998; for the latter the rate was 5.7% in 1990, and 7.7% in 1998 (Newman and Luke, 2000). These changes are further accentuated if one looks at the increases in the numbers of births by plurality in the same time span for women over age 39. Whereas the increase in the singleton births was slow and gradual with time, this rate of increase was eclipsed by the rate of increase in the twins, which was also eclipsed by the exponential rise in the rate of increase in triplet births (Ventura et al, 2000).

Four separate lines of evidence exist to support the contention that mothers of triplets over age 40 are indeed a new obstetric entity, at least in terms of selected outcome characteristics. The first is a private run of the 1995-1997 matched multiple data from the NCHS of the CDC (Martin et al, 2000) provided to the senior author of this chapter by M.S. Amy Branum. In this, nulliparous and multiparous mothers of triplets were grouped in the following aged

categories: 25-29, 35-39, and 40+. Outcome variables were gestational age, total triplet birth weight and individual triplet birth weight. The findings of this study showed that mothers aged 40+ achieved better outcomes in all three outcome categories for nulliparas and multiparas. Of equal importance neonatal death rates (actual numbers and rates per 1000) declined substantially as maternal age increased.

The second is the NIH interpretation of the NCHS matched file (Zhang et al, 2002). This study evaluated, among other things, very preterm birth (<32 weeks), very low birth weight (< 1500gms), perinatal death and infant death. Maternal age categories were 30-34, 35-39, and 40+. Older mothers did significantly better in all of the outcome variables.

The third body of evidence comes from an analysis of more than 3000 sets of triplets in the data base of Matria Healthcare (Marietta, GA), a private firm specializing in assisting physicians to provide various types of home monitoring to high-risk pregnancies (Keith, 2002). The data set contained 171 mothers aged 40 or above who were matched by parity to 342 mothers aged 32-39 and 342 mothers aged 25-29. Among the important findings of this analysis were the following: 1) mothers >40 had only about one third of the number of deliveries <28 weeks versus mothers aged 25-29 (2.3% vs 6.4%); 2) mothers >40 had heavier triplets versus mothers 25-29 (A: $p=0.016$; B: $p=0.01$; C: $p=0.03$); 3) total triplet birth weight was significantly higher for mothers >40 than for mothers aged 25-29 ($p=0.01$).

The final body of data was prepared by Blickstein and Jacques (2002) using the same Matria data set as was used by Keith. Among nulliparas and multiparas the numbers of sets >5000gms increased and the numbers of sets <3000gms decreased as maternal age advanced.

The findings described above are somewhat counterintuitive. At present there is no accepted explanation of why older mothers of triplets appear advantaged in terms of selected obstetric outcomes. Prior pregnancy experience is one potential explanation, but clearly other potential explanations exist. The facts regarding parity effects on uterine size and weight are well known. Until recently, however, they have not been related to potential differences in outcomes. Simply stated, according to Dickinson (1949) the average nulliparas uterus varies in length from 3.2-8.1cms in contrast to the parous uterus, which varies from 5.7-9.4cms. At the same time the average para 0 uterus weighs 6.2gms in contrast to the para 6 uterus whose average weight is 125gms. Lye et al (2001) recently proposed that the uterus is ''remodeled'' as a result of prior pregnancy experience under the influence of hormonal changes. Simply stated, these authors proposed that new myometrial cells proliferate early in pregnancy, subsequently switching from proliferation to hypertrophy as the pregnancy progressed. In such a construct even pregnancy which ends in abortion results

in cell hyperplasia. If this is the case, then one could conclude that subsequent pregnancies may occur in a more efficient uterine structure. To test the likelihood of this possibility, Keith (2002) examined the Matria database (case and controls) for a history of abortion. He found that more women >40 had a history of abortion compared to women aged 25-29 ($p=0.0001$).

Given the data cited above, one can reach five conclusions, albeit tentative, in addition to answering the question posed in the title, which is ''yes''. First, unlike the ovary, the uterus does not lose its' ability to function with age. Second, it is not unclear if older mothers of triplets are advantaged or younger mothers of triplets are disadvantaged. Third, in terms of neonatal outcomes, cautious optimism may be warranted for mothers of triplets over age 40. Forth, our understanding of maternal risks as they relate to age is incomplete, although other data sets document important increases in the known major complications of pregnancy. Fifth, and finally, the upper limit of successfully carrying a triplet pregnancy after age 40 is unknown.

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How do iatrogenic multiple pregnancies complicate perinatal care?

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The human female has been programmed by Nature for mono-ovulation, mono-fetal gestation, and for raising a singleton offspring at a time. Within the infinitesimally short period in Mankind history, starting with the introduction of ovulation induction and followed by assisted reproduction, dramatic changes in multifetal pregnancies occurred. This paper discusses several lines of evidence to support the concept that iatrogenic multiple pregnancies overwhelm the natural capacity and therefore lead to paramount perinatal implications.

In spontaneous pregnancies, only one in 120 (8/1000) deliveries result from a reproductive *disorder*, whereby human females produce more than one leading follicle, ovulates more than one ovum, and give birth to a polyzygotic (DZ, TZ, etc.) conception. There are enough data to support the concept that this disorder characterizes taller, older, parous, and women, who have a familial history (i.e., genetic) and a racial background for twinning. It is unknown if the natural selection of women to have multiples increases the chance of a successful outcome. On the other hand, it is certain that selection for assisted reproduction technologies (ART) does not include any phenotypic characteristic associated with greater chance of a successful outcome.

It is accepted that ovulation induction and ART are the main culprits for the epidemic of multiple gestations seen today. Because of the availability of ovulation enhancing agents and the rather lenient requirements for therapy and registration, data about multiple pregnancy rates following ovulation induction are generally missing and therefore, estimates exist for ART only. In any case, the transfer of multiple embryos and the induction of superovulation have caused a sharp increase in the incidence of multiple gestations and births. Data from developed countries show a 50-80% increase in twin births and 400-800% in triplet and higher-order births within the last two decades.Despite such limitations in hard data, estimations for higher-order multiple births (>triplets) in the United States during 1997 were about 20% spontaneous, 40% following ovulation induction, and 40% after ART. The figure for ART — 40% — is twice the

figure quoted for the years 1990-91, suggesting that this method of conception became an increasingly important etiology for multiples towards the end of the last decade. ART data from the United Kingdom show 25% twin and 5% triplet pregnancies whereas USA data show 32% twin and 6% triplet pregnancies.

In spontaneous twinning, only one in 250 (4/1000) deliveries result from a reproductive *anomaly*, whereby the human female produces one leading follicle, ovulates one ovum, but gives birth to a monozygotic (MZ) twin pregnancy. It is accepted that iatrogenic multiples have an increased rate of zygotic splitting, but the etiology is still obscure. One compelling speculation is exposure of the zona pellucida to biochemical or mechanical trauma, which leads to herniation of the blastocyst and cleavage of the zygote. Overall, a MZ conception is expected in 1:10-14 iatrogenic twins. Zygotic splitting is not only of biologic interest, but is of fundamental clinical importance, mainly because of the confirmed increased morbidity and mortality associated with MZ pregnancies. In a recent population-based survey examining the incidence of twins following single embryo transfer, a 2.3% zygotic splitting rate was found among IVF conceptions, a figure 6 times higher than the 0.4% rate after spontaneous pregnancies as quoted in the literature. Interestingly, similar splitting rates were observed following the transfer of fresh embryos to that of frozen-thawed embryos and in comparing stimulated versus unstimulated cycles.

Taken together, multiples are not only significantly more frequent after ovulation induction and ART, but also include more MZ conceptions.

A second line of evidence to support the concept that iatrogenic multiple pregnancies overwhelm the natural capacity is the association between twinning and increased embryonic, fetal, and neonatal loss. It is quite obvious that embryonic and fetal loss are significantly more frequent among twins, especially iatrogenic. This statement is true for higher order multiples as well.

The third line of evidence relates to the lower growth potential of multiples as compared to singletons, suggested by the fact that multiples are *absolutely* smaller compared with singletons throughout the third trimester. It has been proposed that these growth problems are accentuated in iatrogenic multiple gestations, including those reduced to twins. Taken together, all multiples represent significant growth problems imposed by the maladaptation of the human uterus to carry twins and higher order multiples. The potential nationwide impact of assisted conceptions on outcomes may be appreciated from a recent population-based study that examined the Israel National VLBW Infant Database. Between 1995 and 1999, multiples comprised one third of VLBW infants — 10 times their prevalence in the entire population. Assisted conceptions were responsible for 10% of the singletons, 55% of twins, and 90% of the triplets.

The combination of physiological and pathologies changes increase the

maternal morbidity associated with multiple pregnancies. Morbid states such as hypertensive disorders, eclampsia, complications of treatment for premature contractions, prolonged bed-rest, prolonged hospitalization, and operative deliveries are significantly higher among twin and high-order multiple gestations. The remarkable adaptation, seen in all maternal systems, to the demands of a large ''fetal mass'' at an early gestational age frequently overwhelms the mother's physiologic capacity, leading to relative or absolute failure of adaptation (i.e., insufficiency). Such mechanisms include the enormous change in the cardiovascular system, hematopoiesis, and carbohydrate metabolism that occur during a multiple pregnancy. Many of the maternal complications represent serious pathologic antecedents to mortality, a fact that is often either overlooked or ignored.

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Antagonists for ART without twins: preliminary results in IUI cycles*

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Introduction

The main cause by far of the epidemic twinning observed in the last two decades is the intentional induction of multiple ovulation, either alone or associated with assisted reproductive cycles. Derom et al. (1993) reported that 77% of twins and 72% of triplets born in Belgium were associated with isolated ovarian stimulation. Similarly, a multicentric study in the United States showed that the incidence of multiple gestation was particularly high (29%) among women in whom ovulation had been induced with gonadotrophins, with 9% of high-order multiple pregnancies (Gleicher et al., 2000).

Multiple births are regarded today as a serious complication of pro-fertility treatments, because perinatal mortality is seven times higher among triplets and five times higher for twins (Bergh et al., 1999; Fisk and Trew, 1999). The chance of a triplet pregnancy resulting in a baby with cerebral palsy is 47 times, and for a twin pregnancy eight times that of a singleton pregnancy (Petterson et al., 1993) and the invasive multi-fetal reduction procedure does not improve the outcome of the triplet pregnancy (Leondires et al., 2000). Multiple pregnancy also imposes additional financial, emotional, and logistic burdens on families and health-service providers. These are all solid arguments for preventing multiple pregnancy.

Intrauterine insemination (IUI) in cycles stimulated with gonadotrophins is an accepted, widely adopted policy for aspecifically improving the background fertility of a large proportion of subfertile couples, such as those with unexplained infertility and male subfertility (Crosignani, 2002).

Unfortunately there is no way to reduce the rate of multiple births after induction of ovulation without also reducing the success rate (Collins, 1994).

This pilot study was designed to study the effect of a GnRH antagonist on the quality of IUI cycles stimulated with two low-dose regimens of recombinant FSH.

Patients and treatments

Patients. In the two centers organizing the study (infertility units of the first Department of Obstetrics and Gynecology at Milan State University and at San Raffaele Hospital, Milan) women included had unexplained infertility lasting at least 24 months (normal ovulation, normal tubes, normal male) or the male partner showed a moderate seminal defect (sperm density = 5 million motile sperm).

Randomization. After inclusion in the study the patients were randomized in two groups according to a computer-generated list.

Ovarian stimulation and US monitoring. Starting from the third day of the cycle patients in group A were given recombinant FSH (Puregon, Organon, Netherlands) 50 IU per day and those in group B were treated with recombinant FSH 50 IU on alternate days. Ovarian stimulation in both groups was monitored by daily transvaginal ultrasound (US) scans starting from day 8 of the cycle. The GnRH antagonist Ganirelix (Orgalutran, Organon, Netherlands) at the dose of 0.25 mg per day was started from the day when a follicle = 14 mm in mean diameter was visualised, until human chorionic gonadotrophin (hCG 5000 IU im) was given (leading follicle with a mean diameter = 18 mm).

Cancellation. To prevent multiple gestations, the cycle was cancelled if there were more than two follicles with a mean diameter = 16 mm visible at the time of hCG injection.

Luteal supplementation. No luteal phase supplementation was prescribed since we have previously demonstrated a normal hormonal luteal profile in patients treated with recombinant FSH and GnRH antagonists (Ragni et al., 2001).

Intrauterine insemination. Insemination was performed 34 hours after hCG administration.

Hormone assays. Serum levels of estradiol were assessed daily starting on the first day of GnRH antagonist treatment until the hCG injection. Serum levels of progesterone were determined 0, +2, +4, +6, +8, +10, +12 days after hCG. All hormonal assays were obtained retrospectively and did not influence clinical decisions regarding ongoing cycles. Serum was obtained from blood samples after centrifugation at 800 g for 10 minutes at room temperature and stored at -20C until assay. All samples were analysed simultaneously. Commercially available immunoassays for in vitro quantitative determination (Roche Diagnostics GmbH, Mannheim, Germany) were used for serum estradiol and progesterone.

Results and comments

A total of 69 patients were recruited and 66 cycles were fully evaluable. Mean serum estradiol levels in each group did not rise the day after the GnRH antagonist dose but, then resumed their upward trend. Table 1 shows the folliculogenesis, ovulations and pregnancies associated with the two stimulation schedules.

Table 1. *Clinical outcome of the IUI cycles.*

	Ovarian stimulation	
	Group A daily FSH	Group B FSH alternate days
Cycles	32	34
Large follicles (= 16 mm) mean no./cycle	1.5 ± 0.5	1.2 ± 0.5
Luteal progesterone (ng/ml)	18 ± 6	18 ± 9
Clinical pregnancies per cycle initiated	11	2

In the 66 cycles 13 singleton clinical pregnancies were obtained, 11 in group A and 2 in group B ($p=0.005$). Forty-two cycles were monofollicular (53% in group A and 79% in group B); more than two leader follicles = 16 mm in diameter were observed only in two cases (group A): these cycles were therefore cancelled. One cycle (group B) was cancelled due to low response. Progesterone showed normal ovulatory profiles in all 66 induced cycles.

FSH every 48 hours, despite the normal ovulation indicated by normal progesterone plasma concentrations, is probably associated with a suboptimal rate of fertilization. In fact despite the limitations due to the few cycles studied the pregnancy rate associated with daily FSH seemed better than with alternate-day ovarian stimulation. Considering the 11 pregnancies unexpectedly obtained in group A, the innovative use of a GnRH antagonist seems to improve the fecundability of the oocytes produced by the treatment.

The daily low-dose gonadotrophin and GnRH antagonist regimen induced consistently mild ovarian stimulation with 53% monofollicular cycles and, most important, was constantly associated with singleton pregnancies (Alagna et al., 2002). The GnRH antagonist, as already observed (Ragni et al., 2001), had no detrimental effect on the ovarian stimulation, in either the follicular or the luteal phase.

In conclusion, the remarkably high pregnancy rate associated with monofollicular cycles may be related either to the better quality of ovulation obtained in the medicated cycles or to the proper timing of IUI.

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Consequences of first trimester endocrinology for diagnosis and treatment of pregnancy failure

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Basically progesterone is essential in most animal species and in man for the maintenance of pregnancy (1). Recently, it was also pointed out, that estradiol is also crucial for normal pregnancy development (2). Production and secretion of both are governed by placental HCG.

There are two sources of endogenous progesterone and estradiol during early pregnancy.

1. Corpus luteum
2. Placenta

The first routinely measurable parameter is β-HBG by radioimmunoassay in blood. The first measurable values are found 48 hours after implantation (about day 24 to 26 of the cycle)(3). From there on there is a steep increase of HCG leading to doubling of the HCG concentration within 48 hours (4). If such doubling does not occur a distrurbed pregnancy is indicated, which may be due:

1. ectopic location of the gestation
2. trophoblast abnormality
3. disturbed placentention
4. genetic aberrations

The doubling time of 48 hours last up to a concentration of HCG in blood of 1200 to 2000mIU/ml. Up to 6000mIU/ml the doubling time lasts 72 hours and thereafter 96 hours(4).

Therefore, these doubling times are suitable to evaluate pregnancy development or more precisely trophoblast development and function. Normally β-HCG rises until the tenth week of gestation and decreases thereafter to a

concentration between 10000 to 20000 mIU/ml. In cases of multiple pregnancy the course of the β-HCG levels are similar except that the concentrations are higher (Fig.1).

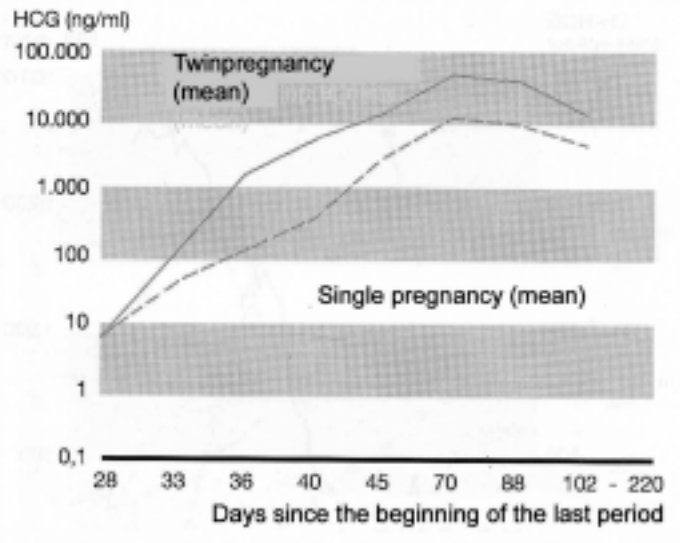

Figure 1: *Course of HCG in normal, single and multiple pregnancies during the first trimester (according to 3)*

The correlation between the length of gestation calculated from the first day of menstruation, the HCG-concentration in blood and urine and the ultrasound findings are shown in Fig.2. Failure of adequate increase of HCG during the first 10 weeks of gestation indicates an abnormality of pregnancy. In case of lack of increase or even decrease of HCG during the first 10 weeks of gestation is associated with pregnancy failure (including ectopic pregnancy). Increased levels of HCG are indicative of twin and multiple pregnancy (Fig.1), but elevated levels can also be associated with molar pregnancy and in rare cases with choriocarcinoma.

Progesterone is of particular importance for implantation and for pregnancy development.

Progesterone dependent effects are:

1. Preparation of the endometrium for implantation (secretory changes)
2. Endometrial dezidualisation
3. Production of a number of endometrial proteins such as uteroglobin, PAPP and PP14. These proteins are influencing the immunological processes by blocking antigen recognition and thereby causing immune suppressive effects.

4. Regulation of the cellular immunity.
5. Stimulation of prostaglandin E2 production which suppresses a number of T-cell reactions.
6. Stimulation of lymphocyte proliferation at the feto-maternal interphase.
7. Suppression of the interleukin 2 increased cellular cytotoxicity.
8. Suppression of T-cell- and killer-cell-activity
9. Shift from the TH1 to TH2 cells
10. Synthesis of the progesterone-induced blocking factor (PIBF)
11. Suppression of matrix metalloproteinases

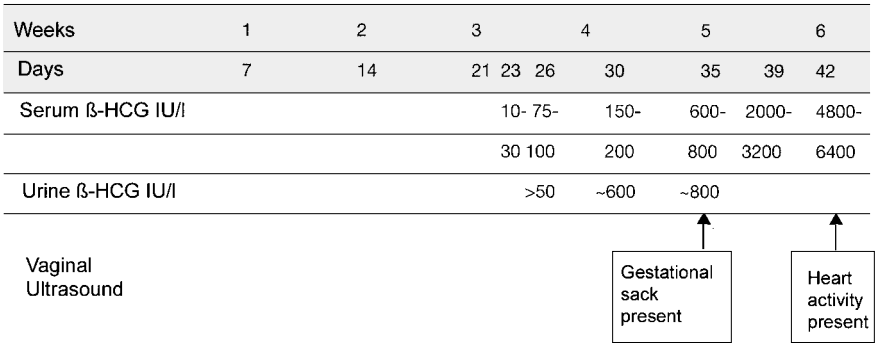

Figure 2: *Correlation of HCG in blood and urine with ultrasound findings (according to 3)*

Therefore, lower levels of progesterone in the corpus luteum phase are causing inadequate development of the endometrium, but are also associated with an insufficient control of the maternal immune system exposing the allogenic conceptus to the reactions of the maternal immune system. In addition, the amount of circulating estradiol seems to be of importance (2). Among the many fold effects of estradiol there is also a positive action of estradiol on the progesterone biosynthesis (11).The ability of progesterone measurements to predict gestational complications was independent of follicular stimulation (10).

Normally the increase of progesterone after implantation due to placental HCG is leveling of towards the 8th to the 10th week of gestation and the rise there after continuously until prior to term (3). For estradiol there is at first a rise, followed by a plateau and than a steep increase around the 7th week of gestation. The increase lasts till term (6). The special profile of progesterone is due to the fact that at first progesterone is biosynthesized and secreted by the corpus luteum, which is stimulated by placental HCG. The life time of such a corpus luteum graviditatis is limited, which has been shown on the one hand biochemically by measuring 17á-hydroxyprogesterone which rises similar to the corpus luteum activity and starts to decline after the 6th week of gestation (3, 6),

since the placenta does not process 17á-hydroxylase, which is however present in the corpus luteum. On the other hand, it was found that lutectomy before the 8th week of gestation leads not only to a step fall of progesterone but to miscarriage (6, 7). Indeed, serum progesterone appears to be the single most specific biomarker for distinguishing viable from nonviable pregnancy (8, 9). Sensitivity and specificity of serum progesterone (cut off level < 45 nmol/l) in predicting nonviable pregnancies was 88.6% and 87.5% respectively) (9). Combining serum progesterone with HCG, specificity improved significantly(8). The ability of progesterone measurements to predict gestational normalicy is independent from normal and chlomiphen citrate stimulated cycles (10).

Further endocrine abnormalities which interfere with normal pregnancy are: thyroid dysfunction, hyperprolactinemia and hyperandrogenemia. All these conditions are associated with corpus luteum insufficiency. Corpus luteum insufficiency is a major cause of spontaneous and recurrent abortion (12) and defective luteo-placental progesterone shift (13,14).

The reason for abortion due to insufficient progesterone and also estradiol is based upon the following abnormalities:

1. Corpus luteum insufficiency either progesterone or progesterone/ estradiol primarily low (4/5 weeks of gestation) or secondarily decreasing until about the 11th week of gestation.
2. Regression of the overstimulated ovaries with decrease of progesterone and estradiol until about the 11th week of gestation.
3. Insufficient placentation or trophoblast function with retarded progesterone increase from the 11th to the 20th week of gestation.

It needs to be clarified to what extend the relative high abortion rate (up to 40%) after stimulated cycles (chlomiphen, gonadotropins) or in combination with ART is related primarily to corpus luteum and /or placental dysfunction.

Therefore, hormonal treatment indications are the following as shown in the table 1 (15, 16).

Table 1: *Hormone treatment of pregnant women with corpus luteum insufficiency and placental dysfunction*

Origin of low progesterone / estradiol	**Hormonal treatment**
Corpus luteum insufficiency a) primary b) secondary	 Progesterone/ progestins Progesterone/ progestin-estradiol combination
Regression of overstimulated ovaries	Progesterone/ progestins Progesterone/ progestin-estradiol combination
Placental insufficiency	Progesterone / progestins Progesterone / progestin-estradiol combination

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Early pregnancy loss, is there any treatment? Can progesterone help?

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Summary

Abortion is one of the most frequent complications during pregnancy. Maintenance of early pregnancy depends on an adequate production of progesterone by the corpus luteum in response to stimulation by trophoblast-derived hCG. The analysis of hormonal profiles in early pregnancy loss cycles demonstrates the critical importance of the timing of implantation for successful pregnancy outcomes. The luteal progesterone surge establishes endometrial receptivity as well as suppression of myometrial contractions and modulation of immune functions. The term 'luteal phase defect' describes situations with inadequate luteal steroidogenesis, e.g. due to an inadequate follicular development or infertility drug-induced. Accepted diagnostic methods are repetitive progesterone serum measurements and endometrial biopsies. The beneficial value of luteal phase support has clearly been demonstrated only in patients undergoing infertility treatment, especially in combination with the use of GnRH agonists.

Statement of the problem

An estimated 12-15 % of all clinical pregnancies end in abortion(1), delineating it as one of the most frequent complications during the course of pregnancy. The incidence of early pregnancy loss — defined as biochemically established pregnancies that are lost around the time of the next menstruation and may not even have been recognized as such — is even higher with estimates around 17-22%(2,3). Repetitive sequences of three or more abortions in one patient are defined as recurrent miscarriage or habitual abortion. Among other factors, endocrinological misbalances appear to be causally related with recurrent pregnancy loss (Fig. 1).

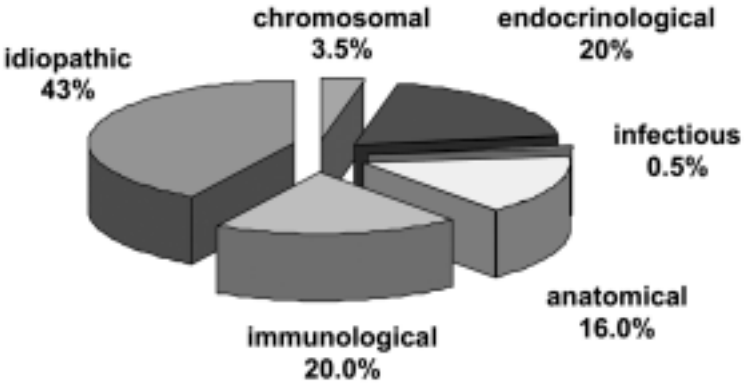

Figure 1. *Frequency of factors associated with habitual abortion. Modified from 4.*

The physiology of the corpus luteum

The source of progesterone in the human is the corpus luteum, which is largely under the control of pituitary-derived luteinizing hormone (LH). Prepared for by an increasing number of LH receptors on the cell surface and initiated by the midcycle LH surge, the granulosa and theca cells differentiate into granulosa- and theca-luteal cells with the capability of producing high amounts of progesterone(5). LH acts through cAMP as a second messenger, thus regulating essential genes for hormone synthesis and luteal development like steroidogenic acute regulatory protein (StAR), the cytochrome P450 cholesterol side chain cleavage ($P450_{scc}$) or 3-β-Hydroxysteroid-Dehydrogenase(6). In non-fertile cycles, regression of the corpus luteum occurs independent of LH pulse frequency or amplitude(7), and most likely due to intraluteal factors regulating LH action. Fertile cycles are characterized by a mid-to-late luteal phase increase of trophoblastic hCG, rescuing the corpus luteum from its programmed senescence. Early studies involving luteectomy have demonstrated, that the corpus luteum is essential in the first weeks of pregnancy, but its function is then replaced by the trophoblast(8). This phenomenon is termed luteo-placental transition or shift and occurs around week 7 of pregnancy.

Progesterone action on the endometrium

Successful implantation depends on the timed receptivity of the endometrium for the blastocyst, which is the biological aim in any menstrual cycle. Endometrial differentiation is cyclically regulated by the ovarian steroid hormones estradiol and progesterone in such, that there is endometrial proliferation in the estradiol-dominant follicular phase and secretory transformation under the influence of luteal phase progesterone. Histological analysis

of the endometrium allows an in- or out-of-phase ''dating'' of endometrial biopsies(9). This classic diagnostic approach has recently been complemented by the analysis of an increasing number of molecules providing a more detailed and significant interpretation of the functional phase of the endometrial cycle(10). Actions of progesterone on the endometrium in terms of support of implantation on the molecular level are based on the upregulation of antiluteolytic agents like interferon (IFN) tau or Osteopontin, and down-regulation of inhibitors of implantation like mucin glycoprotein one (MUC-1)(11). The influence of adhesion and other signaling molecules on the endometrial differentiation process allows speculations about molecular deficits leading to insufficient endometrial reaction even in the case of sufficient circulating progesterone concentrations.

Luteal phase defect

Luteal steroidogenesis is required for the development of endometrial receptivity and maintenance of early pregnancy. The term ''luteal phase defect'' (LPD) has been used to describe conditions with inadequate progesterone production by the corpus luteum and subsequently inadequate development of the endometrium. Disturbances of the luteal phase can be secondary to endocrinological disorders like hypothyroidism or prolactinoma, or to the use of infertility drugs in the follicular phase. In fact, the majority of LPD in unstimulated cycles appears to be rather a resultant of an insufficient follicular maturation, e.g. in polycystic ovary syndrome, than a primary defect in the corpus luteum itself. Treatment of choice would therefore rather be the removal of these underlying causes than the substitution of progesterone itself(12). However, improvement of follicular maturation using infertility drugs, e.g. antiestrogens or gonadotropins, may also be associated with inadequate luteal steroidogenesis(13). Also in stimulated cycles, besides progesterone deficiency, imbalance in ovarian steroid production like a high estrogen-to-progesterone ratio has been associated with pregnancy loss(14). Lacking a clear definition, there has been much controversy about the actual existence and diagnosis of LPD. The two most accepted diagnostic methods include progesterone serum measurements and assessment of endometrial biopsies(15). LPD is generally assumed in patients with progesterone values of less than 10 ng/ml in at least one out of three blood samples during the second phase of the luteal cycle(16), or with biopsies showing insufficient secretory differentiation of the endometrium according to classical criteria 9. In patients with diagnosed luteal phase defect without any other treatable cause, progesterone supplementation appears to be advantageous(12,17), but large-scale randomized studies have not been performed to date.

Hormonal profiles of early pregnancy loss

Several studies have focused on the course of progesterone production in early pregnancies of women without known fertility problems. Early pregnancy loss is recognized by a decline in urinary progesterone metabolites in the late luteal phase, and appears to be critically related to the time of implantation. At the time of implantation, the luteotropic effect of hCG that derives from the conceptus extends the functional life span of the corpus luteum(18). Levels of urinary estrogen and progesterone metabolites are similar in cycles of early pregnancy loss and successful conception cycles up to the time when implantation normally occurs(19). Therefore, in most successful human pregnancies, the conceptus implants 8-10 days after ovulation, with an increased risk of early pregnancy loss in patients with later implantation(20) (Figure 2). At the time of implantation, changes in progesterone metabolites are correlated with the probability of an early pregnancy loss(18). The presence of a progesterone surge at the time of implantation suggests its functional significance, e.g. for the inhibition of uterine contractions or for the modulation of immune functions(21). Based on these studies, ovarian steroid production deficiencies do not appear to be the primary cause of early pregnancy loss in reproductively normal women, but rather the timing of implantation of the conceptus providing a luteotropic signal for the corpus lutum. Whether this can be caught up by exogenous progesterone supplementation has to our knowledge not been thoroughly analysed yet, as these women usually do not present at an infertility clinic which would allow prospective evaluation in the form of clinical studies.

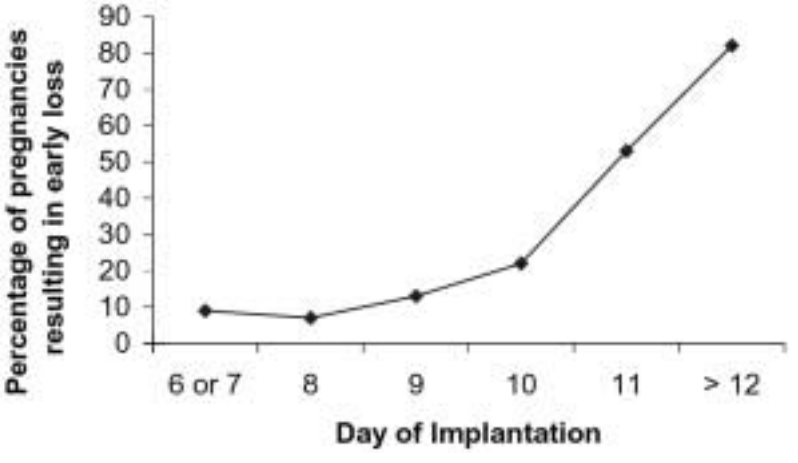

Figure 2. *Timing of implantation in 189 Naturally Occuring Pregnancies and the Risk of Early Loss 20.*

Luteal phase support

The value of luteal phase support has most extensively been investigated in patients undergoing infertility treatment. Corpus luteum insufficiency following ovarian stimulation and ovulation induction has frequently been described in combination with GnRH analoga treatment(22). The pathophysiological explanation for this may not only be the disruption of physiological LH stimulation of the corpus luteum for as long as 10 days after the last dose of the agonist, but also direct effects of the different drugs. The need for luteal supplementation in IVF cycles was evaluated early on(23,24) in large randomized trials, and a recent metaanalysis performed only on cycles where GnRH agonists were used for the prevention of premature luteinization could show a benefit of either progesterone or hCG luteal supplementation versus no treatment for fertility outcomes(25). The advantages of either progesterone supplementation versus hCG stimulation or differences in the route of application have largely been discussed, rendering intramuscular application of progesterone as more effective than intravaginal or oral in the cited meta-analysis. No differences in terms of efficacy were found between progesterone and hCG, but given the increased risk of ovarian hyperstimulation syndrome associated with hCG use, i.m. progesterone has to be favoured.

Conclusions

The production of progesterone by the corpus luteum is necessary for the maintenance of early pregnancy. It is mostly dependent on the adequate hCG stimulation by a timely implanting conceptus. In a subset of patients that suffer from recurrent miscarriages the possibility of a luteal phase defect and its underlying causes have to be carefully evaluated and treated. The benificial use of luteal support regimens with either progesterone or hCG has conclusively been demonstrated in patients undergoing infertility treatment.

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Maternal autoimmune disease: can it affect future generations?

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Autoimmune diseases are familial in occurrence, are polygenetically inherited and show clear female preponderance (1). Selected autoimmune diseases have in recent years been suggested to be the consequence of fetal-maternal, long-term microchimerism (FMM), as fetal cells have been conclusively demonstrated in autoimmune lesions of skin (2) (3) and thyroid (4) many years after pregnancy.

Whether, concomitantly to FMM, there is also maternal-fetal microchimerism (MFM) is unknown, though, of course, cell traffic between fetal and maternal compartments is bi-directional. Indeed, cell traffic in both directions peaks at time of delivery and is greatly influenced by mode of delivery. For example, we know from experience with HIV-infected women that the risk of HIV-transmission to the fetus can be greatly reduced by the performance of a cesarean section (5). The reason for this observation lies in the fact that cesarean sections reduce maternal-fetal cell traffic (MFCT) in contrast to vaginal deliveries. Cesarean sections, however, have exactly the opposite effect on fetal-maternal cell traffic (FMCT), as the immunization experience of Rh-negative mothers has demonstrated (6), who, because of increased FMCT with cesareans, require higher dosages of anti-D immunoglobulin than mothers delivered vaginally.

These observations induced us to consider an experimental model, based on the differences in MFCT and FMCT, in investigating the concept of MFM.

Specifically, our model was based on the assumption that the establishment of microchimerism was dependent on cell traffic. Moreover, it appeared reasonable to assume that the amount of cell traffic might correlate with the probability of microchimerism, i.e. **the more cell traffic, the higher a probability of long-term microchimerism**. Of course, considering the fact that autoimmune diseases were **familial-** and **polygenetically-inherited**, such effects should be most obvious in patient populations at increased risk for

autoimmune diseases, such as first generation offspring of mothers with proven autoimmune diseases. In such populations, one would, under such a hypothesis, expect more cases of autoimmune diseases in offspring delivered vaginally, than in offspring delivered by cesarean section.

Confirmation of this hypothesis would not only strongly support the concept of microchimerism as a pathophysiological cause of autoimmunity, but would, for the first time, present supportive evidence that not only FMM but also MFM may contribute to the prevalence of autoimmune diseases. In addition, such a model could, at least partially, explain the female preponderance for autoimmune diseases (1) since autoimmune disease, based on MFM, would, of course, be similarly distributed between female and male risk. Yet, autoimmune disease due to FMM would exclusively affect females. The combined effect of bidirectional cell traffic and bidirectional microchimerism would then be female preponderance of autoimmune disease.

We tested this model by conducting a 2-generational study of mothers and their offspring in women with autoimmune diseases and in normal controls. This study has been reported elsewhere (7) (8) (9) and, indeed, revealed: 1. A significantly increased risk for autoimmune diseases in offspring of mothers with autoimmune diseases; 2. that women with autoimmune diseases were more often delivered vaginally than controls (trend only) and 3. that offspring from mothers with autoimmune diseases were more often affected by autoimmune diseases, if delivered vaginally, than their control peers. The detailed data have been presented elsewhere (7) (8) (9).

The outcome of our study thus presents indirect evidence for the importance of microchimerism in the pathophysiology of autoimmune diseases. Such indirect evidence is, of course, **not** conclusive and requires substantial confirmation. Yet, the potential implications could be profound, if this model for the generation of autoimmune diseases through MFM were, indeed, to be confirmed. This would then suggest the possibility that, equal to HIV-transmission, cesarean section deliveries could reduce the occurrence of disease in predisposed offspring. While in the case of HIV-infection, the fetal predisposition comes from maternal infection, here it is their genetic predisposition towards autoimmune diseases.

The public health implications from such a discovery would be staggering. Indeed, the reduction of phenotypical expression of genetic risk towards autoimmune diseases could then compensate for the increased prevalence of autoimmune diseases and the increase in female preponderance, which one has to expect, as a consequence of more successful treatment of reproductive risk, due to autoimmune diseases (10).

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ET — Can the technique affect the results?

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Summary

Embryo transfer (ET) is an essential step in assisted reproduction and various modifications have been advanced and practiced in order to perfect it. An evidence-based approach to these modifications revealed that only three procedures have been shown by randomized controlled trials (RCT) to improve implantation rates: performing a trial (dummy) ET before the actual procedure, performing the ET under ultrasound guidance and depositing the embryos 2 cm below the uterine fundus. Randomized controlled trials have also suggested that flushing the cervical canal with culture medium, leaving the catheter for 30 seconds or bed rest after ET are of no value, but the numbers studied need to be increased. The value of routine administration of antibiotics after ET or of using a fibrin sealant has not yet been established and no specific catheter has so far established its superiority over its competitors.

Introduction

Despite numerous developments in the field of assisted reproduction, the implantation rate remains dismally low. It has been estimated that 85% of the embryos replaced during in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) fail to implant [1]. The exact cause of this low implantation rate is unknown but may reside in the technique of embryo transfer (ET), in the efficiency of endometrial receptivity or in the ability of the embryo to invade the endometrium properly.

Various refinements of the technique of ET have been suggested in order to improve the pregnancy and implantation rates. However, many of these suggestions were based on retrospective and/or observational studies. The aim of this work was to evaluate the various factors affecting embryo transfer taking an evidence-based approach.

Materials and methods

A meticulous search of the literature was undertaken for RCTs related to embryo transfer. This consisted of searching the Medline database, the EMBase database, the Cochrane library as well as hand searching relevant publications and proceedings of international congresses. A combination of the following key words were used in the search: embryo transfer, implantation, randomized controlled trial, IVF, ICSI.

We have calculated that in order to improve the pregnancy rate from 25% to 35%, taking 5% as the significance level and accepting a 80% probability of finding a true difference, the least number needed to study was 157 ETs in each arm of the study or the meta-analysis. Similarly, in order to improve the implantation rate from 10% to 15%, the least number needed to study was 316 subject in each arm.

Results

The results of this work may be summarized as follows:

1. Gentle and atraumatic technique.

For obvious reasons, the relationship between the difficulty of ET and the success rates can only be studied by retrospective analysis, as it is not possible to randomize studies intentionally into difficult and easy procedures. Moreover, the definition of embryo transfer is not uniform. Difficult transfers have been variably defined as the presence of blood on the catheter or on the uterine cervix, the retention of some or all of the embryos in the ET catheter, the necessity of changing the catheter or using the volsellum to hold the cervix.

Taken individually, some studies have reported that difficult embryo transfers are associated with lower success rates [2,3,4], while others have not confirmed this observation. [5,6,7] We have conducted a meta-analysis of all these studies, grouping all types of difficult transfers and found that difficult transfers were associated with a significant reduction in the pregnancy rate compared to easy transfers [OR= 0.73, 95% CI (0.55-0.97)] (figure 1).

2. Knee chest versus dorsal position

A single RCT of 100 patients reported that there was no statistically significant difference in the pregnancy rate when the ET was performed with the patient in the knee-chest position compared to the dorsal position. [8] However, this work was not repeated and the number of patients studied cannot exclude a type II error.

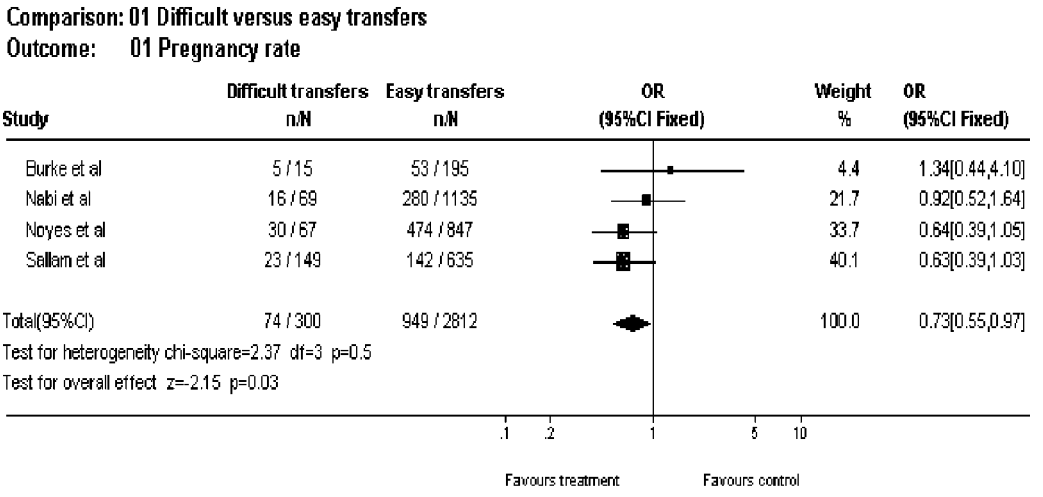

Figure 1. *A meta-analysis of trials studying the relationship between the difficulty of ET and the pregnancy rate in women undergoing assisted reproduction (OR = odds ratio; CI = confidence intervals; n = number pregnant; N = number studied)*

3. Performing a trial embryo transfer before the actual procedure.

Three studies showed that performing a trial (mock or dummy) ET before the actual transfer improved the pregnancy rate, but only one of them was a RCT [9,10,11]. In this study, Mansour et al [9] randomly allocated their 335 patients undergoing ET into two groups. Pregnancy rate and implantation rate were significantly higher in the dummy-transfer group (22.8% and 7.2%) compared to the no-dummy transfer group (13.1% and 4.3%).

4. Embryo transfer under ultrasound guidance.

Performing ET under abdominal ultrasound guidance was first suggested by Strickler et al [12]. This work was followed by numerous publications using ultrasound to accurately position the ET catheter in the uterine fundus and to observe the movement of the embryo-associated air bubble.[13-16]

We have recently conducted a prospective controlled study and found that acute utero-cervical angles were associated with lower pregnancy and implantation rates compared to mild angles. In the same study, we have found that measuring the angle by abdominal ultrasound prior to ET and molding the catheter accordingly, resulted in higher pregnancy (26.25% versus 18.43%; $P<0.05$) and implantation rates (10.72% versus 7.55%; $P<0.01$) [17] (Figure 2).

We have also performed a meta-analysis of RCTs on abdominal ultrasound-guided embryo transfer. A total of 1024 abdominal ultrasound-guided embryo transfers were compared to 1027 with transfers using the ''clinical touch method''. The results showed that abdominal ultrasound-guided ET significantly

increased the clinical pregnancy rate [OR = 1.42 (95% CI = 1.17, 1.73)] and the on-going pregnancy rate [OR = 1.49 (95% CI = 1.22, 1.82)] (figures 3 and 4). There was no effect on the incidence of ectopic pregnancy, multiple pregnancy or miscarriage rate.[18]

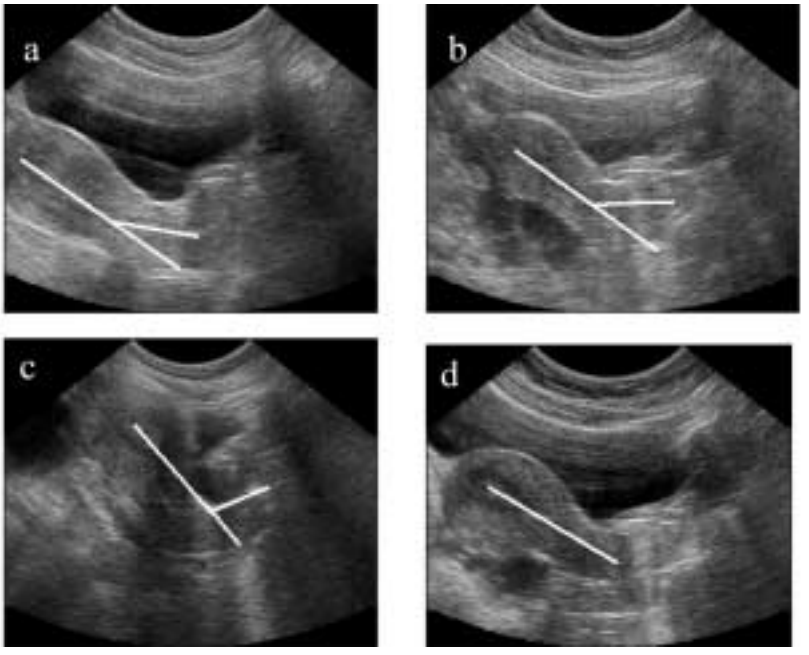

Figure 2. *Measuring the utero-cervical angle on abdominal ultrasonography: (a) Mild angle (<30 degrees), (b) moderate angle (30 to 60 degrees), (c) large angle (>60 degrees), (d) no angle.*

5. Embryo transfer with a full bladder.

A non-randomized trial suggested that straightening the utero-cervical angle by performing ET with a full bladder increases the pregnancy rate [19]. In a different study, Johnson and Bronham [20], suggested to use the volsellum to reduce the uterocervical angle, but other studies have shown that this practice leads to the initiation of uterine contractions which may affect the pregnancy and implantation rates.[21]

6. Removing the cervical mucus prior to embryo transfer.

A non-randomized study on dummy ETs using methylene blue suggested that the removal of the cervical mucus prior to ET prevents expulsion of the dye after the dummy ET, suggesting that removing the cervical mucus might improve the pregnancy and implantation rates[22]. In a different study, Nabi et al [6] analyzed 1204 ET procedures retrospectively and found that embryos were significantly

more likely to be retained when the ET catheter was contaminated with mucus (3.3 versus 17.8%, P = 0.000001). However, there are so far no RCTs on removing the cervical mucus prior to ET.

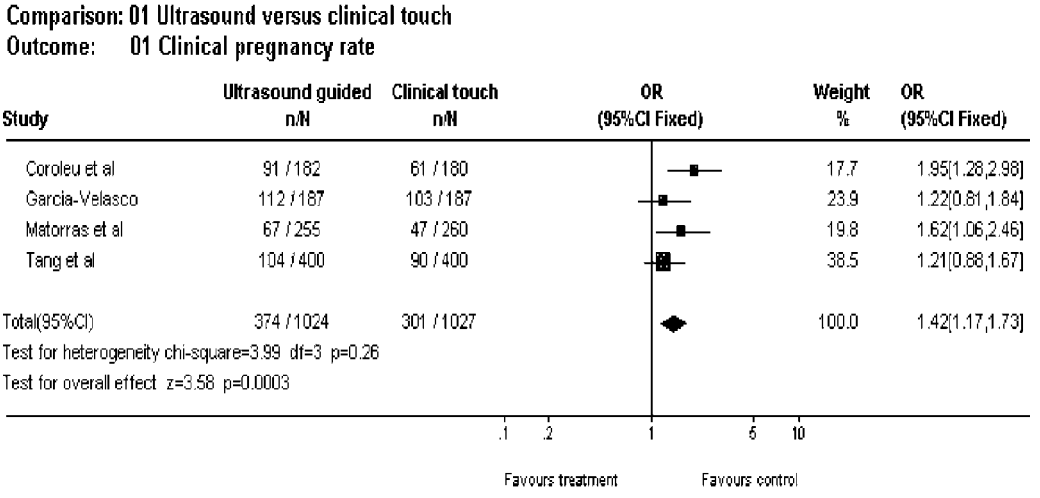

Figure 3. *A meta-analysis of RCTs on ultrasound-guided embryo transfer showing the clinical pregnancy rates (OR = odds ratio; CI = confidence intervals; n = number pregnant; N = number studied)*

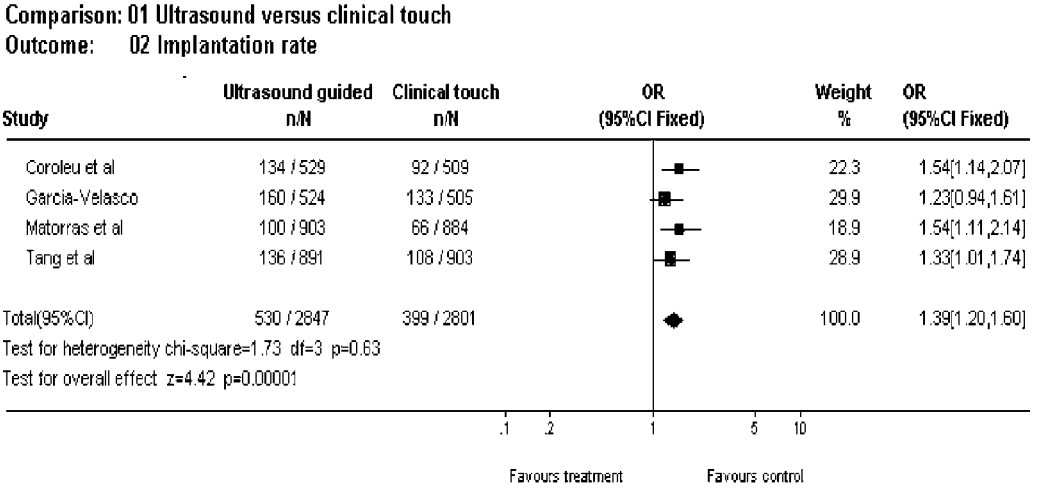

Figure 4. *A meta-analysis of RCTs on ultrasound-guided embryo transfer showing the implantation rates (OR = odds ratio; CI = confidence intervals; n = number pregnant; N = number studied)*

7. Flushing the cervical canal with culture medium prior to embryo transfer
In a non-randomized trial, MacNamee [23] suggested that vigorous flushing of the cervical canal with culture medium prior to ET improves the pregnancy rate from 25.3% to 46.2% and the implantation rate from 8.7% to 17.7%. However, when we conducted a prospective RCT of 110 ETs, we could not confirm these observations [24]. However, the number of patients studied is short of our calculated criteria and the study needs to be extended.

8. Avoiding the use of a tenaculum (volsellum).
In a study by Lesney et al, holding the uterine cervix with a tenaculum (volsellum) was found to stimulate uterine junctional zone contractions [21]. In a different study, Fanchin et al (1998) found that increased uterine contractions at the time of ET were associated with a decrease in clinical and ongoing pregnancy as well as implantation rates, and that the direction of the contractions did not affect ET outcome [25]. In support of these findings, Dorn et al [26] found an elevation in serum oxytocin concentration when the tenaculum was used during ET in 4 out of 5 women undergoing ET. However, the effect of holding the cervix with a tenaculum during ET on the pregnancy and implantation rates has not been studied in a RCT.

9. The type of catheter.
Many studies found that the type of catheter used in ET affects the pregnancy and implantation rates [27-32]. However, in these studies, various catheters were compared and it is difficult to determine which one of them is superior to the others. This can be seen in table I, which shows the results of the 6 RCTs published in the literature.

Table I. *Odds ratios (OR) with 95% confidence intervals (CI) for pregnancy rates in 6 studies comparing various ET catheters*

Catheters compared	OR (95% CI)
Tomcat v/s TDT[23]	5.12 (1.58-16.59)*
Wallace v/s Erlanger[24]	0.56 (0.33-0.94)*
TDT v/s Wallace[25]	0.426 (0.18-0.99)*
TDT v/s Frydman[25]	0.21 (0.09 — 0.48)*
Frydman v/s Wallace[25]	2.01 (1.04-3.88)*
K-soft v/s TDT[26]	1.444 (1.11-1.87)*
Cook v/s Wallace[27]	0.92 (0.56- 1.53)
Soft double (TCC) v/s single rigid (CC)[28]	1.63 (1.14-2.30)*

* = statistically significant ($P<0.05$)

10. Site of embryo deposition

Two RCTs studied the relation between the site of embryo depositing during ET and pregnancy and implantation rates. Nazari et al [33] found that the clinical pregnancy rate after midfundal transfers was higher (14.2%) compared to deep fundal transfer (12.4%), although the difference was not significant. However, the incidence of ectopic pregnancy was higher after deep fundal transfers (1.5% versus 0.4%). In the other RCT, Coroleu et al studied 180 patients and found that the implantation rate was significantly higher when the embryos were replaced 15 mm and 20 mm below the fundus compared to when they were replaced 10 mm only below the fundus ($P<0.05$) [34].

11. Slow withdrawal of the embryo transfer catheter.

It has been suggested that slow withdrawal of the ET catheter might lead to better pregnancy rates as this may prevent uterine contractions. However, in a RCT, Martinez et al [35] studied 100 patients and found no statistically significant difference in pregnancy rates when the catheter was withdrawal immediately or after 30 seconds after embryo deposit. They concluded that either that the waiting interval was insufficient to detect differences, or that the retention time before withdrawing the catheter is not a factor that influence pregnancy rate. However, the number of patients studied is too small to eliminate a type II error.

12. The use of a fibrin sealant.

The addition of a fibrin sealant (glue) to the culture medium containing the embryos during ET was first suggested by Rodrigues et al [36] who tested the system on mouse embryos. A matched control study performed by Bar-Hava et al [37] reported a significantly higher pregnancy rate in the fibrin sealant group compared to the no fibrin group (25.3% versus 14.9%).

Two RCTs were conducted to evaluate the technique and reported contradictory results. While Feichtinger et al [38] reported a pregnancy rate of 18.9% in the fibrin sealant group compared to 17.0% in the control group (not statistically significant), Ben-Rafael et al [39] found a significant increase in pregnancy ($P < 0.05$) and implantation ($P < 0.01$) rate in elderly patients (aged 39-42). Further RCTs are necessary to assess the real value of the technique.

13. Bed rest after embryo transfer.

Bed rest after ET has been found to have no effect on the pregnancy or implantation rates. In a non-randomized study, Sharif et al [40], found that the clinical pregnancy rate per ET was significantly higher in their patients who did not have bed rest after ET, compared to the national data of the UK (30% versus 22.9%). In a RCT, Botta and Grudzinskas [41] studied 182 patients and found no statistically significant differences in pregnancy rates between those who had

24 hours bed rest after ET (24.1%) compared to those who had a 20 minutes rest (23.6%).

14. Routine administration of antibiotics following embryo transfer.
Microbial contamination of the tip of the ET catheter was found to be associated with diminished pregnancy rates after ET [42,43,44]. We performed a meta-analysis of RCTs and found that when microbial cultures from the catheter tips were positive the OR (+/- 95% CI) for diminishing the pregnancy rate were 0.41 (0.27-0.63) (figure 5).

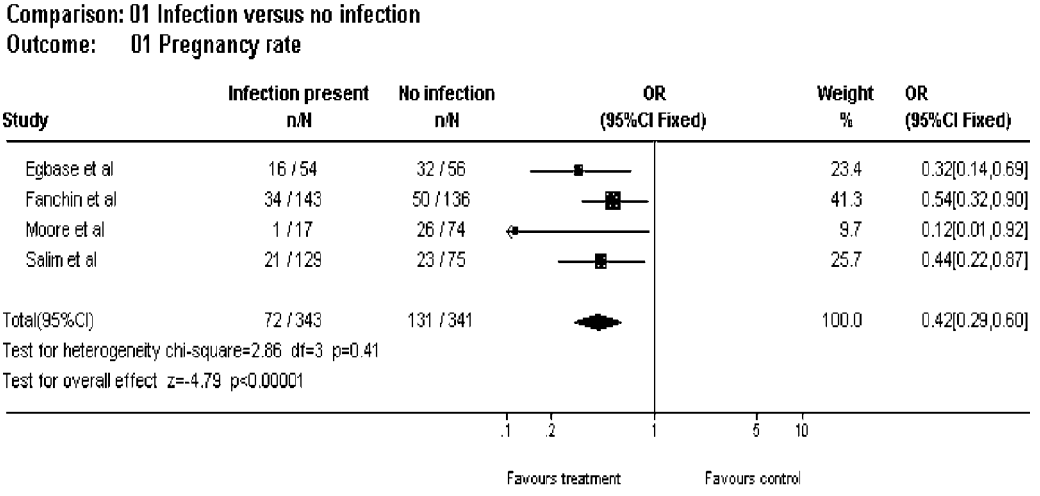

Figure 5. *A meta-analysis of trials studying the relationship between contamination of the ET catheter's tip and the pregnancy rate in women undergoing assisted reproduction (OR = odds ratio; CI = confidence intervals; n = number pregnant; N = number studied)*

However, routine administration of antibiotics following ET is still a matter of debate. In their study, Moore et al [44] found that routine administration of Doxycycline after oocyte retrieval had no substantial impact on the recovery of bacteria. On the contrary, Egbase et al [45] found that the routine administration of prophylactic antibiotics was associated with a reduction in positive microbiology cultures of embryo catheter tips 48h later. No RCT has so far been reported on the effect of routine administration of antibiotics after ET on the pregnancy or implantation rates.

15. Experience of the clinician
It has been suggested that the experience of the clinician performing ET can affect the pregnancy and implantation rates. In a retrospective analysis, Hearns-Stokes et al[46] found that the clinical pregnancy rate varied significantly between

clinicians performing ET (from 17.0% to 54.3%, $P<0.05$). However, this observation has not been confirmed by RCTs.

Conclusions

Embryo transfer remains an elusive step in assisted conception. Only three procedures have been shown by randomized controlled trials to improve implantation rates: performing a trial (dummy) ET before the actual ET, performing the ET under ultrasound guidance and depositing the embryos 2 cm below the uterine fundus. Studies have also shown that difficult ETs and microbial contamination of the ET catheter's tip diminish implantation. RCTs have shown that bed rest after ET, flushing the cervical canal with culture medium and leaving the catheter in situ for 30 minutes are of no value, but more patients need to be studied to exclude a type II error. The value of routine administration of antibiotics after ET or of using a fibrin sealant has not yet been established yet and no specific catheter has so far shown its superiority over its competitors. More RCTs are needed to evaluate the various factors involved in this delicate and essential step of assisted reproduction.

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How many embryos to replace?

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Introduction

The high incidence of multiple pregnancies is the most frequent complication of Assisted Reproductive Technologies (ART) and their obstetrical, neonatal, psychological and social implications are very important. (Bryan et al 2001).

In Spain, as well as in most western countries, there has been a significant rise in the incidence of multiple pregnancies. In 1980, the national incidence of twin pregnancies was 7 in 1000 deliveries and in 2000 this figure was 15 in 1000. As far as triplet pregnancies are concerned, the incidence was 1 in 10000 deliveries in 1980 whereas in 2000 it was 7 in 10000.

Although the major risk is associated with high-order multiple pregnancies, and these pregnancies are almost always the consequence of ovulation induction or artificial insemination and not of IVF, the vast majority of total multiple pregnancies have their origin in IVF.

Recent articles have shown that the elective double embryo transfer (eDET) in cases with good prognosis, due to the high number of high-quality embryos available for replacement, could be useful to reduce the incidence of triplet pregnancy without affecting final pregnancy rates (Templeton and Morris 1998). However, with this policy the incidence of twin pregnancy is elevated ranging between 25-40% and for this reason some Scandinavian groups have proposed the strategy of elective single embryo transfer (eSET) in IVF cycles with several embryos of excellent quality. Their initial experiences have shown that pregnancy rates were unaffected and twin pregnancies have disappeared (Vilska et al. 1998, Martikainen et al. 2001, Gerris et al. 2002).

Our classic policy was to transfer 3 embryos in the majority of the cycles obtaining good pregnancy rates but with an excessively high incidence of multiple pregnancies. We decided to analyze our own data retrospectively in

order to develop a scoring system according to individual circumstances which can be useful to identify patients at high risk for multiple pregnancy in whom the number of embryos to be replaced should be reduced.

Materials and methods

We retrospectively analyzed 227 IVF cycles carried out in our service, all of which had a transfer of 3 embryos on day 2 after ovum pick-up. Out of these cycles, 120 resulted in pregnancy and in 107 a pregnancy was not achieved. Of these pregnancies, 64 were singletons, 39 twins (32.5%) and 17 triplets (14.1%).

The parameters analyzed were patient's age, cause of infertility, number of previous IVF attempts, total number of embryos available and total number of good quality embryos available for transfer.

Once the Multiple Pregnancy Score (MPS) had been validated, we applied it prospectively in 2002 in order to try to reduce the incidence of multiple pregnancy.

Statistical analysis was performed with the SPSS software program, and Chi-square test and Student T test were used to compare quantitative and qualitative variables, and those variables significantly associated with high-order multiple pregnancies were retained for testing in a multivariate regression model. Receiver operating characteristic (ROC) curves were used to determine the cut-off point which best discriminated between multiple and single gestation.

Results

Table 1 shows the comparison between non-pregnancy and single, twin and triplet pregnancies. Univariate analysis found no significant differences in the four groups with respect to the origin of infertility or the number of previous IVF attempts. There were, however, significant differences with regard to age of the patients, total number of embryos available and number of good quality embryos available for replacement. When these three variables were entered simultaneously into the logistic regression model, only age and number of good quality embryos remained statistically significant.

Analysis of receiver operating curves produced the following cut-off values: 34 years of age, 2 good quality embryos and 9 embryos in total available for transfer. According to these results we developed the MPS in which the variables included are age of the patients and number of good quality embryos; each of these variables receives different points: the higher the MPS the higher the risk of multiple pregnancy. Table 2. When MPS values were 5 or 6, transfer of

a single embryo was proposed, 2 embryos in cases of MPS of 3 or 4, and when the value was below 2, 3 embryos could be replaced.

Table 1

	Non pregnant n=107	Singletons n=64	Twins n=39	Triplets n=17	P value
Age (yr) (mean SD)	35,8±4.5	34,6±3,6[a]	33,2±3,6	32,7±2,6[a]	0.000
No. of attempts (mean SD)	1,9±1,1	1,6±0,9	1.7±0.9	2,0±0,9	ns
No. of total embryos available for trnasfer)	6,6±3,8	6,7±3,8[b]	6,9±3,5	9,9±5,8[b]	0.015
No. of good quality embryos for transfer	1,5±1,9	1,4±1,3[c]	3,0±2,4	3,2±1,8[c]	0.000

a = 0.048; b = 0.009; c = 0.000

Table 2: *multiple pregnancy score m.p.s. 2002*

		Points
Number of good quality embryos	0	0
	1	1
	2	2
	3	3
	4	4
Age	< 30	2
	30-34	1
	35-39	0
	40	-1

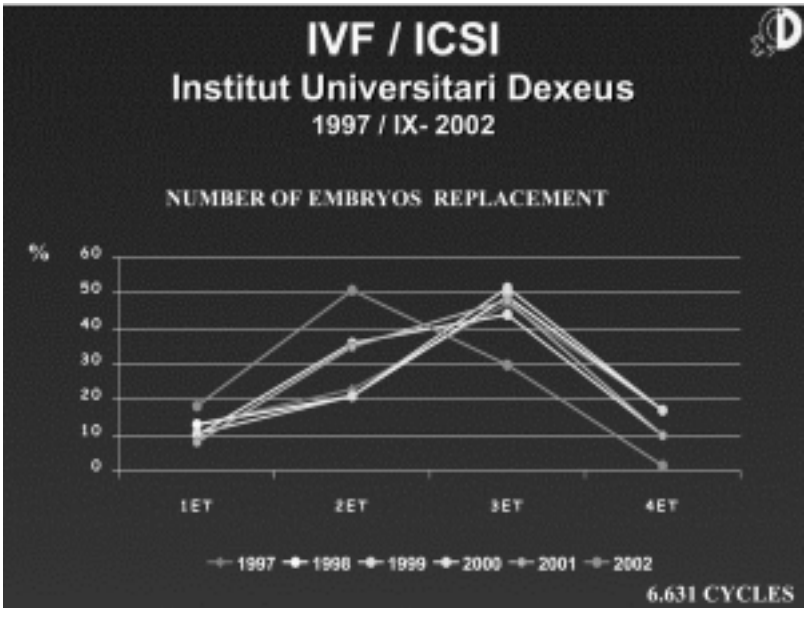
We prospectively applied a policy of elective double (eDET) or single (eSET) in 2002 good prognosis cases whose patients agreed to be included in this trial. In 462 IVF cycles patients received electively 2 embryos resulting in 266 pregnancies (43% pregnancy/replacement) but 118 twin pregnancies were obtained which means that the implantation rate was 57% due to an excessively high twin pregnancy rate of 44%. Apart from results obtained in fresh cycles another 75 pregnancies were achieved after 269 cryoreplacement cycles carried out in the same group of patients reaching a cumulative pregnancy rate after fresh elective double and frozen replacements of 73.8%. Table 3.

Table 3: *fresh elective double embryo transfer + cryoreplacement*

	N	Pregnancies	Pregnancy rate per patient
Fresh replacements	462	266	57.6%
Cryoreplacement	269	75	27.9%
Cumulative pregnancy rate per patient	462	341	73.8%

In order to reduce the high twin pregnancy rate, we performed an elective replacement of a single embryo in 58 cases obtaining 27 pregnancies with a pregnancy and implantation rate of 46.6%. Another 11 pregnancies were obtained after the cryoreplacements carried out in this group of patients, giving a cumulative pregnancy rate after fresh elective single and frozen embryo transfers of 65.5%. No twin pregnancies were observed in this group.

Table 4.



Discussion

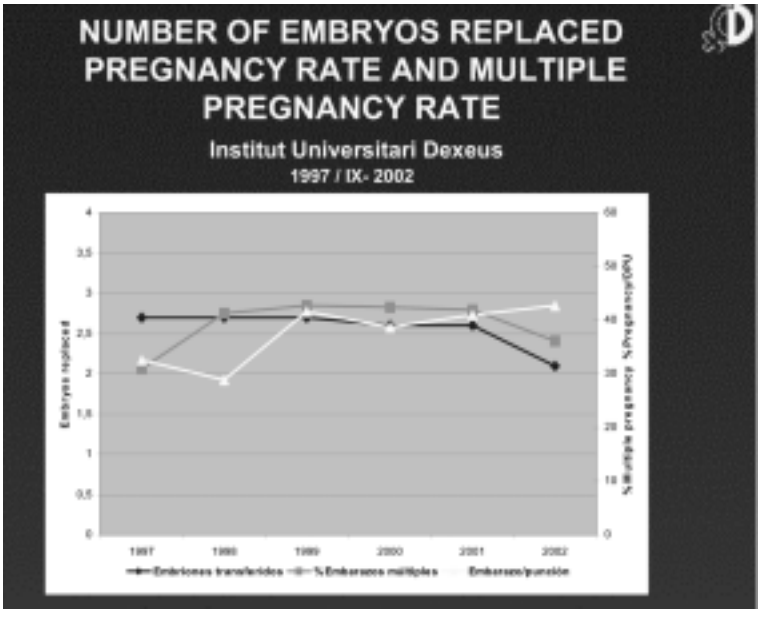
The high implantation rates usually obtained nowadays in our IVF laboratories allow us to introduce our patients into the mentality of elective single or double embryo transfer. Although a complicity exists between the staff and the couple for accepting twin pregnancies as an almost normal pregnancy we cannot forget

that obstetrical and neonatal complications increase exponentially with the number of gestational sacs present. We think that we have succeeded in reducing triplets but the goal today should be to limit twins as much as possible.

Considering the two variables associated with the establishment of multiple pregnancies, embryo quality is even more important than the age of the patient (Terriou et al 2001). It is important to know that other aspects such as infertility aetiology and rank of attempt did not have enough discriminatory power to be clinically useful but the total number of embryos available, although not significant, is interesting in order to have more good embryos to select one or two for eSET or eDET (Murdoch et al). In the last years we increased the percentage of cycles in which 1 or 2 embryos were replaced electively and transfer of 4 embryos disappeared. Table 4.

For an adequate development of such clinical strategies several items are mandatory: a correct patient selection, a performing IVF programme, an excellent embryo freezing program and above all a well-trained staff strongly convinced of the benefits of limiting the incidence of twin pregnancy. We believe that it is already time to progressively increase the percentage of cycles with a single embryo electively replaced; by doing so we reduced the mean number of embryos replaced in our IVF programme, multiple pregnancy rates have decreased, and what is most important — the final pregnancy rate has not been affected. Table 5.

Table 5.



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Andropause- fact or fiction?

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In distinction to the course of reproductive aging in women, with the rapid decline in sex hormones and expressed by the cessation of menses, men experience a slow and continuous decline of: Testosterone, Dehydro Epi Androsterone (DHEA) Thyroid stimulating Hormone (TSH), Growth Hormone (GH), IGF1, and Melatonin. In addition Sex Hormone Binding Globulin's (SHBG) increase with age resulting in further lowering the concentrations of free biologically active androgens. Parallel to the endocrine changes, aging is associated with a decrease in muscle and bone mass, progressive failure of body functions and particularly with an increasing lack of physical strength and mobility. Due to Osteoporosis, muscle weakness, cognitive and visual impairment aging men are at a greater risk for injuries, accidents, vertebral and hip fractures. This can be and often is associated with a progressive decline in independence and quality of life, leading eventually to a prolonged dependence on others. An increase in body fat, with fat redistribution from peripheral to central stores, also occurs in aging men parallel to the decrease in androgens and GH. The increase in central fat stores increases health risks such as diabetes, hypertension and cardiovascular disease. A decrease in sexual function and an increase in fatigue and depression often parallels the decrease of bioavailable androgens. Interventions such as hormone replacement therapy may alleviate the debilitating conditions of secondary partial endocrine deficiencies by preventing the preventable and delaying the inevitable. It has been demonstrated that interventions, such as hormone replacement therapies may favorably influence some of the pathological conditions in aging men, by preventing the preventable and delaying the inevitable. A comprehensive medical, psycho-social and life-style history, a physical examination and laboratory testing is essential for the diagnosis and management of acquired hypogonadism in aging men. Acute, chronic or inter current diseases must be taken into consideration prior to initiating any hormonal substitution therapy. Hormone substitution should only be performed following evaluation of risk factors and after having ascertained that there are no contraindication (e.g. Prostate cancer). Treatment must be monitored periodically with clinical and

laboratory examinations by physicians with basic knowledge and clinical experience in the diagnosis and treatment of aging men. In the Era of Evidence Based Medicine, we have to acknowledge that data on hormone replacement therapy (HRT) in the aging male, is mostly circumstantial, based on experience in treatment of transitional or chronic endocrine deficiencies in young men due to disease or experiments of nature. However Over the past several years, there has been an increasing interest in evaluating whether male HRT might be beneficial for certain older men in preventing or delaying some aspects of ageing, and a number of prospective studies on hormone replacement therapy in the aging male were performed and demonstrated beneficial effects. With an exponential increase in the aging population, there is an urgent need to obtain more information for men. In light of this, basic, clinical, socio-economic and epidemiological research needs to be intensified and public awareness of medical knowledge needs to be increased .This will necessitate a quantum leap in multi-disciplinary and internationally coordinated research efforts supported by inter-governmental, governmental, commercial and voluntary sectors.

Anti-Aging strategies using vitamins and "natural" products

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Introduction

The most important precaution to be taken in aging people is to prevent or treat life-threatening diseases. This requires a physical ''check-up'' to be performed at regular intervals, by a trained (team of) physician(s). At the other hand, life style should be adapted. The lower energy requirements must be compensated by a lower calorie intake and by moderate physical exercise. Vigorous exercising is not recommended, except in trained athletes. Stress should be avoided and a possible depressive state must be diagnosed and treated.

Nutritional supplements (Nutriceuticals) aim at preventing common diseases including cardio- vascular disease, heart failure, degeneration of the central (and peripheral) nervous system, bone demineralisation, joint arthrosis, and prostate diseases. Also, ''wear and tear'' can be slowed down by protecting DNA against oxidative damage, by maintaining membrane fluidity, and by counteracting the damage caused by toxic agents in the environment.

Protection of the brain and Central Nervous System (CNS)

Degeneration of brain function is related to several causes. Cerebro-vascular insufficiency, decreased metabolic activity, and inflammatory plaque formation are well-recognised mechanisms of diminished brain function, and development of dementia. The extract of *Vinca Minor* has successfully been used for several decades to improve blood supply to the brain (Solti et al, 1976; Hadjiev and Yancheva, 1976; Karpati and Szporny, 1976), and to enhance glucose uptake and metabolism (Tesseris et al, 1975; Vamosi et al. 1976; Matcovics et al, 1991).

Hormone replacement therapy by oestrogens or their precursors in the female and testosterone in the male has been proven to reduce inflammatory plaque formation. A similar effect can probably be obtained by shifting the phospholipid content toward Omega- 3 poly-unsaturated fatty acids. The latter can be materialised by combining the intake of linseed oil oil, rich in Alpha-

linolenic acid, with antioxidants, Zinc and Vitamin B6 (Christophe et al, 1998, see below).

Antioxidants

Selenium Methionine, Vit. C, Ubiquinone Q10 and Tocopherol exert strong antioxidant effects, particularly when given in a balanced proportion.

Vitamin C is water-soluble and, aside from its intrinsic antioxidant effect, regenerates Vit E. However, high concentrations of Vit C may display an intrinsic pro-oxidative effect through activation of, among others, the Fenton reaction. This will occur preferentially in persons with the haptoglobin type 2-2 or 2-1. Excessive Vit C intake may, therefore, exert a damaging rather than protective effect in some persons.

Antioxidant combinations can delay the oxidation of LDL cholesterol. The oxidised LDL is the initiator of vascular plaque formation. Delaying oxidation of LDL will reduce plaque formation and may prevent, or reduce atheromatosis of the blood vessels. This effect is reinforced by the supplementation with poly-unsaturated fatty acids that can decrease the level of LDL cholesterol in blood.

Ubiquinone Q10 supports contractility of the cardiac muscle. Environmental toxicants such as certain poly-chlorinated bisphenols (PCB's), dioxins and furanes inhibit the oxido-reductase activity of natural Ubiquinone. In addition, the intake of statin drugs that are commonly used to reduce hyperlipemia, while inhibiting the activity of the HMG-Coenzyme A reductase, also inhibit the production of Ubiquinone Q10 in the liver. This then may result in heart failure. Hence, there is a need to supplement this antioxidant (Navarro et al., 1998).

There are several forms of Vit E (tocopherol). The synthetic alpha-d-tocopherol is most commonly used (Hartman et al. 1998), but recent studies suggest that gamma-tocopherol (Giovannucci, 2000) may display a much stronger effect in detoxification (Cooney et al. 1993) and in preventing prostate cancer (Tran and Chan, 1992; Nomura et al., 1997; Heinonen et al, 1998; Chan et al.; 1999, Moyad et al. 1999, Helzlsouer et al. 2000, Freeman et al. 2000). At the other hand, excessive supply of alpha tocopherol reduces the gamma-tocopherol concentration in blood (Handelman et al. 1985; Behrens and Madere 1986; Behrens and Madere, 1987; Handelman et al. 1994).

Therefore, formulations should provide a nutural vitamin E mixture containing one third of alfa-tocopherol and twothirds of gamma-tocoferol. In addition, natural Vit E has been proven to exert a stronger and more long-lasting antioxidant effect at the level of the cell membrane.

Vitamin B

It is well known that Vit B1, B6 and B12 have a neurotrophic effect. These vitamins protect the central and peripheral nervous systems, and counteract

neuropathy that commonly occurs in sub-clinical degree, as a result of exposure to toxic agents in the environment. In addition, Vit B6 directs fatty acid metabolism toward long chain, omega-3 poly-unsaturated fatty acids, which have an anti-inflammatory effect and maintain fluidity of the cell membrane. Vit B9 (folic acid) supports the contractility of the heart muscle.

Protection of the prostate

The extract of *Serenoa Repens (also called sabal serrulata)* inhibits the 5-alpha-reductase enzyme that converts testosterone to 5-alpha-dihydrotestosterone (DHT)(Weisser et al. 1996; 1997). The latter is ten times more potent an androgen than testosterone. Five-alpha-reductase activity is exceptionally high in the prostate, locally generating high concentrations of 5-alpha-DHT. Inhibition of the 5-alpha-reductase by means of Finasteride (Proscar, MSD), prescribed to patients with Benign Prostate Hyperplasia (BPH), reduces the prostate volume. The extract of *serenoa repens* has the same effect as Finasteride (Wilt et al. 2000; Sokeland, 2000), and it has been proven to be similarly potent in reducing prostate volume in BPH patients (Romics et al, 1993; Kondas et al. 1996; Stepanov et al. 1999; Boyle et al, 2000; Vacherot et al. 2000; Bayne et al. 2000), respectively preventing BPH (Cristoni et al. 2000). Also, the *serenoa* extract induces apoptosis of prostate cells in vitro. In contrast to Finasteride, the extract of *serenoa* does not suppress the level of prostate specific antigen (PSA) in blood, which leaves the possibility of using this marker of prostate cancer.

In addition, the *serenoa repens* extract may prevent frontal male pattern hair loss (Hoffmann and Happle, 2000), similar to treatment with low dose Finasteride (Drake et al. 1999; Leyden et al. 1999; Hogan and Chamberlain, 2000; Granel, 2000); Therefore, it should be considered to prescribe *serenoa repens* extract in all aging men, including in those men that receive hormone replacement therapy with testosterone.

Low Vit E levels in smokers have been related to an increased risk for prostate cancer (Eichholzer et al. 1999). In several large scale, double-blind and controlled trial, Vit E was shown to reduce the incidence of prostate cancer. These studies were performed with alpha-tocopherol. However, recent data suggest gamma-tocopherol to be the most active substance in this respect. This ''new player'' seems to reduce the risk of prostate cancer by up to 70%. In synergism with other antioxidants, gamma-tocopherol reduces the oxidative damage to DNA. This may be the major mechanism of its anti-cancerous effect (references, see above).

Phyto-oestrogens for men

Certain preparations that aim at preventing prostate cancer contain soya extracts, mostly composed of the phyto-oestrogens Genisteïn and Daïdzeïn.

There is some indirect epidemiological suggestion that the life-long intake of food rich in soya products may reduce the prevalence of prostate cancer. Also, genisteïn, in particular, was shown to inhibit prostate cancer cell-growth in vitro. However, data on controlled prospective population studies are lacking, and the effect of these phyto-oestrogens is questionable when they are taken later in life. In addition, phyto-oestrogens increase the concentration of sex hormone binding globulin in blood of men. This then lowers the free fraction of testosterone, which is already clearly decreased in the ageing male. In this way phyto-oestrogens may promote the signs and symptoms of andropause. Furthermore, several publications highlight possible toxic side effects of Genisteïne (including stunted growth in children). For these reasons the use of food supplementation containing soya-derived oestrogens is controversial, particularly in aging men.

Seed oils and lignanes

Rather than recommending soya-derived phyto-oestrogens, it is suggested to complement the intake of antioxidants with oil extracted from linseed or flaxseed. These oils are rich in the essential fatty acid: alpha linolenic acid (18:3omega3) and also contain particular lignanes.

In the human intestine, the lignanes are converted into *enterolacton*, which is a rather strong aromatase inhibitor, and a very weak phyto-oestrogen. Because of its dominant aromatase inhibiting effect, enterolacton reduces the endogenous production of the strong endogenous (natural) oestrogens, namely oestradiol and oestriol. Therefore, the sum of the activity of entreolacton is to reduce the oestrogen load. This is favourable since the increased aromatase activity in ageing men, together with the resulting relative hyper-oestrogenism, are held responsible for increasing the risk of coronary heart disease and, together with 5-alpha-DHT, for prostatic hyperplasia (BPH).

Female Formulation

There is no need for a 5-alpha-reductase inhibitor in aging females, who should rather receive hormone substitution preventing menopausal symptoms and osteoporosis. Hormone replacement therapy with oestrogens or dehydro-epi-androsterone (DHEA) adds potential risks to their proven benefits. The long-term use of these hormones meets with increasing scepticism. The extract of *dioscorea villosa* may offer an interesting alternative. This extract (from the root of Wild Yam) contains Sarsasapogenin and *Diosgenin*, the chemical structure of which is similar to that of natural progesterone (Komesaroff et al. 2001). In the adrenals, progesterone is derived from delta-5-pregnenolone that is also the precursor of DHEA via the intermediate substance 17-alpha-pregnenolone

(l'Allemand and Biason-Lauber 2000). The extract of *dioscorea villosa* seems to influence the biosynthesis and the receptor binding of particular adrenal steroids. The extract of *dioscorea* mitigates menopausal complaints, and may protect against endometrial cancer. Indeed, *diosgenin* inhibits cell division in the G1 stage and increases apoptosis. Furthermore, *diosgenin* was found to protect against fragility of the bones resulting from osteoporosis in experiments using ovariectomized rats.

Through its interesting mechanism of action, the extract of *dioscorea villosa* can influence the effects of the natural steroid hormones produced by the adrenals, and it can, to some extend, compensate for the (ovarian) hormone deficiency in the aging female. It does, however, not display the potentially hazardous side effects of oestradiol, since we have proven *diosgenin* neither to display any intrinsic oestrogenic activity (so it is not a phyto-oestrogen) nor to bind to the human oestrogen receptor.

Complementary plant extracts and protective substances

In very old persons complementary support can be provided by certain plant extracts. For example, the *extract of Crataegus* exerts cardiotonic, inotropic effects. Deterioration of the cartilage and development of arthrosis deformans can be counteracted by the regular intake of acidic Chondroitin and glucosamine. The extracts of the bark of particular trees (Pinus Maritima, Salix) reduce inflammation and pain at the level of the articulations and tendons (teno-synovitis). It has been documented that these extracts exert effects which are similar to those of non-steroidal-anti-inflammatory drugs (NSAI), among other things by inhibiting the cyclo-oxygenase (COX) enzyme.

Conclusion

Well balanced supplementation with particular vitamins and plant extracts (Nutriceuticals), as well as certain seed oils containing omega-3 poly-unsaturated fatty acids, may protect the aging person from, or at least delay the occurrence of, diseases that commonly affect their quality of life. The immediate subjective effects of such supplementation are not spectacular. However, long- term benefits can be made objective by means of sophisticated biological tests. It is the long-term effects in preventing health deterioration that are aimed at by these supplements. Food supplementation, together with the implementation of essential life style adjustment, can be recommended based on solid scientific data. Nevertheless, long-term follow-up studies (which are ongoing at this time) are needed to empirically prove the projected beneficial effects.

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Most uteri can be taken out vaginally

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Purpose

For the gynaecologist hysterectomy is an operative procedure demanding a high standard of surgical dexterity; however, for the patient this operation often symbolises the disintegration of her womanhood and may be the starting point of a history of pain. Although this is seldom the case, hysterectomy has to be performed with the greatest respect and care. In benign indications, which comprise 80% of the hysterectomies performed at the present time, patient-tailored hysterectomy should be performed. Within this concept the supracervical approach comes back into discussion. We definitely feel that endoscopic hysterectomies are playing a relevant role in the treatment of benign disease and may play a relevant role in the treatment of malignant uterine disease. The classic intrafascial supracervical hysterectomy technique may be applied in over 60% of all indicated cases for benign uterine disease. This assertion is based on the following principles: The cardinal ligament is not transsected and the normal anatomical relation of the pelvis remains including the blood supply by the descending branches of the uterine artery. The amount of healthy tissue destruction is greatly reduced and the urethers are not endangered. The vaginal cuff heals quickly and the paracervical/fascial network of nerves remains intact. The excision of the paravaginal and paracervical network of nerves and the ligation of the uterine artery reduces the sensory sexual feelings in this area and may cause alterations in proper bladder and rectum function. This iatrogenic destruction of important aspects of a woman's life is seldom discussed.

Methods

Supracervical hysterectomy allows the gynaecological surgeon to prevent this destruction of the pelvic floor by performing hysterectomy either by laparotomy, pelviscopy or vaginally. Indications for laparoscopic supracervical hysterectomy with or without coring of the inner cervix are given in cases of adenomyosis, uterine fibroids, therapy resistant menorrhagias and endometrial ablation. This type of hysterectomy can be combined with suspension operations, pelvic floor

repair and genital prolapse surgery. In a comparison of six different types of hysterectomy performed at our department, the 600 pelviscopic CISH interventions demonstrated the shortest blood loss, the shortest operation times, the shortest hospitalization time and the shortest post operative morbidity with the lowest complication rates. The CISH method minimalizes the operative procedure whether performed by laparotomy, pelviscopy or vaginally. The preservation of the anatomical relations of the pelvic floor including the blood supply of the descending branches of the uterine artery is in keeping with the ideals of minimal invasive surgery, providing a better quality of life for the patient.

Results

The usual justification for performing total hysterectomies is the additional removal of the cervical transformation zone. At CISH this is achieved through the coring of the cervical tissue cylinder using the calibrated uterine resection tool which leaves the pelvic floor intact. The indication for total hysterectomy as a prophylactic operation is no longer valid and may be considered as an overtreatment.

Conclusions

Supracervical laparoscopic hysterectomy, with and without coring of the inner cervix, is a surgical concept which demands more attention.

Myomectomy during cesarian-section. Time to reconsider?

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Background

Myomectomy during cesarean section is strongly discouraged in all the leading textbooks despite the lack of any direct evidence supporting the approach. Leiomyomata are the most common gynecologic tumors, with a reported incidence of 20-25%. Fibroids are the most common indication for hysterectomy, accounting for over 200,000 hysterectomies per year in the United States. Fibroids affect mainly women in their childbearing years and may be asymptomatic or cause a variety of symptoms, including menometrorrhagia, dysmenorrhea, pelvic pain, reproductive failure, and compression of adjacent pelvic viscera. The estimated prevalence of fibroids in pregnancy is 1-4% (1,2).

Management depends on the goal of therapy. Hysterectomy is most often used for definitive treatment, and myomectomy when preservation of childbearing ability is desired. Intracavitary and submucous leiomyomata can be removed by hysteroscopic resection, whereas intramural and subserosal fibroids are usually removed by laparotomy. Although laparoscopic myomectomy is also technically feasible today, it apparently has an increased risk of uterine rupture during pregnancy. Gonadotropin-releasing hormone-agonist induces hypogonadism and can reduce the volume of leiomyomata, but its severe side effects and association with prompt recurrences makes them useful only for short-term goals such as reversing anemia or shrinking an intracavitary tumor prior to hysteroscopic resection. New approaches such as myolysis and uterine artery embolization are currently being evaluated and may offer more options providing their safety in women desiring fertility is established (3).

According to Te Linde (4) '' Myomectomy delivery in conjunction with cesarean section is contraindicated. If there is a pedunculated subserous fibroid attached to the uterus with a small pedicle, suturing and excision of the pedicle may be done easily. However, the removal of intramural myomata from the

pregnant uterus is inadvisable due to recognized difficulty in controlling blood loss''. Bleeding may be profuse and lead to hysterectomy. According to other textbooks, myomas resected during pregnancy may show bizarre nuclear changes often resembling sarcoma. Furthermore, they often undergo remarkable involution after delivery when myomectemy is much safer (5,6). Our computer medline search yielded only one study supporting this finding, where in large intramural leiomyomas found at cesarean section became pedunculated postpartum, making them more amenable to myomectomy (7). Otherwise, we were able to identify very few articles concerning myomectomy during cesarean sections, with the majority reporting favorable results. In 1989, Burton and colleagues (8) reported on 13 successful cesarean myomectomies with the sole complication of intraoperative hemorrhage. They concluded that surgical management of leiomyomata during pregnancy (and cesarean section) is safe in carefully selected patients. Four years later, Hsieh et al (9) reviewed 47 incidental cesarean myomectomies. The procedure added only 11 minutes to the operation time, 112 ml to the operative blood loss and a half-day to hospitalization time. There were no wound infections or serious morbidity. Exacoustos et al (1) reported on nine myomectomies performed during cesarean delivery. Of which three were complicated by severe hemorrhage necessitating hysterectomy. They emphasized the importance of ultrasound findings of myoma size, position, location, relationship to the placenta, and echogenic structure in identifying women at risk of myoma-related complications. Michalas and colleagues (10) described a patient in whom 8 fibroids obstructing the lower part of the uterus were removed during cesarean section in the 39th week of pregnancy. There were no maternal or fetal complications.

In 1999, Dimitrov and co-workers (11) in Bulgaria conducted a prospective study to evaluate whether myomectomy could be performed on a routine basis during cesarean section. In a comparison of 21 women in whom myomectomy was done during cesarean section and 162 consecutive women after cesarean section without myomectomy, they found that myomectomy during cesarean section increased hemorrhage by 10%. Placental disorders (abruptio placentae and placenta previa) were the main cause of the overall increase in blood loss. There were no postoperative complications. The authors concluded that irrespective of the number and magnitude of the myomas, myomectomy during cesarean section is a feasible option. The same year, Omar and colleagues (12) described 2 large uterine myomas located in the anterior aspect of the lower segment of the uterus complicating pregnancy at term. Myomectomy in both instances allowed delivery of the fetus through the lower segment, making vaginal delivery in subsequent pregnancies possible. In the most recent study of this issue, Ehigiegba et al (13) assessed the intra- and postoperative complications of cesarean myomectomy in 25 pregnant women. Five required

blood transfusions and none required a hysterectomy. They concluded that with adequate experience and the use of high dose oxytocin infusion (intra- and post-operatively), myomectomy at cesarean section is not as hazardous as many now believe.

For the last 7 years we have been performing planned myomectomy during cesarean section in cases in which the fibroid is known to be large enough to require surgery in the future or is the cause of malpresentation. Meticulous attention is directed to hemostasis, with enucleation using sharp dissection with Metzenbaum scissors and adequate approximation of the myometrium and all dead spaces to prevent hematoma formation. An experienced surgeon performed the first operations, but the procedure is now more common and is performed by different surgeons including residents.

In the light of the inconclusive data in the literature, we conducted the present retrospective analysis.

Objective

To assess the intra- and postoperative complications of cesarean myomectomy.

Methods

After completion of the CS, an interlocked suture was temporarily placed on the uterine incision without closing it. This also allowed working from within or from the outer part of the uterus without significant bleeding from the incision. Myomectomy was performed using sharp dissection. Oxytocin drip was given during and after the enucleating the fibroid. .

The files and operative records of 32 consecutive patients who underwent cesarean myomectomy between 1997 and 2001 were reviewed for demographics, indication for cesarean section, emergency or elective procedure, and characteristics of the fibroids. Outcome measures were type of anesthesia, type of incision, intraoperative blood loss, need for blood transfusion, intra- or postoperative complications, and duration of hospital stay.

Results

Thirty-nine myomas were removed from the 32 patients in 15 elective and 17 emergency procedures. Indications for cesarean section were obstetric (breech presentation, more than one previous cesarean section, etc) in 26 women. Of the reminder 3 had tumor previa, 1 degenerative myoma, and 2 previous myomectomy with uterine cavity penetration. Ninety percent of the myomas were subserous or intramural and 10% were submucous. Average size (largest

dimension) was 6 cm (1.5-20), with 26 myomas > 3 cm and 11 > 6 cm. Four sections (12.5%) were classical and the reminder low segmental. Three operations were done with regional anesthesia (9.3%) and the reminder with local anesthesia (spinal block). The difference in hemoglobin and hematocrit levels before and 12 hours after the operation was statistically significant compared with a group of women who underwent cesarean section without myomectomy ($p<0.05$); however only 4 patients required blood transfusion. Re-operation was done in one patient with 2 large myomas and excessive bleeding and in another because of a hematoma below the scar. None of the patients required hysterectomy. Six patients had postpartum fever (18.7%). Average duration of hospitalization was 5.7 days, with 5 patients requiring more than 6 days. There was no correlation between complications or duration of hospital stay and patient age, gravidity, parity or indication for cesarean section.

Conclusions

Myomectomy during cesarean section is feasible. Meticulous attention to hemostasis with enucleation using sharp dissection with Metzenbaum scissors and adequate approximation of the myometrium and all dead spaces to prevent hematoma formation can increase the safety of the procedure. Despite the lack of prospective randomized studies we believe that myomectomy during cesarean section is an easy and safe procedure when done appropriately. The old dictum discouraging cesarean myomectomy should be reassessed.

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Interstitial cystitis: what is it and why should it interest the gynecologist?

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Summary

Interstitial cystitis (IC) is a chronic, severely debilitating disease of the urinary bladder marked by exacerbations and remissions. Excessive urgency and frequency of urination, suprapubic pain, dyspareunia, chronic pelvic pain and other symptoms are often seen in IC. The condition is diagnosed following certain inclusion and exclusion criteria. There is, however, an overlap of signs and symptoms between IC and various gynecologic disorders. The potassium sensitivity test is positive in 80% of patients, but may also be positive in patients with chronic pelvic pain of gynecologic origin. Oral treatments of IC include pentosan polysulfate, tricyclic antidepressants and antihistamines. Intravesikal therapies use hydrodistention, dimethyl sulfoxide, heparin, and others. Surgical approaches include sacral nerve root stimulation, bladder augmentation or cystectomy. IC may be a common unrecognized cause of pelvic pain in gynecologic patients and deserves greater consideration in the differential diagnosis of pelvic pain.

Background

Interstitial cystitis (IC) was first recognized clinically in 1887, but is still poorly understood and often resistant to treatment. IC has consistently been reported to be more prevalent in women than in men. For the average patient with IC, there is a 4-year delay between the time the first symptoms occur and the diagnosis and 2 to 5 doctors are consulted before the correct diagnosis is made. This demonstrates both the difficulty of diagnosis and how infrequently this condition is suspected.

In a study by Leppilahti et al. the prevalence of urinary symptoms related to IC in women in Finland and randomly selected 2,000 study participants 18 to 71 years old from the Finnish population register was estimated (Leppilahti et al., J Urol 2002). The prevalence of urinary

symptoms was evaluated using the validated O'Leary-Sant interstitial cystitis symptom and problem index questionnaire, which was mailed to subjects. Women with high (12 or greater) symptom and problem scores, including nocturia 2 or greater, pain 2 or greater, and excluding urinary infection and pregnancy, were considered most likely to have IC. The response rate after 2 mailings was 67.2% (1,343 respondents). After further exclusions 1,331 women (66.6%) comprised the final study group. Of these 1,331 respondents 11 (0.8%) reported severe symptoms and problems (12 or greater), including 6 (0.45%) who fulfilled the criteria for probable IC. The authors found that the prevalence of urinary symptoms corresponding to probable IC is 450/100.000, which is an order of magnitude higher than previously reported (Leppilahti et al., J Urol 2002).

Etiology

IC is an inflammation (irritation with increased presence of immune cells) of the tissues of the bladder wall, with no known infectious (bacterial, viral, or fungal) cause. Up to 450,000 people in the US have IC but only 20,000 to 40,000 cases actually being diagnosed. It is accepted today is that the etiology of IC is multifactorial. The causes are:

1. Bladder epithelial permeability
2. Mast cell activation
3. Inflammation (Infection, autoimmune process)
4. Toxic urine components
5. Occult infection
6. Neuropathic changes

Symptoms

The onset of symptoms is normally characterized by a rapid increase in pain intensity over several months. Symptoms include suprapubic pain, burning sensations in the bladder and genitals, urinary frequency and urgency, bladder spasms and dyspareunia, are then characterized by periods of remission and exacerbation. The typical patient with IC has a maximal bladder capacity of 350 ml while awake and needs to void up to 60 times per day and every 20-30 minutes at night.

Diagnosis

The definitive diagnosis is based on reported urinary symptoms, absence of other urological or gynecological diseases, the presence of petechial hemor-

rhages or Hunner's ulcers confirmed by cystoscopy and bladder distension, the potassium sensitivity test and symptom scores. A list of inclusion and exclusion criteria has been published by the National Institutes of Health-National Institute of Diabetes and Kidney diseases (NIH-NIDDK) (Gillenwater et al., J Urol 1988). The original intent of these criteria was to ensure standardization of patients enrolled into IC studies. When used as guidelines for clinical diagnosis, these criteria tend to miss all but advanced IC and miss approximately 2/3 cases when strictly applied (Hanno et al., J Urol 1999).

Inclusion criteria:

1. Urinary frequency/urgency and pelvic pain of chronic duration
2. Presence of glomerulations and/or Hunner's ulcers on cystoscopy and hydrodistention (Peeker et al., J Urol 2002).

Exclusion criteria:

1. Diagnosis of bacterial cystitis within a 3-month period
2. History of bladder calculi of current/active ureteral or urethral calculi
3. Genital herpes within the past 12 weeks
4. History of uterine, cervical, vaginal, or urethral cancer
5. Symptomatic urethral diverticulum
6. History of cyclophosphamide use, history of any type of chemical cystitis
7. History of tuberculous cystitis
8. History of pelvic irradiation
9. History of benign or malignant bladder tumors
10. Active vaginits

The diagnosis of IC is supported by the potassium stimulation test (Parsons et al., J Urol 1998). Positive potassium sensitivity is known to be associated with a bladder mucosa dysfunction present in most individuals with IC. The rationale of the test is based on the assumption that the bladder mucosa is leaky. Instillation of 40 ml of 0.4 M KCl is performed and left for 5 minutes. Patients rate the degrees of pain and urgency on a scale from 0 to 5 with a score greater or equal 2 considered to be positive.

Therapy

The management of patients with IC remains a challenge because no single agent has proven universally effective (Rovner et al., Urology 2000). As with any treatment algorithm, it is reasonable to begin with conservative treatment before surgical procedures are applied (Lukban et al., Urol Clin North Am 2002). Therapeutic options include:

1. Conservative treatments: diet, clothing, hygienic practices, and stress reduction, heat or ice, exercise, physical therapy, and bladder holding protocol
2. Hydrodistention of the bladder
3. Glycosaminoglycans (Hwang et al., Urology 1997; Parsons et al., J Urol 1993)
4. Dimethyl sulfoxide
5. Treatments directed against mast cells
6. Amitriptyline
7. Bacillus Calmette-Guerin (Peters et al., J Urol 1997; Peters et al., J Urol 1998; Peeker et al., Urol 2000)
8. Immunosuppression (cyclosporine, oral hydroxychloroquine and intravesical doxorubicin)
9. Antibiotics (Warren et al., J Urol 2000)
10. Pain control (Transcutaneous electrical nerve stimulatio=TENS)
11. Surgical treatments: bladder denervation processes like cystolysis, sympathetic denervation, selective sacral rhizotomy, laser therapy; bladder augmentation or even cystectomy with urinary diversion (van Ophoven et al., J Urol 2002)
12. Treatments for associated (pelvic pain) conditions (endometriosis, irritable bowel syndrome, vulvodynia, levator muscle pain and others)

Why should interstitial cystitis interest the gynecologist?

The following conditions are possibly correlated to IC or serve a differential diagnosis: Endometriosis, irritable bowel syndrome, vulvodynia, levator muscle pain, sensitive skin, fibromyalgia, migraine headache, medication hypersensitivity, and many others (Myers et al., Clin Obstet Gynecol 2002).

Chung et al. determined the value in the initial laparoscopic and cystoscopic evaluation of avoiding the unnecessary delay in diagnosing the "evil twins" of chronic pelvic pain syndrome, endometriosis and IC (Chung et al., JSLS 2002). A total of 60 women (19 to 62 years) underwent concurrent laparoscopy, cystoscopy, and hydrodistentions by a gynecology and urology team. Fifty-eight patients (96.6%) were diagnosed with IC by the presence of glomerulation and terminal hematuria according to NIH criteria. A diagnosis of endometriosis was found in 56 patients (93.3%). Biopsy-confirmed active endometriosis was found in 48 patients (80%). In the IC patient group (58), 54 patients had a diagnosis of endometriosis (93.1%), and 47 patients had biopsy-confirmed active endometriosis (81%). In the group of 56 patients with a diagnosis of endometriosis, 54 patients were found to have IC (96.4%). In the group of 48 patients with active

biopsy-confirmed endometriosis, 47 have IC (97.7%). Eighty percent were found to have endometriosis and had numerous previous operations. Many patients failed to respond to multiple therapies. In some cases pain persists even after a hysterectomy. The authors showed the high prevalence and association of IC and endometriosis. Thus, both laparoscopic and cystoscopic examinations should be performed with the patient anesthetized in the initial evaluation and treatment of chronic pelvic pain syndrome (Chung et al., JSLS 2002).

On the basis of clinical experience with more than 5,000 patients with IC, Parsons et al. have designed a pelvic pain and urgency/frequency (PUF) symptom scale that gives balanced attention to urinary urgency/frequency, pelvic pain, and symptoms associated with sexual intercourse (Parsons et al., Urology 2002). They used the intravesical potassium sensitivity test (PST) to validate the PUF scale in urologic patients suspected of having IC, gynecologic patients with pelvic pain, and controls. The PST was positive in 74% of patients with a PUF score of 10 to 14, 76% of those scoring 15 to 19, and 91% of those scoring 20 or higher. All controls' PUF scores were less than 3, and the rate of positive PST in controls was 0%. High PUF scores appear to correlate directly with a higher likelihood of positive PST in both urologic patients suspected of having IC and gynecologic patients with pelvic pain. The PUF appears to be a valid tool for detecting IC in these two populations, as well as in the general population. Use of the PUF alone may prove to be an accurate method for detecting IC (Parsons et al., Urology 2002).

Clemons et al. evaluated the Interstitial Cystitis Symptom Index and Problem Index as a screening tool for IC in women with chronic pelvic pain (Clemons et al., Obstet Gynecol 2002). Women (n=45) were questioned about lower urinary tract symptoms, administered the Interstitial Cystitis Symptom Index and Problem Index, and rated pain symptoms on a 0-10 visual analogue scale. Cystoscopy with hydrodistension and bladder biopsy was performed at the time of laparoscopy. Interstitial cystitis was diagnosed if women had a combination of: 1) urgency, 2) frequency or nocturia, and 3) positive cystoscopic findings. Seventeen women (38%) were diagnosed with IC. A score of 5 or more on the Symptom Index had 94% sensitivity and 93% negative predictive value in diagnosing interstitial cystitis. On multivariable analysis, an elevated Symptom Index score of 5 or more (odds ratio 9.4) and an elevated dyspareunia score of 7 or more (odds ratio 5.5) were risk factors for IC. In that sample of women with chronic pelvic pain, the prevalence of interstitial cystitis was 38%. Independent risk factors for the diagnosis of IC were an elevated Symptom Index score and an elevated dyspareunia pain score. For women with chronic pelvic pain, screening for IC should be performed (Clemons et al., Obstet Gynecol 2002).

Parsons et al. determined the prevalence of IC in a large number of gynecologic patients with pelvic pain versus control subjects, as indicated by a

positive result on a potassium sensitivity test (Parsons et al., Am J Obstet Gynecol 2002). Of 244 patients with pelvic pain, 197 patients (81%) had a positive result from a potassium sensitivity test. Urologic symptoms were reported by 84% of patients, but only 1.6% of the patients had received an initial diagnosis of IC. None of the 47 control subjects were tested positive with the potassium sensitivity test. The authors concluded that IC may be a common unrecognized cause of pelvic pain in gynecologic patients and deserves greater, if not primary, consideration in the differential diagnosis of pelvic pain (Parsons et al., Am J Obstet Gynecol 2002).

In conclusion, IC remains a challenge in terms of diagnosis and therapy. Gynecologists and urologists should work together when dealing with patients having "chronic pelvic pain".

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Role of Estrogens in older patients with recurrent urinary tract infections (UTI)

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Summary

Although urogenital complaints, such as recurrent lower urinary tract infections (UTI), and dysuria, are commonly encountered symptoms in elderly women, few women have taken part in randomised studies of estrogen therapy for this condition. This is a paradox as compared to the often cited beneficial effect of estrogen in reducing the UTI incidence. Nevertheless, in postmenopausal women, hormone replacement therapy using a topical estrogen normalizes the vaginal flora and greatly reduces the risk of vaginal atrophy. Similarly, vaginal estrogen administration seems to be effective for preventing recurrent urinary tract infections (UTI).

Introduction

Urogenital complaints, such as recurrent lower urinary tract infections (UTI), and dysuria, are commonly encountered symptoms in elderly women and have been shown to have widespread human and social implications. The incidence of UTI has been reported to be as high as 20% in women living at home and sometimes over 50% in institutionalised patients. Furthermore, elderly women with a history of UTI have been reported to have a higher overall mortality rate than their age-matched peers (1). The role of preventive measures to avoid recurrent UTI is therefore essential.

Pathophysiological changes, which may account for the increase of UTI include impairment of bladder emptying, poor perineal hygiene and both faecal and urinary incontinence. These problems are often encountered in institutionalised patients. Alterations in the vaginal flora associated with estrogen deficiency (vaginal atrophy) are also thought to place women at risk of UTI, particularly if they are sexually active. There is a rise in vaginal pH and fall in the number of lactobacilli, allowing colonisation by gram-negative bacteria which act as uropathogens.

Several authors have reported that the transition to menopause is accompanied by changes in the paraurethral connective tissue and that these changes are modified by estrogen replacement therapy. For instance Falconer et al obtained biopsies from the paraurethral tissue from pre and postmenopausal women (some of them treated with estrogen). These authors observed that the collagen concentration was almost doubled in postmenopausal biopsies compared to premenopausal peers. The collagen organization was also modified: the proteoglycan/collagen ratio was significantly decreased. Estrogen replacement therapy resulted in decreased collagen concentrations, and reversal of this ratio almost to premenopausal levels, suggesting that estrogen replacement therapy tends to restore the metabolism of the genitourinary connective tissue to premenopausal conditions (2).

The question remains, however, whether estrogen replacement therapy is efficient in preventing UTI. The exact role of the menopause as an etiological factor in the development of UTI may be exagerated. Indeed some authors reported that analyses of mid-stream urine specimens indicate an increase in the proportion of pathological results increases as women age, but there is no specific change in the rate of infection occurring at or following the menopause **(3).**

What evidence can we find in observational?

Orlander et al conducted a case-controled study involving 3,616 patients from 276 practices aged 50-69 years, and had experienced a first recorded UTI. These patients were compared to 19,162 controls. The authors found that women using estrogens for at least 1 year had an increased risk of being diagnosed with a UTI compared to non-users, (crude odds ratio (OR) 1.9 ; 95% CI 1.5-2.2). All of this excess risk was observed in women with intact uteri, OR 2.1 (CI 1.7-2.7). Hysterectomized women had no increased risk, OR 1.1 (CI 0.8-1.5). Since an association does not mean that there is a causal effect, the authors suggested that the observed differences in UTI incidences between estrogen users and non users may be explained by prescribing biases (4).

Foxman et al conducted a case-controled study to explore the role of health behavior and sexual and medical history on urinary tract infection (UTI) risk among otherwise healthy women aged 40-65. Cases and controls were recruited from nine practices and clinics in Michigan and a single clinic in Israel. In both countries, several factors were reported significantly more often among UTI cases than among controls: a UTI within the past 12 months, incontinence symptoms, a recent episode of more than 30 minutes of cold hands, feet, back or buttocks, and recent antibiotic use. In their study, cases were less likely than controls to report recent estrogen use, but the results were only statistically

significant in Michigan. Sexual activity during the previous 2 weeks and having ceased menses were modestly, but not statistically significantly, protective. These authors concluded that risk factors for UTI among women 40-65 differ from those for younger women and that these differences cannot be attributed solely to changes in menopausal status (5).

What do the trials tell us

Initial small uncontrolled studies using oral or vaginal therapy produced encouraging results, suggesting that estrogen replacement therapy may provide a certain protection against recurrent UTI (6-8). Thereafter, several randomised studies assessed the effect of either systematic or local estrogen use: Kirkengen et al (1992) evaluated, in 40 women aged 66-91 years (median age 78 years) the effect of estriol 3 mg p.o./ day versus placebo for four weeks (=phase 1), followed by 1 mg / day for eight weeks (=phase 2). The authors found that both oestriol and placebo significantly reduced the number of infections per week in both periods, compared with the pretreatment period, underscoring the need for randomized control trials. There was no difference between estriol and placebo treatment in the first period. In the second period, however, oestriol treatment was significantly more effective than placebo ($p = 0.05$). Vaginal pH at the end of the study was significantly different between the two groups ($p < 0.05$) (9). Raz and Stamm (1993) studied 93 postmenopausal women with a history of recurrent UTI in a randomized, double blind, placebo-controlled trial of a topically applied intravaginal estriol cream. They evaluated patients monthly for 8 months. Vaginal cultures were obtained at enrolment and after 1 and 8 months. The incidence of UTI in the group given estriol was significantly reduced compared with that in the placebo group (0.5 vs. 5.9 episodes per patient-year, $P<0.001$). Lactobacilli were absent in all vaginal cultures before treatment and reappeared after 1 month in 61% of the 36 estriol-treated women compared with none of 24 placebo recipients ($P<0.001$). The prevalence of Enterobacteriaceae fell from 67 to 31% in estriol recipients but was virtually unchanged in the placebo recipients ($P<0.005$). Likewise, the vaginal pH fell in the estriol group (mean, 5.5 at entry to 3.6), whereas it remained unchanged in the placebo group. There appeared to be a relation between vaginal colonization with lactobacillus and UTI in that three of 23 estriol-treated women who were colonized with lactobacillus after therapy developed UTI compared with seven of 13 who were not colonized (10). These data convincingly demonstrate that in postmenopausal women, replacement topical estrogen normalizes the vaginal flora and greatly reduces the risk of UTI.

Similarly, Erikesen (1999) reported a multicenter randomized open study assessing an estradiol-releasing silicone vaginal ring compared to an untreated

control group. The proportion of women remaining free of urinary tract infection was significantly higher in a group of 53 women assigned to receive the estradiol- vaginal ring (Estring 2 mg) than in the control group (n=55) (P =.008). After 36 weeks of study, the cumulative likelihood of remaining free from disease was approximately 45% in the women with the vaginal ring compared with approximately 20% in the control group. Again, the estradiol ring lowered vaginal pH, and the time to first recurrence was effectively prolonged after local estrogen treatment. Vaginal and, to a lesser extent, urethral mucosal cells were significantly more mature in the treated group (11).

Nevertheless all studies did not conclude that estrogen therapy is efficient in reducing UTI: Cardozo et al (1998) evaluated the effect of oral estriol (3 mg per day) or placebo for six months in 72 postmenopausal women older than 60 years of age (mean 73,2 years) suffering from recurrent urinary tract infections. They found that the study was difficult to conduct because of its design and the age of the participants. Still they were unable not show that estriol was superior to placebo in the prevention of recurrent urinary tract infections, but both estriol and placebo improved urinary symptoms during the trial. These authors concluded that the power of the study might have been too low to detect a significant difference between the groups. Alternatively, 3 mg per day of oral estriol may have been either the wrong dose or the wrong route of administration for this indication (12).

Similarly, Brown et al reported negative results. They evaluated the effects of hormone therapy on urinary tract infection frequency using the data from the HERS study, a randomized, blinded trial, assessing 0.625 mg of conjugated estrogens plus 2.5 mg of medroxyprogesterone acetate or placebo on coronary heart disease events among 2763 postmenopausal women aged 44-79 with established coronary heart disease. They curiously found that Urinary tract infection frequency was higher in the group randomized to hormone treatment, although the difference was not statistically significant (odds ratio [OR] 1.16, 95% CI 0.99-1.37). The other statistically significant risk factors for urinary tract infections in multivariable analysis included: women with diabetes on treatment (insulin OR 1.81, 95% CI 1.40, 2.34), oral medications OR 1.44, 95% CI 1.09, 1.90), poor health (OR 1.34, 95% CI 1.14, 1.57), childbirth (OR 1.38, 95% CI 1.00, 1.90), vaginal itching (OR 1.63, 95% CI 1.07, 2.50), vaginal dryness (OR 1.30, 95% CI 1.04, 1.67), and urge incontinence (OR 1.51, 95% CI 1.30, 1.75). Urinary tract infections in the previous year were strongly associated with a single urinary tract infection (OR 7.00, 95% CI 5.91, 8.29) as well as multiple urinary tract infections (OR 18.51, 95% CI 14.27, 24.02) (13).

The negative finding of this study may be explained by the fact that the amount of oral hormone that reaches the vaginal mucosa may be too low to affect the colonization of uropathogens or produce a lubricating effect.

Nevertheless, this finding is puzzling since both bacteriuria and urinary tract infections are more common in patients with diabetes than in patients without diabetes. In a more recent publication of the HERS study it has been revealed that fasting glucose levels increased significantly among women assigned to placebo but did not change among women receiving hormone therapy. The incidence of diabetes was 6.2% in the hormone therapy group and 9.5% in the placebo group (relative hazard, 0.65 [95% CI, 0.48 to 0.89]; P = 0.006) (14).

Conclusion

In postmenopausal women, replacement therapy using a topical estrogen normalizes the vaginal flora and greatly reduces the risk of vaginal atrophy. Similarly, vaginal estrogen administration seems to be effective for preventing UTI. However, it is important to note that although UTI is a very common affection and may result in high morbidity, few women have taken part in randomised studies of estrogen therapy for this condition and at least two reports assessing estrogen replacement therapy (orally) have failed to show any advantage of estrogen over placebo (15). This is a paradox as compared to the often cited beneficial effect of estrogen in reducing the UTI incidence.

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Perinatal and neonatal dilemmas at the limit of viability

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Results of the care of the neonate born at the edge of viability in this past decade has led to a major ethical dilemma. This dilemma is a result of four factors

1. Increased survival of neonates born at the threshold of viability (22-25 week gestation).
2. A persistently reported high morbidity, especially neurodevelopmental, amongst (30-50%) of the survivors.
3. As result, a profound impact on the infant, family, health care system and society at large.
4. Inadequate and incomplete data to serve as a basis for providing proper guidance to the physicians and counseling to the families.

The dramatic increase in survival is a result of both obstetric and neonatal factors

1. Use of prenatal steroids to accelerate fetal lung maturation
2. Improved perinatal management of high-risk mother and fetus
3. Use of postnatal surfactant
4. Improved neonatal ventilation techniques (high frequency, NO)
5. More optimal neonatal nutrition

Survival statistics during this decade from selected national networks or complete national data sets has noted the following:

Neonatal mortality data-selected sites

Gestational age	NICHD 1995-96	UK 1995	ISRAEL 1996-200
22 Weeks	79	100	100
23 Weeks	70	89	93
24 weeks	50	74	75
25 Weeks	25	46	48

NICHD: National Institutes of Child Health and Development Neonatal Network (12 sites)
UK: United Kingdom national data

Outcome data from NICHD Network noted the following morbidity at 18 months among survivors who were born at birth weight <1000 gm. (Vohr)

25% had abnormal neurological exam
17% were diagnosed as cerebral palsy
29% had abnormal Bayley exam
11% had hearing impairment
10% had vision impairment

Similar data were noted from follow up of a European born population i.e.:

Moderate-Severe morbidity at 23 weeks was 56%, at 24 weeks 53%, and at 25 weeks 45%.

This degree of neurodevelopmental morbidity is not surprising as at this critical point in gestation neuronal migration to the cerebral cortex is just completing its journey and, as yet, neither axons and dendrite growth nor synaptic formation has occurred to any extent, leaving the brain in a most vulnerable condition. Apparently the separation from the mother and placenta and her supply of neurotrophic and neuroprotective agents is clearly not in the infant's favor.

An analysis was performed on the outcome of of all Israel infants born in the State of Israel from 1996-2000. The information was received from the Israel National Neonatal Network, a volunteer organization of all the newborn services in Israel. Results were as follows:

	22 weeks	**23 weeks**	**24 weeks**	**25 weeks**
Live births	101	243	423	481
Treated	34 (34%)	165 (68%)	389 (92%)	470 (98%)
Survivors % of LB (% Of survivors)	1 1 (3%)	10 4% (6%)	86 20% (22%)	204 42%
Severe morbidity	0 0	6 (60%)	51 59%	124 61

Neurological morbidity was 20% in all age groups while pulmonary morbidity was 40% at 23 weeks and only 11 % at 26 weeks

Discussion

The Israeli results confirm the trend of the last decade i.e. improving mortality but a plateau of morbidity statistics. Informal surveys have noted that physician in most European countries are willing to withhold treatment in many infants born at this gestational age, particularly between 22-24 weeks gestation, there are some geographic differences (Netherlands 95%, Italy only 35%). The

willingness to withhold care of the newborn born in Israel is much less than in Europe 35% vs. 75% and reflects the religious input on parental desires and doctors practices.

The various International Guidelines for the need to resuscitate and/or threat borderline viable infants are summarized below:

1. Japan 1991 Eugenic Protection Act 1991 Treat all infants' over22 weeks
2. International Guidelines of AAP and AHA (2000) Non initiation of resuscitation is appropriate or infants below23 weeks or 400 grams
3. Netherlands (2001) No need to offer active intensive care before 25th week
4. Candian Pediatric and Ob Gyn Societies 1994 reconfirmed in 2000
   a. <22wk (<154 days) compassionate care
   b. 22 (154-160 days) Active treatment only upon request of fully informed parents
   c. 23-24 weeks (161-174 days) Per parents wishes modified by condition on birth
   d. 23-26 weeks (175-188 days) Resuscitate and treat all except those with fatal anomalies

Recent statements of the American Academy of Pediatrics and American College of Obstetrics confirm this approach of parental preferences in the decision to treat or not

There remains however, the dilemma of the physician who know that a significant resuscitation and/or long trial of hospitalization will result all too often with a severely brain damaged infant. Therefore we should acknowledge that what is in the best interest of the infant may not match what is the best interest of the family and society and their in lies the dilemma for the physician

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Pre — and postnatal inflammatory events in chronic lung disease of preterm infants

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Key words: chronic lung disease, bronchopulmonary dysplasia, lung injury, proinflammatory cytokines, oxygen radicals, proteases

Chronic lung disease (CLD) of preterm infants is associated with a significant inflammatory response of the airways and the interstitium of the lungs characterized by the presence of neutrophils and macrophages immunoreactive for numerous cytokines as well as an arsenal of humoral mediators. Besides proinflammatory / profibiotic cytokines and toxic oxygen radicals, potent proteases may be responsible for acute lung injury and may considerably affect normal alveolization and pulmonary vascular development in preterm infants with CLD (1). Various pre- and postnatal events have been identified as possible risk factors for inducing inflammatory changes in the immature lung: Exposure to chorioamnionitis (2), postnatal systemic and pulmonary infections (3), persistent ductus arteriosus (3), inappropriate resuscitation, initiation and duration of mechanical ventilation especially with large tidal volumes and no positive end-expiratory pressure (1, 4, 5). In general, an imbalance between pro- and anti-inflammatory factors can be considered as a hallmark of lung injury, and may considerably affect normal alveolarization and pulmonary vascular development by inducing growth arrest of the immature lung.

Inflammatory cells and adhesion molecules

Airway secretions of infants with CLD have been shown to contain a number of chemotactic and chemokinetic factors which are responsible for the recruitment of neutrophils and monocytes/macrophages (6). In addition, increased airway concentrations of L-selectin and intercellular adhesion molecule (ICAM-1) as well as increased plasma levels of ICAM-1 and selectins have documented in babies with CLD (7, 8). These studies provide indirect evidence for the recruitment of circulating neutrophils into pulmonary tissue and airways. Moreover, there is growing evidence that immediately after the initiation of mechanical ventilation in infants with respiratory distress syndrome (RDS) a

systemic activation of the clotting system and fibrinolysis takes place (9, 10, 11), and that inflammatory signals responsible for neutrophil sequestration in the pulmonary vascular bed induce a temporary depression of circulating neutrophils (12).

Cytokines

During the inflammatory process which is only partly understood several mediators may have direct detrimental effects on structures of lung tissue by affecting cell integrity and inducing apoptosis. Besides interleukin-8 (IL-8), other proinflammatory cytokines such as tumor necrosis factor-á (TNF-á), interleukin-1 (IL-1) and interleukin-6 (IL-6) seem to be important mediators in the early inflammatory response (1). A transient overexpression of IL-1 in rat lungs by intratracheal adenoviral gene transfer was recently shown to be accompanied by a local increase of TNF-á and IL-6 and a vigorous acute inflammatory response with evidence of tissue injury (13). The infiltration by TNF-á positive macrophages in pulmonary tissues of preterm infants who had died from RDS was found to be associated with a striking loss of endothelial basement membrance, and interstitial glycosaminoglycanes (14). The increased levels and enhanced mRNA expression of proinflammatory cytokines present in the airways and pulmonary tissue of preterm infants may reflect an inability to regulate inflammation through an adequate expression of the antiinflammatory cytokine interleukin-10 (IL-10) (15). In general, an imbalance between proinflammatory and antiinflammatory factors can be considered as a hallmark of lung injury.

Other inflammatory mediators

Neutrophils and macrophages attracted to the site of injured tissue can cause severe lung damage by release of potent proteases such as elastase and collagenase. The presence of free elastase activity and oxidative inactivation of $á_1$-proteinaseinhibitor which protects the alveolar capillary unit from autolytic proteolysis has well been documented (1, 16). The protease-antiprotease imbalance as well as toxic oxygen radicals released by phagocytes and generated by tissue bound xanthine oxidase may play a central role in lung injury (17). Recently, it was demonstrated that due to high concentrations of free iron detected in airway samples of infants with respiratory distress syndrome hydroxyl radicals are generated (18, 19). By now, the possible role of oxidative stress and its toxic effects on lipid peroxidation as well as on disruption of extracellular matrix through oxidative upregulation of metalloproteinases is well established (20, 21, 22). Reduced antioxidant capacity and low concentrations of

tissue inhibitor of metalloproteinases seem to predispose the preterm infant to issue damage.

One of the most important features in RDS and CLD is the increased alveolar capillary permeability which is pathognomonic for the early stage of CLD, and it is clearly associated with a deterioration of lung function. Several factors may have detrimental effects on the microvascular permeability: inflammatory cells and various mediators including toxic oxygen radicals, proteolytic enzymes, cytokines, and lipid mediators which modulate the vascular perfusion in the inflamed area. Lung injury leads to an induction of transforming growth factor-β (TGF-β) which limits some of the inflammatory reactions and plays a key role in mediating tissue remodeling and repair (23). If the reparative processes are exaggerated fibrotic changes in pulmonary tissue will ensue. This is typically associated with increased levels of TGF-β and its receptors, and overexpression of TGF-β has been shown to be predictive for the development of chronic lung disease (24). The complex interaction between mediators of inflammation and fibrosis has still to be defined.

Chorioamnionitis and antenatal events

Epidemiological data suggest a strong association between chorioamnionitis and the development of chronic lung disease (CLD). Increased concentrations of cytokines in human amniotic fluid TNF-, IL-1, IL-6 and IL-8 which were released during chorioamnionitis were clearly identified as risk factors of CLD (25). These proinflammatory cytokines seem to be important mediators in the early inflammatory response by recruiting and activating inflammatory cells and by inducing pathways of lung injury. In addition, increased levels of IL-1 and IL-6 in fetal cord blood — indicating a systemic fetal inflammatory response during chorioamnionitis — were shown to be an independent risk factor for the development of CLD (26).

Systemic and pulmonary reactions

Recently, an upregulation of vascular cell adhesion molecules (VCAM) by both venous and arterial endothelial cells in umbilical cord vessels has been observed in response to chorioamnionitis (27). This finding most likely reflects an activation of endothelial cells by cytokines present in the fetal circulation; as a consequence of VCAM expression migrational events of inflammatory cells may be initiated. Furthermore, a pronounced inflammatory response of lung tissue has been demonstrated in human fetuses exposed to chorioamnionitis. This inflammatory reaction was characterised by a marked infiltration of neutrophils and macrophages as well as an increased expression of IL-8 mRNA

mainly located in the bronchoalveolar epithelium and — in a scattered pattern — in the interstitial tissue (28). During this process neutrophils and monocytes were shown to be activated (29).

In animals similar observations have been made. Following a single intraamniotic injection of a high dose of endotoxin inflammatory cells were recruited to the chorioamnion and the lung which both expressed mRNA for various proinflammatory cytokines. Similarly, a systemic inflammation was noticed within 5 hours. However, cytokine expression was found to be more pronounced in lung tissue and inflammatory cells persisted much longer in the lung compared to the chorioamnion. Even with low doses of endotoxin an inflammatory response was observed (30).

Recent findings provide strong support for the hypothesis that expression of vascular endothelial growth factor (VEGF) is decreased in lungs of infants with BPD, and that impaired VEGF signalling may contribute to an inhibition of vascular growth (31, 32).

In a case-control study infants were at greatest risk for CLD when they had exposure to both chorioamnionitis and either mechanical ventilation > 7 days or postnatal infection (33).

Conclusion

These data indicate that a complex cytokine-induced lung injury responsible for CLD may begin prior to birth in a subset of preterm infants exposed to chroioamnionitis. The antenatal events may prime pulmonary tissue such that minimally injurious events in the postnatal period provoke an excessive locally inflammatory response in infants with RDS and CLD which could affect normal alveolarization and pulmonary vascular development and promote growth arrest of the immature lung.

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Do developmental therapies influence the outcome of very premature infants?

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Premature infants, particularly those born with a birth weight of less than 1750 gram and at a gestational age of 34 weeks or less have been noted to have significant neurodevelopmental morbidity. It has been argued, that, in addition to the neurological abnormalities that occur secondary to tissue hypoxia, intercranial hemorrhage, nutritional deprivation or infection the environment of the nursery, in of it self, is damaging to the infants vulnerable and immature. nervous system. Underlying this assumption however is two contradictory hypotheses. The first postulates that the infant suffer from the deprivation of the sensory stimuli it was programmed to receive in utero, while the second hypothesis postulates that the premature infant is overwhelmed by the highly stressful nursery environment and is further damage by sensory overload. Thus, a variety, and at times contradictory myriad of intervention therapies have been introduced into the care plan of premature infants ranging from minimal handling to structured massage and kinesthetic stimulation. In addition, no consistent evidence based data has emerged that clearly indicates any long-term developmental benefit from any of the proposed interventions, though tactile stimulation programs seemed to provide the most positive outcome. We thus chose to study the effect of skin-to-skin (Kangaroo Care KC) in a large group of high risk premature infants. Kangaroo care was chosen as the intervention program as it appears to incorporate both modulated sensory input while minimizing otherwise overwhelming environmental stimuli

Methodology

Two groups of infants were studied;

Study 1 was a group of 70 premature infants with a mean birth weight of 1230 grams (range 540-1650) and mean gestational age of 30.2 weeks (range 25-

33 weeks). Thirty five infants received kangaroo care and a matched group of 35 infants received standard nursery care.

Study 2 analyzed the results of kangaroo care in 73 infants who were matched with 73 infants receiving standard care. The mean birth weight of the study population was 1270 gram (range 530-1720 gram) and mean gestational age was 30.7 weeks (range 25-34 weeks)

Exclusion criteria for both studies were IVH grade 3 -4, perinatal asphyxia, metabolic or genetic diseases or maternal ingestion of illicit drugs. Kangaroo care, skin-skin-skin contact, was provided for a minimal of one and half hours a day for minimum of 2 weeks. Outcome measures were recorded at 37 weeks gestation prior to discharge and at 3 and 6 months corrected age. Vagal tone was assessed by calculating the amplitude of the respiratory sinus arrhythmia utilizing a computerized based system. State was assessed by structured observation and neurobehavior was assessed by Brazelton exam. Maternal-infant interaction was videotaped and analyzed with computerized frame-frame analysis program. Six month infant function was measured by Bayley II Scale

Results

Study 1. Significant improvement in infant state organization vagal tone, habituation and orientation was noted in those infants treated with kangaroo care. Treated infants spent a greater period of time in quiet sleep and alert wakefulness states and significantly less time in active sleep. KC treated infants had mature habituation patterns, oriented better to their environment and had more mature autonomic nervous system function (more respiratory sinus arrhythmia)

Study 2. Mother-infant interaction was significantly better in KC treated dyads. Mothers touched their infants more, had more visual contact with them and had better affect. Treated infants were more alert and had less gaze aversion. At 6 months treated infant scored better on the Bayley exam

Infant mental and psychomotor development and mother infant interaction at 6 months

	KC n=66		Control n=67		
	Mean	S.D.	Mean	S.D.	
MDI	96.4	7.2	91.8	9.8	**Sig <0.01**
PDI	85.5	18.4	80.5	13.3	**Sig < 0.05**
MAT SENS	4.2	0.6	3.9	0.8	**Sig <0.05**
INF SOC	2.4	0.7	2.3	0.9	**NS**

Mat Sens= Maternal sensitivity; MDI = Mental developmental index; Inf Soc = Infant social involvement; PDI= Psychomotor developmental index; Sig = significant

Discussion

Kangaroo care apparently provides a controlled and modulated stimulation that integrates tactile, vestibular, and proprioceptive stimulation that mimics the intrauterine environment while minimizing the excessive visual and auditory stimulation from the nursery. As a result kangaroo care leads to maturation of the autonomic system, facilitates better maternal-infant interaction and results in significant improvement in neurodevelopmental outcome. It's positive developmental influence is a result of a direct effect on the neurophysiological organization of the infant's nervous system and indirectly by improving maternal interactive behavior

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Postnatal steroid therapy for chronic lung disease in preterm infants — a continuing dilemma

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Abstract

Postnatal steroid therapy has been widely used for the prevention and treatment of Chronic Lung Disease (CLD) in preterm infants. Recently, evidence has accumulated to suggest that the risks of therapy may outweigh the possible benefits. In 2002, the American Academy of Pediatric and the Canadian Pediatric Society issued guidelines for use. While clarifying a confusing situation, these guidelines have given rise to much controversy. This review will summarize current information on the clinical trials of steroids and also consider the issues surrounding the guidelines.

Introduction

Postnatal steroid therapy for the prevention and treatment of CLD in preterm infants has recently been clouded in controversy. Emotions tend to run high when the suggestion is made that a widely used, powerful and perhaps life-saving therapy may cause brain damage and perhaps should not be used routinely.

In this review, I will present the most up-to-date information on short and long term outcomes from randomized controlled trials (RCT's) of systemic steroid therapy. In addition, I will discuss the current AAP/CPS guidelines and their implications for clinical practice.

However, first of all, it is necessary to remind ourselves of the history of this saga.

Historical perspective

It has long been recognized that corticosteroid therapy in the perinatal period has the potential to influence many body systems, most notably, the lungs and the brain. Shortly after the recognition of the central role of the surfactant

system in the etiology of Respiratory Distress Syndrome (RDS)[1], adrenal corticosteroids were noted to enhance surfactant synthesis and lung development.[2,3] As a result, studies of postnatal steroid therapy were undertaken both before and after the advent of mechanical ventilation.[4-7] These studies showed no appreciable benefit and follow-up data from one study showed increased incidence of intraventricular hemorrhage, EEG abnormalities and lower scores on the locomotor section of the Griffith developmental scale.[8,9

The next important development in this history was the recognition of the central importance of inflammation in the development of bronchopulmonary dysplasia (BPD) (or chronic lung disease (CLD)).[10,11] This provided a powerful rationale for the use of anti-inflammatory steroids in either the prevention or the treatment of established CLD. A large number of both controlled and descriptive studies of the use of the synthetic steroid dexamethasone followed, and many clinicians were impressed by the rapid improvement in lung function seen in many infants during the days following initiation of therapy.14§12 It seemed a logical and reasonable deduction that, if the intervention resulted in short-term improvement in the infant's pulmonary status, this would most likely translate into reduced risk for CLD and even mortality. Thus, although short-term side effects such as hyperglycemia, hypertension and gastro-intestinal hemorrhage were widely observed, these were felt to be a reasonable price to pay for improved lung function, and thus systemic dexamethasone became the mainstay of pharmacologic therapy for CLD during the 1980's and 1990's.

However, in the late 1990's, information began to accumulate suggesting that the risk-benefit ratio for steroid therapy may not be so favorable.[15-17] Systematic review of the many studies revealed that steroid therapy is associated with serious short-term effects such as intestinal perforation, growth failure and hypertrophic cardiomyopathy as well as increased risk for long-term morbidity such as cerebral palsy and developmental delay.[18-22] Presentation of some of the more adverse findings at major neonatal meetings was followed shortly by an extensive review of the issue by a combined committee of the American Academy of Pediatrics and the Canadian Pediatric Society. In 2002, this body published guidelines that have subsequently become a source of much heated debate.[23

Current State-of-the-Art

Scientific background

Evidence to support a possible neurotoxic effect of postnatal steroid therapy has come from both in vitro and in vivo laboratory studies, together with imaging studies of the developing brain in human neonates. Dexamethasone restricts general somatic and specific brain growth in rats, an effect that may be

mediated by insulin-like growth factors.[24,25] Sophisticated 3-dimensional MRI studies of brain volume have shown dexamethasone to be associated with restriction of gray matter volume in preterm neonates.[26[This may be a dose-dependent effect.[27

A number of mechanisms have been proposed to explain the direct effect of dexamethasone on the brain. Reduced brain cell division and myelination, inhibition of neurogenesis and abnormal neuronal migration have been reported in rats.[28-30] Dexamethasone has also been shown to influence the permeability of the blood-brain barrier in perinatal lambs,[31] and both the glucocorticoid and NMDA receptors are altered by postnatal steroids in rat and sheep.[32,33

In summary, the available scientific evidence supports but does not conclusively prove the possibility of a direct neurotoxic effect for postnatal steroid therapy in human preterm neonates.

Clinical studies

There are now 37 published RCT's of postnatal steroid therapy, encompassing 4303 infants, which were considered to be of appropriate methodological quality to be included in the meta-analyses on the Cochrane Collaboration. These have been updated a number of times, most recently this year.[12-14] The current review includes information from published reports and also follow-up data obtained directly from the investigators. Dr's Halliday, Ehrenkrantz and Doyle are to be commended for this important contribution that allows very complex large amounts of information to be made easily understandable to the busy clinician.

As steroids have been used for prevention, treatment or both, the studies have been divided into 3 groups according to the age of the infants at the start of therapy — early — before 96 hours, moderately early — 7-14 days, and delayed — more than 3 weeks of age. The relative risk for each of the major reported outcome variables are presented in Tables 1 (short-term) and 2 (long-term). Therapy in all 3 groups was shown to significantly facilitate earlier extubation, reduce the need for late steroid therapy and reduce the incidence of CLD at 36 weeks gestational age. However, mortality to discharge was unchanged in all groups, although mortality at 28 days was reduced in the moderately early group of trials (RR 0.44, 95% confidence interval 0.24-0.80). Short term adverse effects such as gastrointestinal hemorrhage and perforation, hyperglycemia and hypertension were increased in steroid-treated infants in all groups. Hypertrophic cardiomyopathy and growth failure were increased in the few studies that reported these outcomes.

The main reason for the current update of the meta-analyses was the availability of additional long-term follow-up data from a total of 19 of the 37

trials. As shown in Table 2, the risk for cerebral palsy was increased in infants from early but not from moderately early or late trials. However, there was no increase in the risk for the combined variable of death or survival with CP. Also, the increase in CP did not correlate with increased major neurosensory deficit in any of the groups.

These findings are somewhat similar to those that formed the basis of the AAP/CPS guidelines, although, as more data has accumulated, it is unclear as to why the possible neurotoxic effect of steroids appears only in the trials of early postnatal therapy.

Table 1: *Relative risk and 95% confidence intervals for major short-term outcome variables in randomized controlled trials of postnatal steroid therapy. Abbreviations: CLD — Chronic Lung Disease; PVL — Periventricular Leucomalacia; ROP — Retinopathy of Prematurity; N/A — not available or insufficient data.*

Outcome variable	Early	Moderately-Early	Delayed
Failure to extubate (7 days)	0.76 (0.88-0.66)	0.62 (0.84-0.46)	0.69 (0.82-0.58)
CLD 36 weeks	0.69 (0.80-0.60)	0.62 (0.82-0.47)	0.76 (1.0-0.58)
Mortality	1.02 (1.17-0.90)	0.66 (1.09-0.40)	1.03 (1.50-0.71)
GI hemorrhage	1.90 (2.66-1.35)	1.74 (2.98-1.02)	1.13 (1.73-0.74)
GI perforation	1.98 (2.95-1.32)	N/A	N/A
Hyperglycemia	1.36 (1.51-1.23)	1.51 (1.90-1.20)	1.42 (2.07-0.97)
Hypertension	1.84 (2.21-1.54)	2.73 (5.95-1.25)	2.61 (5.26-1.29)
Infection	1.01 (1.14-0.90)	1.35 (1.71-1.06)	1.03 (1.40-0.77)
Cardiomyopathy	4.33 (13.4-1.40)	3.29 (7.20-1.50)	N/A
Growth Failure	6.67 (19.6-2.27)	N/A	N/A
PVL	1.37 (2.05-0.91)	N/A	N/A
ROP	0.86 (1.02-0.73)	1.01 (1.70-0.61)	1.52 (2.12-1.09)
Late Dex therapy	0.50 (0.71-0.35)	0.70 (0.78-0.63)	0.40 (0.57-0.28)
Home oxygen	0.75 (1.07-0.53)	0.67 (3.71-0.12)	0.66 (0.92-0.47)

Table 2: *Relative risk and 95% confidence intervals for major long-term outcome variables in randomized controlled trials of postnatal steroid therapy. Abbreviations: CP-Cerebral Palsy.*

Outcome variable	Early	Moderately-Early	Delayed
Cerebral Palsy	1.69 (2.38-1.20)	0.83 (1.74-0.39)	1.20 (1.85-0.77)
Major Neurosensory disability	1.16 (1.52-0.89)	0.89 (2.10-0.38)	1.13 (1.75-0.73)
CP or death	1.16 (1.34-1.00)	0.83 (1.23-0.55)	1.05 (1.34-0.82)
Abnormal neuro exam	1.81 (2.47-1.33)	N/A	1.90 (3.33-1.08)
Blindness	1.92 (5.54-0.66)	0.40 (1.96-0.08)	1.44 (4.78-0.43)

The AAP/CPS Guidelines

After extensive review of the available evidence, the committee made the following recommendations in February 2002¤

1. The routine use of systemic dexamethasone for the prevention or treatment of CLD in infants with VLBW is not recommended.
2. Use of systemic dexamethasone should be limited to randomized, double-masked, controlled trials, in which the primary outcome should be survival without long-term developmental impairments.
3. Long-term neurodevelopmental assessment of infants from previous trials is strongly encouraged.
4. Clinical trials investigating the use of alternative anti-inflammatory corticosteroids, systemic and inhaled, are required before additional recommendations can be made.
5. Outside the context of a randomized, controlled trial, the use of corticosteroids should be limited to exceptional clinical circumstances (e.g. an infant on maximal ventilatory and oxygen support). In those circumstances, parents should be fully informed about the known short- and long-term risks and agree to treatment.

The post-guidelines era

Although the committee attempted to carefully balance the risks and benefits of steroid therapy, the resulting guidelines have sparked much controversy in both printed media and informal discussion.

1. "The guidelines went too far". The opinion has been proposed that the risk benefit-ratio, particularly for moderately early and delayed therapy, is reasonable and that therefore there is no need to restrict steroid therapy to "extreme clinical circumstances".[34] However, if so, it has been suggested that therapy should be limited to a brief course aimed at facilitating extubation (reduced by therapy in all 3 groups of trials). In addition, an alternative to high dose (0.5-1.0 mg/kg/day) intravenous dexamethasone should be used. The possible alternatives that have been suggested include a lower dose of dexamethasone (0.05-0.2mg/kg/day), intravenous hydrocortisone, inhaled steroids such as budesonide, betamethasone or fluticasone and oral betamethasone.[35-38] There is some evidence to support each of these forms of therapy, but none so far have been proven effective and safe in large trials that include the outcome variable of survival without neurodevelopmental disability.
2. "Catch 22 — when to treat". If early treatment is definitely not recommended and if treatment is to be restricted to "extreme clinical circumstances" and, if only moderately early therapy comes close to

reducing mortality, then by the time most infants are sufficiently sick they will mostly be in the "delayed" age group and may miss the peak positive effect. In contrast, it has been suggested that "extreme clinical circumstances" are vaguely defined thus effectively allowing clinicians to treat infants who are at relatively low risk for CLD or mortality.

3. "Why do steroids need informed consent?" It has been suggested that the insistence on informed consent for this therapy was inappropriate and is, in effect, an abdication of the responsibility by the clinicians who decide on all other therapies, such as surfactant, nitric oxide and antibiotics.[39] In addition, this may be the start of a slippery slope — today dexamethasone, tomorrow ampicillin! However, fully informing parents and obtaining their consent can be seen as the fulfillment of the parents' central role as surrogate decision makers for their child.[40] This is particularly so when a difficult decision needs to be made weighing the relative risks for death and neurologic impairment.
4. "More studies may never be done". In view of the risks inherent in the current risk-benefit ratio, and taking into consideration the medico-legal climate, it is likely that clinicians will be unwilling to either design or participate in future studies of systemic steroid therapy. For example, the DART study, which is a randomized controlled trial of low-dose short course dexamethasone in ventilator-dependent infants, has encountered major problems in recruiting study centers.[41
5. "If we can't use Dex, let's try ...". The fear of using dexamethasone in the context of caring for a sick, tiny infant with severe CLD, may invite the uncontrolled use of other unproven, experimental therapies, such as nitric oxide, prostacyclin and others. Hopefully, however, the neonatal community will have learnt from the steroid story and will carefully introduce only well-tested, evidence-based interventions.

Summary

Neonatologists must continue to search for alternative therapies for infants with CLD. In the meantime, despite their limitations, the AAP/CPS guidelines provide the most realistic, balanced advice for the neonatal community. So far, the updated scientific and clinical data do not justify any substantive change in this approach.

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Synthetic Humanized Surfactant — A Promise for the Future

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Pulmonary surfactant is a complex mixture of lipids and proteins where phospholipids compromise 90% of the content, of which DPPC (dipalmitoyl phosphatidylcholine) predominates (~65% of lipid content). The protein component consists primarily of four surfactant lipoproteins (SP-A, SP-B, SP-C and SP-D) along with serum-derived proteins. SP-A and SP-D are hydrophilic and are involved in host defense and activating immune functions in the lung. SP-B and SP-C are hydrophobic and are primarily responsible for the biophysical function of surfactant to lower surface tension in the lung. Data now demonstrate that SP-B is most important in lowering and maintaining surface tension and thus the most important surfactant protein for promoting respiration.

Sinapultide (also referred to as KL4, where ''K'' represents the water soluble amino acid lysine and the ''L'' the fat-soluble amino acid leucine) is 21-residue synthetic peptide modeled after human surfactant protein B (SP-B). This is accomplished by increasing inter- and intramolecular ordering of the phospholipid layer which results in a dramatic lowering of surface tension- comparable to natural surfactant.

The combination of the biophysical activity of the phospholipids and SP-B are well accepted as being the basis of the key pharmacological activity of pulmonary surfactant — lowering of surface tension. There is now a large body of literature associating the loss of surfactant function with a wide variety of respiratory diseases, including Acute Lung Injury (ALI), Acute Respiratory Distress Syndrome (ARDS), Meconium Aspiration Syndrome (MAS), respiratory distress syndrome (RDS), allergy, asthma, bronchiolitis, COPD, cystic fibrosis, otitis media, rhinitis/sinusitis.

Studies have demonstrated that surfactant replacement therapy (SRT) would be a viable pharmacological approach for patients suffering from respiratory diseases. To date, successful product development has been limited to respiratory distress syndrome (RDS), because the only available source of protein containing surfactant has been through the use of extracts of animal

(porcine, bovine) lungs. These animal-derived products have a number of drawbacks which prevents development for a broad range of diseases- among these drawbacks are: limited supply; variation in content; inability to formulate; high-cost to manufacture; the risk of exposure to infectious agents, and potential immunogenicity of animal proteins.

Surfaxin (lucinactant; KL4-Surfactant) is a humanized, proprietary aqueous suspension containing the 21-residue synthetic peptide, **sinapultide,** and the lipids dipalmitoylphosphatidylcholine (DPPC) and palmitoyl-oleoyl phospatidylglycerol (POPG), and the fatty acid palmitic acid (PA). This formulation mimics the activity of endogenous human lung surfactant without the problems and potential risks associated with the use of animal-derived exogenous surfactants. Preclinical studies with KL4-Surfactant in animal models of MAS, RDS and ARDS have shown marked improvement in lung compliance and gas exchange.

A phase 1/2 clinical trial in forty-seven (47) premature infants diagnosed with RDS has shown that Surfaxin improves gas exchange and pulmonary function when administered within 4 hours after birth. Further, there were no serious, unexpected or adverse Surfaxin-related events in any of the treated infants. A Phase 3 landmark, pivotal trial in RDS started in 2001. A phase 1B clinical trial in ARDS demonstrated the safety of the novel bronchoscopic lavage procedure (lung wash) and a Phase 2B trial in ARDS is currently ongoing. A Phase 2 clinical trial in MAS babies demonstrated improved oxygenation and an average savings of 3 days on mechanical ventilation and a Phase 3 pivotal trial in MAS has been initiated.

The promise of KL4-surfactant includes:

- Ability to formulate as an instillate, wet aerosol or dry powder aerosol; only complete surfactant with this potential
- Highly active peptide-mimic of human Surfactant Protein B, physiologically most important surfactant protein; potentially more active than other surfactants
- More resistant to proteolytic degradation and oxidation; potentially longer activity than other surfactants
- Demonstrable anti-inflammatory capability; potentially inhibit inflammatory component of respiratory diseases
- Not animal-derived; overcomes difficulties of animal-extract products and no potential transmission of animal-derived diseases
- Consistent production of quality, stable, large quantities and cost

Debate: All breeches at term should be delivered by elective Caesarean Section — Pros

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Summary

Caesarean section is recommended for the delivery of the singleton breech fetus at term if the breech is footling, if the fetus has a hyperextended head, if the fetus is large (>4000g), or if there is an anomaly or condition that may result in a mechanical problem at delivery.[1] However, most breech pregnancies at term are without these risk factors. Should an elective or planned caesarean section also be recommended for these women? Three randomised controlled trials have compared fetal, neonatal and maternal outcomes for the selected singleton breech fetus at term, delivered either by a planned caesarean section or delivered after a planned vaginal birth.[2-4] The results of these trials indicate that planned caesarean section substantially reduces the risk of fetal or neonatal death and serious neonatal morbidity, but does so at the expense of a moderate increase in maternal morbidity during the postpartum period.[2-5] Elective or planned caesarean section may also reduce the risk of urinary incontinence in women 3 months after the birth.[6]

Women with a singleton fetus in breech presentation at term should be informed of these findings and their choice in terms of delivery method should be respected.

Introduction

Caesarean section is recommended for the delivery of the singleton breech fetus at term if the breech is footling, if the fetus has a hyperextended head, if the fetus is large (>4000g), or if there is an anomaly or condition that may result in a mechanical problem at delivery.[1] However, most breech pregnancies at term are without these risk factors. Should an elective or planned caesarean section also be recommended for these women? The risks associated with planned vaginal

birth for the selected singleton breech fetus at term have been determined primarily by retrospective and prospective cohort studies which have compared outcomes following elective caesarean section with those following planned vaginal birth (emergency caesarean section or vaginal delivery). Meta-analyses of these studies have found significant reductions in risk of fetal or neonatal death and neonatal and infant morbidity with an elective caesarean section.[7-8]

Many clinicians have been reluctant to accept that the findings of better outcomes with elective caesarean section in non-randomised studies are true. This is because non-randomised studies suffer from selection bias. That is, the better outcomes with elective caesarean section may be due to healthier infants being selected for that method of delivery. As well, many of these studies have not described in detail the care of women during labour and the skill and experience of the clinicians at delivery. These factors may be associated with the risk of adverse fetal or neonatal outcome.[9]

In many teaching centres, caesarean section rates for breech presentation at term have been increasing. In these settings, obstetrical trainees have less chance to acquire the skills necessary to undertake a vaginal breech delivery.[10] These younger obstetricians who have been trained in these settings have been turning to elective caesarean section as their preferred method of delivery for women with a singleton breech presentation at term. Thus, women with breech pregnancies have been losing the option of a safe vaginal breech delivery.

The Term Breech Trial Collaborative Group felt some urgency, therefore, to prove the relative safety of planned vaginal birth compared with planned caesarean section for the well selected woman with a singleton breech presentation at term, if this was true. For, if planned caesarean section was not better than planned vaginal birth, there was a need to attempt to reverse the trend towards elective caesarean section, whilst older more experienced clinicians were still available to teach and assist newer trainees. Prior to embarking on a large randomised controlled trial, obstetricians from Canada and other countries, recognised for their skill and experience in vaginal breech delivery, in their own settings, were invited to participate in a consensus conference at which the details of the Term Breech Trial protocol were agreed.[9]

The Term Breech Trial[2,6]

In the Term Breech Trial, eligible women were randomly allocated, using a centrally controlled computerised randomisation service, to planned caesarean section or to planned vaginal birth (Table 1). Women in the planned caesarean section group underwent caesarean section at 38 weeks gestation. Women in the planned vaginal birth group were cared for in labour according to the protocol that was agreed at the pre-trial consensus meeting.[9] In brief, management was expectant until spontaneous labour began, unless an

indication to induce labour or undertake a caesarean section developed; the fetal heart rate was monitored either using continuous or intermittent electronic fetal heart rate monitoring or using intermittent auscultation; augmentation of labour with intravenous oxytocin was acceptable as long as the clinician was confident that there was no evidence of feto-pelvic disproportion; adequate labour progress in the first stage of labour was defined as a rate of cervical dilatation of 0.5 cm/hour after the onset of active labour, and in the second stage, as descent of the breech to the pelvic floor within 2 hours of full dilatation, with delivery being imminent within 1 hour of beginning active pushing; the choice of epidural analgesia was determined by the woman and her care providers; the method of delivery was by assisted or spontaneous breech delivery with control of the aftercoming head usually either with the use of forceps or the Mauriceau-Smellie-Veit manoeuvre. Babies in breech presentation, who were delivered vaginally were attended by an experienced clinician, defined as someone who considered him/herself to be skilled and experienced at vaginal breech delivery, confirmed by the individual's Head of Department.

Table 1. *Summary of the randomised controlled trials comparing planned caesarean section and planned vaginal birth for the singleton breech presentation at term included in the Cochrane Review.*

Author/Year	Selection Criteria	Planned CS	Planned VB
Collea[4] 1980	Frank breech, 36wks, singleton, EFW:2.5-3.8kg, cx 7cm, no hyperextension of fetal head	N=93 Booked for CS under supervision of senior obstetric resident	N=115 Xray pelvimetry, oxytocin for augmentation & induction, delivery by or supervised by senior obstetric resident
Gimovsky[3] 1983	Non frank breech in labour, GA:36-42wks, EFW:2-4kg, cx<7cm, no hyperextension of fetal head	N=35 Elective CS	N=70 Xray pelvimetry, continuous EFM, oxytocin prn, supervision by chief resident or obstetric staff
Hannah[2] 2000	Frank or complete breech, 37 wks, singleton, EFW: <4kg, no hyperextension of fetal head, no feto-pelvic disproportion, no evidence of a condition that might cause a mechanical problem at delivery, no contraindication to labour or vaginal delivery	N=1043 CS after 38 weeks	N=1045 IA or EFM, induction of labour prn, augmentation of labour with caution, limits to length of labour, skilled and experienced clinician at delivery

CS = caesarean section; VB = vaginal birth; EFW = estimated fetal weight; Cx = cervix; GA = gestational age; EFM = electronic fetal heart rate monitoring; IA = intermittent auscultation

The Term Breech Trial recruited 2088 women from 121 centers in 26 countries between January 1997 and April 2000.[2] The findings were a large statistically significant reduction in risk of perinatal or neonatal death or serious neonatal morbidity with a policy of planned cesarean section (1.6% vs 5.0%, p<0.0001), relative risk [95%CI]: 0.33 [0.19 — 0.56]), and a significantly reduced risk of perinatal/neonatal death in the planned caesarean section group (0.3% vs 1.3%, p = 0.01; relative risk [95% CI]: 0.23 [0.07 — 0.81]). Extensive subgroup analyses revealed that the reduction in risk of the combined outcome of perinatal/neonatal death or serious neonatal morbidity with planned caesarean section was similar for subgroups of women as defined by the following baseline variables: maternal age, parity, type of breech presentation, gestational age, presence of labour, presence of ruptured membranes, estimated size or weight of fetus, method of assessing fetal size or weight, method of assessing adequacy of pelvis, method of assessing attitude of fetal head, previous attempt at external cephalic version, standard of care provided by the centre, or total number of women recruited in the centre. However, the reduction in risk of the combined outcome of perinatal/neonatal death or serious neonatal morbidity with planned caesarean section was not similar for the subgroups defined by the country's national perinatal mortality rate (PMR). The reduction in risk was much greater in countries with a national PMR of 20/1000 (0.4% vs 5.7%, p = 0.0000002; relative risk [95%CI]: 0.07 [0.02 — 0.29]), than in countries with a national PMR of > 20/1000 (2.9% vs 4.4%, p = 0.13; relative risk [95%CI]: 0.66 [0.35 — 1.24]).

When vaginal breech deliveries following prolonged labour, labour induced or augmented with oxytocin or prostaglandins, those with a footling or uncertain type of breech presentation at delivery, those not having an epidural anaesthetic, and those without a skilled and experienced clinician present at the birth, were excluded from the analysis, the risk of the combined outcome of perinatal/neonatal death or serious neonatal morbidity with planned caesarean section, compared with planned vaginal birth was 1.5% vs 2.9%, 1-sided p = 0.05, relative risk [95% CI]: 0.50 [0.24 — 1.03].

There were no significant differences in maternal death or serious morbidity between the planned caesarean section and planned vaginal birth groups (3.9% vs 3.2%, p = 0.35; relative risk [95% CI]: 1.24 [0.79 — 1.95]). There was one maternal death in the planned vaginal birth group. Mothers completed a structured questionnaire at 3 months postpartum. Women in the planned caesarean section group experienced a lower risk of urinary incontinence (4.5% vs 7.3%, p = 0.02) than women in the planned vaginal birth group.[6]

Cochrane Review of Randomised Controlled Trials

The Cochrane meta-analysis of randomised controlled trials found a significant reduction in perinatal or neonatal death (excluding lethal malformations) with

planned caesarean section [Relative Risk (95% CI) = 0.29 (0.10 — 0.86)], a significant reduction in infants with an Apgar score <7 at 5 minutes with planned caesarean section [Relative Risk (95% CI) = 0.32 (0.17 — 0.83)], and a significant reduction in perinatal or neonatal death or short term neonatal morbidity with planned caesarean [Relative Risk (95% CI) = 0.0.31 (0.19 — 0.52)] (Table 1).[5] The meta-analysis also found that a policy of planned caesarean was associated with higher rates of maternal morbidity (Relative Risk [95%CI]: 1.29 [1.03, 1.61]).[5] The data from randomised controlled trials on the risk of maternal death from planned caesarean section compared with planned vaginal birth are too few to comment on this important outcome. However, observational data suggest that the risk of maternal death is unlikely to be increased with a policy of planned caesarean, because planned vaginal birth for the singleton fetus in breech presentation is associated with a high rate of emergency caesarean section.[11]

Conclusions

How a woman gives birth should be decided by the woman after discussion with her midwife or physician. To make this decision, she must have access to the highest level of evidence as to risks and benefits. The currently available evidence from randomised controlled trials indicates that an elective or planned caesarean section is the best approach for the outcome of the baby. Elective or planned caesarean section may also reduce a woman's risks of urinary incontinence later in life. Each woman will need to determine if these benefits justify an increased risk of immediate postpartum morbidity for herself and the possibility of unknown risks in future pregnancies. If the woman values the experience of a vaginal birth, she will also need to determine if the benefits of an elective/planned caesarean section are large enough to be willing to forgo this experience. A woman's choice in terms of delivery method should be respected.[12]

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All breeches at term should not be delivered by elective caesarean section

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Summary

Management of vaginal breech delivery continues to cause controversy and disagreement among obstetricians. Although randomised studies suggest that a policy of prelabour caesarean delivery is safer for the infant, these trials have several deficiencies, which render their results inconclusive. On the other hand, careful case selection in many institutions in developed countries with appropriate facilities and experienced obstetricians in attendance renders vaginal breech delivery a safe procedure for the infant and a less morbid experience for the mother.

Introduction

About 3-4% of singleton fetuses will present by the breech after 37 weeks' gestation. Because perinatal mortality and morbidity tends to be increased in association with vaginal breech delivery, the proportion of breech babies delivered by caesarean section has been rising progressively in developed countries during the past 50 years. Three randomised trials comparing planned vaginal breech delivery with planned prelabour caesarean section have now been published, the largest and most recent showing a poorer perinatal outcome among infants planned for vaginal delivery (1). On the other hand, meta-analysis of these three trials shows that early serious maternal morbidity is increased in association with caesarean delivery (2). Nevertheless, the results of the Term Breech Trial have accelerated the trend towards attempted prelabour caesarean section for term breech presentations. The results of this trial have been accepted by some obstetricians as an incontrovertible indication for changing their practice.

Appropriate selection for vaginal breech delivery

No reasonable obstetrician would advocate vaginal delivery for all term breech fetuses. Macrosomia and fetal neck extension are likely to increase mechanical difficulties at delivery; fetal growth restriction and footling breech presentation will predispose to intrapartum hypoxia and augmentation of uterine activity risks fetopelvic disproportion.

The axiom of ''a breech presentation combined with any other significant antepartum complication'' (e.g. growth restriction, maternal diabetes, pre-eclampsia, antepartum haemorrhage) is a useful rule for initial selection for planned prelabour caesarean delivery. Thereafter, the need for induction or oxytocin augmentation of dystocic labour, fetal macrosomia or hyper-extended attitude or simply slow progress during labour — defined as failure of the cervix to dilate at a rate of 1 cm per hour or a second stage exceeding 60 minutes — provide reasonable criteria for emergency caesarean intervention. Evidence of presumptive intrapartum fetal distress, based on cardiotocography, even without comfirmatory blood pH estimation, will select out those fetuses otherwise prone to hypoxic injury if labour progresses to vaginal breech delivery. Perhaps of greatest importance is the involvement of an experience obstetrician, who is a competent breech deliverer, in the management of breech labours, as the most reliable guarantee of a successful fetal and maternal outcome.

Using such a stringent and criterion-referenced management plan for term breech presentation, several authors publishing from countries with low background perinatal mortality and morbidity rates have continued to report equally good perinatal outcomes for both abdominally and vaginally delivered breech infants (3,4,5,6). In a consecutive series of almost 700 breech pregnancies published three months ago, Giuliani et al (7) reported no significant differences in serious neonatal morbidity between vaginally and caesarean delivered breech infants; the incidence of low cord pH values (<7.00) was equal in both groups and developmental delay on longer term follow-up did not differ significantly; half their breech presentations delivered vaginally.

Difficulties with breech randomised trial evidence

The longest randomised trial of term breech presentation (1) which concluded that prelabour caesarean delivery was safer in terms of perinatal outcome had several deficiencies which may lend doubts to its conclusions. Several practices were employed in the management of breech labour which would not be widely practised, if at all, in obstetric units in developed countries; these included augmentation of dystocic breech labour with oxytocin, antepartum estimation of fetal weight based on clinical assessment alone (rather than using ultrasound)

and protracted criteria for the duration of labour — such as permitting the active second stage to exceed 60 minutes.

Because it became necessary in the Term Breech Trial to enrol 121 obstetric centres in 26 different countries, in order to recruit adequate patient numbers, the levels of clinical facilities, technical support and obstetric and midwifery expertise inevitably varies widely among trial participants. Appropriate modern management of breech labours requires adherence to strict clinical criteria within individual institutions.

Although the conclusions and recommendations of the Term Breech Trial in favour of prelabour caesarean delivery were based primarily on differences in perinatal mortality, most of the breech deaths were unrelated to difficult vaginal delivery and one of the caesarean deaths was secondary to respiratory distress. Indeed, perinatal mortality within participating centres based in countries with low national perinatal mortality rates was similar whether breeches were delivered vaginally or abdominally (8). When the outcomes between caesarean and vaginally-delivered breech subsets were labour was not induced or augmented with oxytocin and where an experienced obstetrician supervised the vaginal breech deliveries — as also happened in the well-selected series reported by Irion et al (6) and Giuliani et al — the advantages of caesarean section melted away (9).

It should also be remembered that 6% of breech infants who were planned for elective caesarean delivery in the Trial actually presented at an advanced stage of labour, so that vaginal delivery was unavoidable, and that two of these 59 infants had an adverse outcome, presumably due to unpreparedness and lack of expertise of the birth attendants.

Conclusion

Despite randomised trial evidence based on appropriate methodology, the heterogeneity of the patients and obstetric units studied means that the results must be treated with considerable scepticism. Widespread evidence exists to support a policy of selective term vaginal breech delivery supervised by experienced obstetricians in units with facilities for rapid resort to intrapartum caesarean section when indicated. Skill at vaginal breech delivery must also be maintained in order to manage women who opt for vaginal delivery, following antepartum counselling and also to deal with those women planned for caesarean section unexpectedly in breech labour too late for abdominal delivery. There is no question that all breeches at term can be delivered by prelabour caesarean, even if such a practice were justified or desirable.

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Evidence-Based Perinatal Medicine: What is Evidence?

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The meteoric rise of Evidence-Based Medicine is a clear indication of the great need in medicine for a systematic approach in appraising the increasing volume of clinical literature. An important development is the classification of evidence levels, and grades of recommendations for clinical guidelines. According to the RCOG, evidence is classified as follows:

Ia Evidence obtained from meta-analysis of randomised controlled trials.
Ib Evidence obtained from at least one randomised controlled trial.
IIa Evidence obtained from at least one well-designed controlled study without randomisation.
IIb Evidence obtained from at least one other type of well-designed quasi-experimental study.
III Evidence obtained from well-designed non-experimental descriptive studies, such as comparative studies, correlation studies and case studies.
IV Evidence obtained from expert committee reports or opinions and/or clinical experience of respected authorities.

This in turn affects the *grading* of clinical guidelines:

Grades of recommendations

A Requires at least one randomised controlled trial as part of a body of literature of overall good quality and consistency addressing the specific recommendation. (Evidence levels Ia, Ib)
B Requires the availability of well-controlled clinical studies but no randomised clinical trials on the topic of recommendations. (Evidence levels IIa, IIb, III)
C Requires evidence obtained from expert committee reports or opinions and/or clinical experiences of respected authorities. Indicates an absence of directly applicable clinical studies of good quality. (Evidence level IV)

This uni-dimensional hierarchy places meta-analyses of RCT's at the apex, and observational studies at the base of the ladder. These are the foundations of EBM, but do the stand on solid ground ?

A classic instance of the potential flaws of this classification system is the discovery of the teratogenic effects of thalidomide. This sedative drug was introduced on a large scale in Europe in the late 50's. The syndrome of phocomelia and its link to thalidomide were described as case series and observational studies by McBride and Lenz. As a result of these publications we no longer prescribe thalidomide, on the basis of what would be regarded as ''Level III'' evidence.

Another example is the relationship between poor perinatal outcome and maternal smoking. Few clinicians would dispute the fact that smoking during pregnancy is harmful. Yet, what evidence do we have to support this fact? Scores of retrospective human observational studies and animal studies is all there is. Again, low grade ''level III'' evidence. No randomized trial on whether smoking causes fetal growth restriction o preterm labour has ever been performed, or would ever be allowed by an Ethics Committee. Hence a causal hypothesis between cigarette smoking and poor pregnancy outcome in humans is theoretically not established.

There was a time when the justification for medical intervention in obstetrics was questioned by radical midwives and other groups. The efficacy of standard medical care during pregnancy and delivery cannot be ethically evaluated using a randomized trial design. The paper by Kaunitz (1984) is a widely quoted observational study which equates to an ''experiment of nature''. It examined the maternal and perinatal mortality of members of the Faith Assembly in Indiana (USA), and compared it with non-members in the same county. This sect is unique in that it prohibits any medical intervention during pregnancy and delivery; its members are mostly white, with high educational levels. The study reported a MMR of 872 / 100 000 amongst members c/w 9/100 000 for non-members. Apart from religion, there were no demographic differences between

members and non-members of the sect. The design of this study is virtually free of bias, and its strength would be almost equivalent to that of a RCT.

Another type of evidence that is relatively neglected — in fact not classified — is decision-analysis. The superiority of cesarean section in preventing poor neonatal outcome in the management of breech presentation was predicted long ago by a decision analysis paper published by Bingham and Lilford in1987. However, clinical practice did not change until confirmation by a large randomized trial by Hannah et al (2000). Decision analysis is often misunderstood by clinicians. Numerous instances of neonatal injury may have been prevented if the weight of this evidence had been recognized at an earlier stage.

The current grading of evidence places undue emphasis on the *type* of

evidence and the randomized controlled trial. It fails to recognize that the *quality* of clinical studies is an important dimension that is independent of *type*. A well-conducted observational study should carry far more weight than a poorly designed randomized controlled trial. Likewise, case series and case reports are the *only* way to describe new disease entities, or new side effects of treatments. Often, they generate hypotheses, whose strength may require evaluation by randomised trials or more formal observational studies, such as case-control designs.

Clinical Significance of an Antenatal Nuchal Cord

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Summary

Antenatal nuchal cords are very common and their incidence rises with advancing gestation yet they are an infrequent cause of perinatal mortality and morbidity. They appear to come and go at random but a few persist and perhaps it is within this group that morbidity occurs while mortality is usually linked to complex entanglements with multiple loops around the neck, trunk and extremities. Antenatally, the presence of a single loop of nuchal cord, even if it persists for 4 or more weeks, is not associated with an increased incidence of abnormalities in the non-stress test, biophysical profile or umbilical velocity wave profile but fetuses with a persistent single loop in late gestation do demonstrate evidence of mild asymmetric growth retardation at birth and some of these appear to have subtle neurodevelopmental deficits at 1 year of age.

Introduction

As discussed elsewhere,(1,2) clinical studies in the 1950's, 60's, 70's and 80's established that the incidence of nuchal cords at birth rose with advancing gestation reaching an incidence between 25 and 30% at term. Both their occurrence and the number of loops was related to cord length and they were more frequently found in Caucasian offspring. Although they were not associated with an increase in perinatal mortality, the incidence of multiple neonatal morbidities of varying severity (mild acidosis and low Apgar score to severe cerebral palsy) were slightly increased in babies born with multiple loops, a single tight loop or a true knot. In the 90's several investigators noted that some of these newborns were growth restricted as well suggesting that nuchal cords might be associated with restricted nutrient availability in late gestation.(3,4) The only study which attempted long-term followup of the

offspring noted that pediatric neurological exams were normal at 1 year of age,(2) and, in the absence of information to the contrary, it was assumed that there were no long-term sequella which lead to a conservative clinical approach to the problem.

Nonetheless, the advent of routine antenatal ultrasound exams in obstetrics lead to multiple reports indicating that antenatal nuchal cords were extremely common (up to 40 + % in late gestation) but that many did not persist until the onset of labor.(5-8). This suggested the possibility that some of the occasional morbidity associated with nuchal cords (such as growth restriction and cerebral palsy) probably had their genesis prior to the onset of labor.

These findings lead us to initiate one retrospective and one prospective blinded study designed to assess the relationship between the presence of a nuchal cord at delivery and neurodevelopmental outcome at 1 year as well as a detailed evaluation of the natural history of an antenatal nuchal cord prior to birth and its, relationship, if any, to long-term neurodevelopmental outcome.(1,9)

Materials and Methods

The retrospective study (1) used a previously gathered follow-up data base to assess the effects of a nuchal cord at birth on birth weight and neurodevelopmental outcome at 1 year. Standard morphometric assessment was performed at the time of birth and the Bayley Scales of infant development were used to assess neurodevelopmental performance at 1 year.

The prospective study used an observational physican and patient-blinded design in which serial ultrasound and biophysical assessments were conducted in the second and third trimester.(9)

Results

Fetuses who were born with a symptomatic nuchal cord were significantly lighter (170 gm) at birth than those without a symptomatic cord. Although neurodevelopmental scores at one year of age were in the normal range in both groups, they were significantly lower in fetuses with a symptomatic cord especially in those cases with either a persistent (diagnosed by ultrasound 4 or more weeks prior to labor) or very tight (had to be cut to effect delivery)nuchal cord

The incidence of antenatal nuchal cords rose with advancing gestation but persistent nuchal cords were rare and there was no association between the presence of an antenatal nuchal cord and fetal biometry, fetal heart rate patterns or the biophysical profile prior to labor.

Conclusions

These data indicate that the isolated ultrasound finding of a single nuchal cord loop appears to be a normal part of intrauterine life that, in most instances, does not cause detectable clinical evidence of fetal stress prior to labor. However, in a minority of persistent cases there is evidence of intrauterine growth restriction and/or subtle effects on neurodevelopment. It appears that most of the severe outcomes associated with cord entanglement occur in cases with complex entanglements and/or true knots.

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Controversies in Neonatology; The Prevention of Early Onset Group B Streptococcal Infection

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Background

Group B streptococcus (Streptococcus agalactiae) is a gram-positive coccus that has been recognized as a significant cause of neonatal morbidity and mortality over the last 30 years. Early onset Group B *Streptococcus* (GBS) disease is defined as disease that occurs within the first 72 hours of life. Prior to the implementation of guidelines early onset GBS infection had an incidence of 1.3-3.7 per 1000 live births with males and females equally affected[1] (Figure 1).

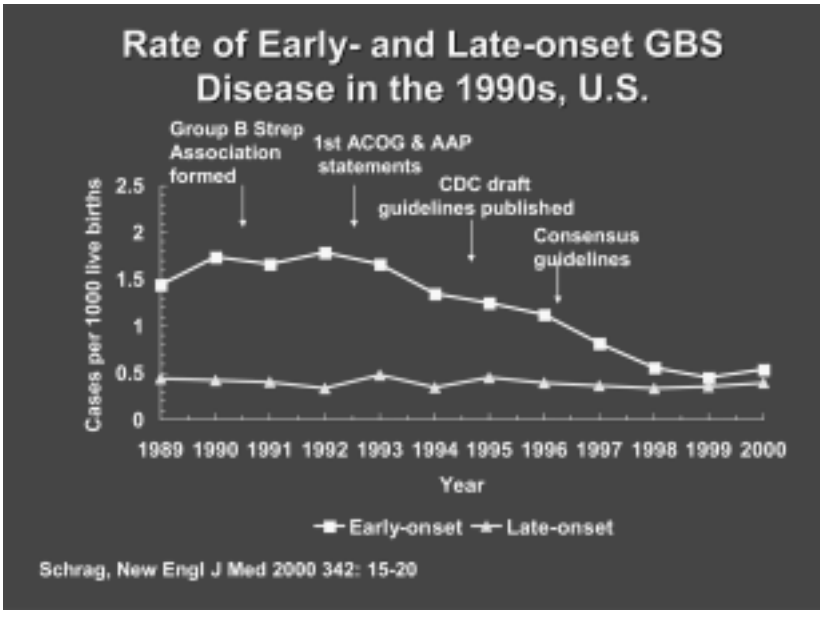
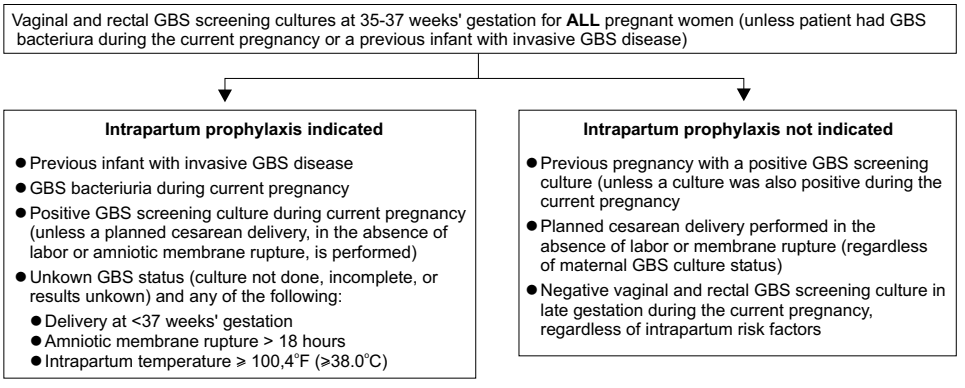


Figure 1

The GBS organism is acquired via vertical transmission from a colonized woman. It has been reported that 15-30% of all pregnant women are asymptomatically colonized with GBS in the vaginal or anorectal regions. [2] Vertical transmission ranges from 30-75% and is increased in the presence of high levels of colonization.[3] Despite this high transmission rate, only 1-2% of infants will go on to develop invasive disease.[1] Symptomatic presentation occurs within the first 12-24 hours of life, indicating that the infection must be acquired *in utero*.

Disease may be manifest by septicemia, asymptomatic bacteremia, pneumonia, and meningitis. Septic shock and neutropenia may also be present. With the advances in therapeutics, mortality rates which were as high as 50-70% in the 1970's and 13-37% since 1980[3], have been reduced so that about 5% of infected babies die in the late 1990's.[4] However, there is still a 25-50% incidence of permanent neurologic sequelae in survivors of GBS meningitis.[2]



1 If onset of labor or rupture of amniotic membranes occurs at <37 weeks' gestation and there is a significant risk for preterm delivery (as assessed the clinician), a suggested algorithm for GBS prophylaxis management is provided (Figure 3).

2 If amnionitis is suspected, is suspected, broad-spectrum antibiotic therapy that includes an agent known to be active against GBS should replace GBS prophylax.

Figure 2: *Indications for intrapartum antibiotic prophylaxis to prevent perinatal GBS disease under a universal screen strategy based on combined vaginal and rectal cultures collected at 35-37 weeks' gestation from all pregnant women*

Prevention of Perinatal Group B Streptococcal Disease

There have been many efforts to prevent early onset GBS disease. Antepartum antibiotics, as well as prophylactic postpartum treatment of the newborn, have not been shown to be effective in lowering the incidence of neonatal sepsis.[3] Intrapartum chemoprophylaxis has been shown to effectively reduce vertical

transmission, and thus decrease the rate of neonatal sepsis and asymptomatic colonization.[6]

Intrapartum chemoprophylaxis is the only strategy that has been proven to be effective and safe in preventing early onset GBS infection. The CDC/AAP/ACOG jointly developed guidelines based on a focused management approach to women known to be GBS colonized (as determined by screening culture at 35-37 weeks' gestation), or with identified risk factors significant for GBS transmission[7,8] (Figure 2).

The guidelines recommended the administering of intrapartum antibiotics to colonized women and those identified at risk (intrapartum fever, preterm labor, or membrane rupture of 18 hours or more) and provided an approach to the evaluation of their newborn infants on the basis of gestational age and symptomatology (Figure 3).

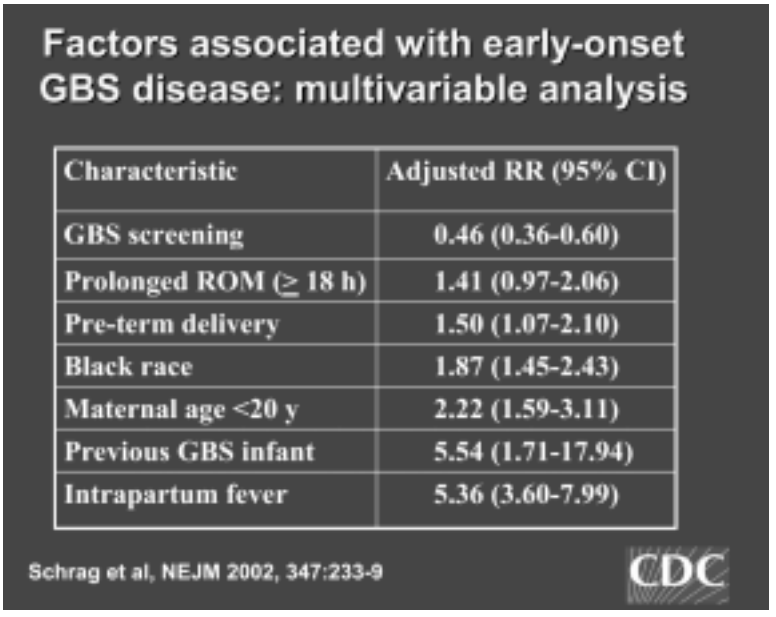


Figure 3

The incidence of group B streptococcal disease in babies less than a week old declined by over 70% in the 1990s, coinciding with increased use of intrapartum antibiotic prophylaxis. In 1999, three years after the release of CDC guidelines in 1996, the incidence began to plateau. Studies conducted after the issuance of the 1996 guidelines prompted re-evaluation of prevention strategies by CDC. Compelling evidence for a strong protective effect of the screening-

based strategy relative to the risk-based strategy led to a new recommendation in 2002 for universal prenatal screening for group B strep colonization by vaginal-rectal culture at 35-37 weeks gestation. In light of emerging clindamycin and erythromycin-resistant group B strep isolates, second line agents for penicillin-allergic women were revised. A number of additional issues related to management of threatened preterm delivery, planned cesarean section deliveries in group B strep colonized women, group B strep bacteriuria, management of newborns exposed to intrapartum chemoprophylaxis and culture collection and processing methods are also addressed. The 2002 guidelines for perinatal group B strep prevention are comprehensive and replace the 1996 guidelines. (11)

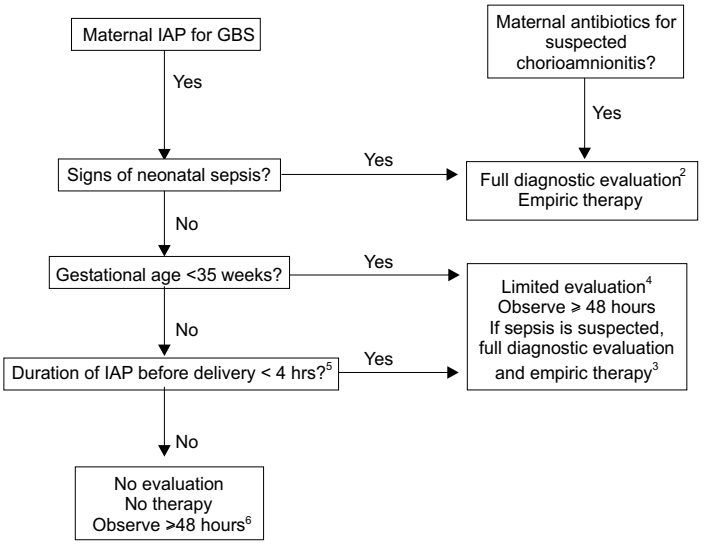
The main differences and similarities between the 2002 revised and previous 1996 guidelines:

Differences:

- Recommendation of universal prenatal screening for vaginal and rectal group B strep colonization of all pregnant women at 35-37 weeks' gestation
- Updated prophylaxis regimens for women with penicillin allergy
- Detailed instruction on prenatal specimen collection and expanded methods of group B strep culture processing, including instructions on susceptibility testing
- Recommendation against routine intrapartum antibiotic prophylaxis for group B strep colonized women undergoing planned cesarean deliveries without preceding labor or membrane rupture
- A suggested algorithm for management of patients with threatened preterm delivery
- An updated algorithm for management of newborns exposed to intrapartum antibiotic prophylaxis (Figure 4)

Although some important changes have been instituted, many recommendations remain the same:

- Penicillin remains the first line agent for intrapartum antibiotic prophylaxis, with ampicillin an acceptable alternative
- Women whose culture results are unknown at the time of delivery should be managed according to the risk-based approach; the obstetric risk factors remain unchanged (i.e., <37 weeks' gestation, duration of membrane rupture >18 hours, or temperature >100.4 F (>38.0°C))



1. If no maternal intrapartum prophylaxis for GBS was administered despite an indication being present, data are insufficient on which to recommend a single management strategy.
2. Includes complete blood cell count and differential, blood culture, and chest radiograph if respiratory abnomalities are present. When signs of sepsis are present, a lumbar puncture, if feasible, should be performed.
3. Duration of therapy varies depending on results of blood culture, cerebrospinal fluid findings, if obtained, and the clinical course of the infant. If laboratory results and clinical course do not indicate bacterial infection, duration may be as short as 48 hours.
4. CBC with differential and blood culture.
5. Applies only to penicillin, ampicillin, or cefazolin and assumes recommended dosing regimens (Box 2).
6. A healthy-appearing infant who was >38 weeks' gestation at delivery and whose mother received >4 hours of intrapartum prophylaxis before delivery may be discharged home after 24 hours if other discharge criteria have been met and a person able to comply fully with instructions for home observation will be present. If any one of these conditions is not met, the infant should be observed in the hospital for at least 48 hours and until criteria for discharge are achieved.

Figure 4: *Sample algorithm for management of a newborn whose mother received intrapartum antimicrobial agents for prevention of early-onset group B streptococcal disease or suspected chorioamnionitis. This algorithm is not an exclusive course of management. Variations that incorporate individual circumstances or institutional preferences may be appropriate.*

- Women with negative vaginal and rectal group B strep screening cultures within 5 weeks of delivery do not require intrapartum antimicrobial prophylaxis for group B strep, even if they develop obstetric risk factors (i.e., <37 weeks' gestation, duration of membrane rupture >18 hours, or temperature >100.4 F (>38.0°C))

- Women with group B strep bacteriuria in any concentration during their current pregnancy or who previously gave birth to an infant with early-onset group B strep disease should receive intrapartum antimicrobial prophylaxis

"Although universal screening for GBS colonization is anticipated to result in further reductions in the burden of GBS disease, the need to monitor for potential adverse consequences of intrapartum antibiotic use, such as emergence of bacterial antimicrobial resistance or increased incidence or severity of non-GBS neonatal pathogens, continues, and intrapartum antibiotics are still viewed as an interim strategy until GBS vaccines achieve licensure". (11).

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Multiple pregnancy and cerebral palsy — the effect of prematurity or more?

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It is now accepted that the risk of cerebral palsy is significantly increased in multiples compared to singletons. It is possible to summarize published information on four accepted points.

- The higher the number of fetuses the greater the prevalence of cerebral palsy. An exponential relationship exists between the number of fetuses and the average cerebral palsy rate per 1000 survivors. The average risk is 1.5, 8.6, and 34.6 for singletons, twins, and triplets, per 1000 survivors.
- Multiple and singleton pregnancies have similar risks for cerebral palsy until term, and, although LBW and preterm birth are the most significant risk factors for cerebral palsy, twins weighing more than 2500 g or delivered after 37 weeks have an excess risk over that of singletons.
- Single fetal demise invariably increases the risk of cerebral palsy in the survivor(s), especially in monochorionic twins.
- In comparison, other factors, such as zygosity, twin-twin transfusion syndrome, growth restriction, birth weight discordance, and mode of delivery, are less powerful contributors to the risk of cerebral palsy in multiples.

Simply stated, over-representation of multiples among premature and low birth weight infants is the most important risk of long-term neurological morbidity but the importance of the combination of these points cannot be overlooked. The potential nationwide impact of assisted conceptions on outcomes may be appreciated from a recent population-based study that examined the Israel National VLBW Infant Database. Between 1995 and 1999, multiples comprised one third of VLBW infants — 10 times their prevalence in the entire population. Assisted conceptions were responsible for 10% of the singletons, 55% of twins, and 90% of the triplets. American data showed that ART confers a 2.6 times greater risk of being LBW, whereby infants conceived with ART accounted for 0.6% of all infants born to mothers >20 years of age, but for 3.5% (6-fold increase) of LBW and 4.3% (7-fold increase) of VLBW infants. Thus, ART conceptions, irrespective of plurality, seem to be associated with an increased likelihood for low birth weight and hence, of increased risk of neurological morbidity.

In a population-based retrospective cohort study comparing neurological problems in Swedish children born after IVF with matched controls, the former were 70% more likely to need rehabilitation or remedial services. The risk for cerebral palsy was almost 4-times increased in children born after IVF, a sequela largely due to the high frequency of multiples, LBW, and prematurity among IVF children. The data confirm a model that suggested a significantly lower estimated cerebral palsy rate (2.7 / 1000 neonates) after spontaneous pregnancies as compared with transfer of three embryos (OR 6.3), two embryos (OR 3.3), and transfer of three embryos in which all triplets have been reduced to twins (OR 3.8). Similar estimations suggested that iatrogenic multiples contribute 8% to the annual number of cerebral palsy cases in the USA.

Because birth weight and gestational age are related, it is expected that similar findings will be observed regarding gestational age. Japanese and Australian researchers found a strong relationship between the risks of cerebral palsy in twins. When comparing twin to singleton births, the relative risk of cerebral palsy was greatest, and significant, only for twins delivered at 37 weeks or more. The data therefore implicate that multiple and singleton pregnancies have similar risks for cerebral palsy until term, and *although LBW and preterm birth are apparently the most significant risk factor for cerebral palsy, the disadvantage of twins becomes evident near term.* The excess risk for cerebral palsy beyond 37 weeks may suggest that 'term' occurs earlier in twins, in accord with observations that twins experience increased morbidity compared with singletons, are at increased risk of death and severe handicap after reaching 38 weeks' gestation. This also constitutes a strong argument to deliver twins by 38 weeks' gestation. Based on different risks in twin A and B, it is also possible that intra-partum factors may influence cerebral palsy rates in multiples. Finally, it should be stressed that the most powerful specific cause of cerebral palsy in twins is death of a co-twin. This is represented by the much-increased rate of brain damage in survivors of monochorionic and like-sex pregnancies, but also in cases of unlike-sexed pairs. Regrettably, data are present for gestational ages usually registered as births and are poorly recorded for embryonic demise of a co-twin.

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GBS: who, when, and which screening approach should be used for GBS screening and intrapartum therapy?

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Introduction

Group B *Streptococcus* (GBS) is usually a commensal bacterium that asimptomatically colonizes the vaginal or rectal areas of 10-30% of pregnant women (1). In these women, GBS can cause preterm labor, chorioamnionitis, postpartum endometritis, postpartum wound infection and sepsis (2). At birth, approximately 50% of infants who are born to colonized mothers will also become colonized on their mucosal surface and the skin. The vast majority of colonized newborns remain free of symptoms, whereas approximately 1% will develop invasive disease (3).

Neonatal GBS infection, a major cause of morbidity and mortality, occurs in two distinct syndromes of early- and late-onset diseases (3). The majority of infections in newborns occurs within the first week of life (early-onset), while late-onset infections occurs in infants aged > 1 week, with most infections evident in the first 3 months of life. Newborns with early-onset GBS disease acquire the organism intrapartum from their mothers, who are colonized in the genital tract, or from ascending spread of the organism into the amniotic fluid. Perinatal transmission can occur across intact membranes (4). The pathogenesis of late-onset disease is less well understood. Although some cases probably reflect acquisition of the organism during the passage through the birth canal, nosocomial and community sources may be involved in GBS transmission (5).

The most common clinical syndromes of GBS disease in newborns include sepsis, meningitis, pneumonia, cellulitis, osteomyelitis, and septic arthritis. In some cases, also endocarditis (6) and epiglottitis (7) have been observed. Bloodstream infections, with or without pneumonia, are the main manifestation of GBS disease in infants, and are evident in 89% of cases (8). Meningitis is less common, occurring in approximately 10% of neonatal infections (8). In addition

to acute illness due to GBS, which is itself costly, GBS infections in newborns can result in death, disability, and in rare instance recurrence of infections. Recent studies on death among newborns suggest fatality ratios ranging from 4 to 6% for cases identified by population-based surveillance during the 1990s (8, 9). Surviving infants may have long-term development disabilities, such as mental retardation or hearing or vision loss. Clinical trials have demonstrated the effectiveness of intrapartum prophylaxis in the prevention of early-onset neonatal GBS infection (10, 11), while antibiotic treatment performed during pregnancy can not prevent a subsequent vaginal colonization from the rectum. Current guidelines for prevention of neonatal GBS disease recommend that health care providers use either a risk-based or a screening approach to identify candidates for intrapartum antibiotic prophylaxis (11).

Risk-based and GBS culture-based screening methods

The most important predictive circumstance that leads to neonatal GBS disease is the exposure of the newborn to the microorganism in the birth canal. In addition to colonization with GBS, other factors increase the risk for infection, such as those related to increased host susceptibility and those associated with a large inoculum of the microorganism (12). An infant's susceptibility to GBS is increased when the level of anticapsular antibody to the infecting serotype is low. This is the case of low maternal antibody levels (13) or when infants, whose mothers had adequate antibody levels, were born before 34 weeks gestation, since transplacental transport of immunoglobulin G is reduced early in gestation. The inoculum of GBS can be large either because maternal colonization is particularly dense or because of obstetric complications allowing multiplication of bacteria at the time of labor (14). Ascending spread of GBS or increase of the inoculum to which the infant is exposed may be facilitated by some obstetric manipulations, such as intrauterine monitoring, chorionic villus sampling, and numerous vaginal examinations (15). Maternal bacteriuria due to GBS is likely indicative of a large inoculum present in the genital tract and is also associated with a higher risk for early-onset GBS disease (16).

Among the various risk factors, prevention strategies recently promoted by the Centers for Disease Control and Prevention (CDC), American College of Obstetricians and Gynecologists (ACOG), and American Academy of Pediatrics (AAP) focused on six clinical and microbiologic characteristics of at-risk mothers. These factors are maternal GBS colonization late in pregnancy, previous delivery of an infant with GBS disease, maternal GBS bacteriuria during pregnancy, onset of labor or membrane rupture before 37 weeks, prolonged membrane rupture (>18 hours), maternal amnionitis evidenced by intrapartum fever.

The risk-based approach provides intrapartum antibiotics to mothers in the presence of one or more of the risk factors listed above. The screening-based approach provide intrapartum antibiotics to women with GBS colonization identified by late gestation cultures and to those without culture results who are delivering at <37 week gestation.

Numerous studies have documented that the accuracy of prenatal screening cultures in identifying intrapartum colonization status can be enhanced by careful attention to the time of cultures, the anatomic site swabbed and the precise microbiologic methods used for culture and detection of organisms. Since the most accurate cultures are those collected within 5 weeks of delivery (17), and since preterm delivery can be addressed empirically if negative culture results are not yet available, collection of prenatal cultures at 35 to 37 weeks' gestation is now recommended by CDC to improve the sensitivity and specificity of women who remained colonized at the time of delivery. Swabbing both the lower vagina and rectum provides a significantly higher yield than does collection of vaginal samples alone. These may be collected by use of a single swab (swabbing the vagina first) or with two separate swabs. If two swabs are used, both should be inoculated in a single broth for culture, since the site of isolation is not important for clinical management, and laboratory costs can thereby be minimized. Because vaginal and rectal swabs are likely to yield diverse bacteria, use of selective enrichment broth, such as Todd-Hewitt broth supplemented with either gentamycin and nalidixic acid or with colistin and nalidixic acid, is recommended to maximize the isolation of GBS and avoid overgrowth of other organisms. After enrichment, the broth must be subcultured on blood agar plates to identify organisms suggestive of GBS (i.e. narrow zone of b-hemolysis, gram-positive cocci, catalase negative). Typycal colonies without hemolysis should also be further tested. Organisms can be then identified as GBS by antigen detection tests (latex agglutination tests or genetic probes) or by the CAMP test. When direct agar plate is used instead of selective enrichment broth, as many as 50% of women who are GBS carrier have false-negative results (18). The screening method is sensitive, but also time-consuming, since identification of positive samples requires a minimum of two days, and sometimes three days. Therefore, more rapid detection assays are desirable. Different media have been evaluated that identify the growth of GBS by the production of a carotenoid pigment, with a good sensitivity and specificity, sometimes within 4-18 hours (19, 20). In our experience, the use of a commercially available medium (Instant Granada Medium, Biomedics, Madrid, Spain) led to a rapid (~ 18h) and low-cost GBS identification from vaginal specimens, with 100% of both sensitivity and specificity (21). However, it must be considered that, although very infrequently, nonhemolytic, nonpigmented GBS have been implicated in cases of neonatal

disease (22). Thus, methods that do not rely on either hemolysis or pigment production must be used to detect them.

Although the guidelines for the prevention of GBS perinatal disease recommend the risk-based and screening approaches as equally acceptable, the debate continues over whether both strategies are equally effective. A recent study provided the first large scale of direct comparison of the strategies. By incorporating population-based surveillance for early-onset GBS disease into a sample survey of a population of over 600,000 live born infants, this analysis found that the screening approach was 50% more effective than the risk-based approach at preventing perinatal GBS disease (23).

Antibiotic susceptibility

GBS isolates remain susceptible to penicillin, and this is the agent of choice for GBS prophylaxis and therapy. Reports of penicillin-tolerant isolates have not been accompanied by evidence that this

in vitro observation has clinical relevance (24). Ampicillin is an acceptable alternative to penicillin, but penicillin is preferred because it has a narrower spectrum of antimicrobial activity, and may be less likely to select for resistant organisms. In contrast, the proportion of GBS isolates with in vitro resistance to clindamycin and erythromycin have increased since 1996. The prevalence of resistance among invasive GBS isolates in the USA and Canada ranged from 7 to 25% for erythromycin, and from 3 to 15% for clindamycin (25). A study performed in a German university hospital from 1997 to 1999 reported that 12% of the isolates from adult women were resistant to erythromycin, and 7% to clindamycin (26), while in Italy a 9.5% resistance to erytromycin was evidenced (27).

A greater concern about antimicrobial resistance associated with GBS is whether increased use of antimicrobial prophylaxis in obstetric care for GBS prevention will lead to the emergence of antimicrobial resistance among other perinatal pathogens. Although GBS isolates thus far seem resilient to antibiotic pressure from penicillin or ampicillin, perhaps other enteric organisms such as *Escherichia coli* will become increasingly resistant as the obstetric use of antibiotics increases. Contradictory results have been obtained so far. For example, whereas a Californian study found that the number of cases of other types of sepsis increased (28), in another study performed in USA from 1996 to 1999 the number of non-GBS sepsis cases remained very stable (29).

Ante-intrapartum chemoprophylaxis

The use of intrapartum prophylaxis with antibiotics to prevent perinatal group B streptococcal infections has led to a 70 percent decline in the incidence of group

B streptococcal disease during the past decade. However, despite this dramatic decrease, early-onset group B streptococcal disease (in infants less than seven days old) remains a leading infectious cause of illness and death among newborns in the United States, resulting in approximately 1600 illnesses and 80 deaths annually. Surviving infants may have long-term developmental disabilities, such as mental retardation or hearing or vision loss. (30, 31)

As said above, the Centers for Disease Control and Prevention, the American College of Obstetricians and Gynecologists, and the American Academy of Pediatrics have all published guidelines recommending that health care providers attending pregnant women implement 1 of 2 possible (24, 32, 34) algorithms for the potential prevention of early onset neonatal sepsis with group B streptococci. Both strategies recommend the treatment of pregnant women who are prematurely delivered of infants at <37 weeks' gestation. For the patient at term, one approach employs the use of cultures obtained between 35 and 37 weeks' gestation, with treatment during labor for all patients with a positive result. The other approach does not use cultures but reserves treatment for patients who have risk factors during labor.

Although it is generally accepted that intrapartum chemoprophylaxis is an effective measure, for the prevention of early onset Group B Streptococcus (GBS) neonatal sepsis no strategies have been demonstrated effective in absolute terms (33). Moreover it is well known that the widespread use of antibiotics leads to the selection of resistant strains; indeed, it has been reported that the increased administration of antenatal Ampicillin is associated with a decrease in GBS sepsis, while at the same time being associated with an increase in early-onset non-GBS disease, especially E.Coli (34-35). As an alternative, the antimicrobial agent chlorhexidine has been proved effective in reducing puerperal and neonatal colonisation by GBS. Indeed, Burman et al. (36) have demonstrated that vaginal chlorhexidine flushings in labour bring about a 2- to 3-fold reduction in the number of newborns transferred to the neonatal intensive care unit, with respect to placebo. Furthermore, chlorhexidine reduces overall admissions due to respiratory disorders and infections in carriers of GBS.

The specific action of vaginal chlorhexidine on perinatal GBS transmission was first evaluated by Christensen et al. (33). In their pioneering study, carried out on women with intact membranes, vaginal washing with the antiseptic agent reduced neonatal colonisation by 90%, although other methods of applying chlorhexidine in the vagina of GBS carriers, such as the lubrication of gloves employed for obstetric examination, gave no results (37).

On the assumption that chlorhexidine is as efficacious as ampicillin , after a pilot observational study (38), Facchinetti et al (39) published a randomised, controlled trial to compare the efficacy of chlorhexidine vaginal flushings vs

ampicillin i.v. on neonatal GBS vertical transmission. They enrolled 244 single pregnant women presenting at term with a previous vaginal swab positive for GBS. Rupture of membranes (ROM), when present, must not have occurred more than 6 hours previously. Women with any gestational complication (i.e., hypertension, diabetes, cholestasis, IUGR), with a newborn previously affected by GBS sepsis or whose cervical dilation was greater than 5 cm were excluded.

Randomisation was performed on the basis of hospital records: even numbers were treated with 2 grams of Ampicillin (AMP, prepared by Pharmacia & Upjohn) i.v. on admission, then 1 gram i.m. every 6 hours until delivery. Odd numbers underwent vaginal flushing with 140 ml chlorhexidine (0.2%) (CLX, prepared by Istituto Ganassini S.p.A., Milan), the treatment being repeated every 6 hours until delivery. The irrigation solutions were dispensed in sterile bottles. Flushings were performed during active labour by midwives who inserted the nozzles high into the vaginal fornix and slowly discharged the contents of the bottle. Neonatal swabs were taken once at birth, in 3 different sites (nose, ear and gastric juice). Both ear and nasal swabs were taken immediately after birth, while gastric juice was collected within 24 hours.

The analysis of data was carried out on 108 women treated with AMP and 109 with CLX. No local or systemic undesired effects were recorded. The overall rate of GBS colonisation was 13.8% with no differences between the AMP(12.4%) and CLX (15.6%) groups. The overall rate of E. Coli was 4.6% and it was significantly more frequent in the AMP (7.4%) than in the CLX (1.8%) group (χ^2=3.87, P=0.049). Clinical features such as maternal age, weeks at delivery, labour more than 8hrs, ROM at entry, maternal leukocytosis, birth-weight and Apgar score of negative and colonized newborns were similar.

These findings demonstrate that in singleton pregnancy, at term, intrapartum vaginal flushings with a chlorhexidine-based solution are able to significantly prevent neonatal GBS colonisation, and confirm an interim analysis of the same study (40). After the initial report on the efficacy of chlorhexidine in preventing neonatal morbidity associated with GBS (37) few studies have been carried out on the vaginal use of such an antiseptic agent in antepartal women.

In a study performed in Malawi, the cleansing of the birth canal with chlorhexidine was associated with reduced puerperal infections. More importantly, the authors found a significant reduction in neonatal sepsis as well as a decrease in neonatal mortality due to infectious causes (41).
It would be extremely helpful to have a low cost, easy-to-use method for the disinfection of the birth canal, in order to prevent peripartal transmission of infecting agents (HIV included). chlorhexidine flushings seem to fulfil this need, at least as far as the vertical transmission of GBS at term is concerned.

Recommendations

As stated above, the risk based approach is not a reliable method for the prevention of perinatal GBS disease. Thus, it should be desirable to achieve a consensus about a GBS colonization prenatal screening. According to recommendations from CDC, together with AAP and ACOG (30), as well as recommendations of Spanish Society of Obstetricians and Gynecology, Spanish Society of Neonatologists, Spanish Society of Infectious Diseases and Clinical Microbiology and Spanish Society of Chemotherapy (42), we suggest that obstetric-care practitioners, together with supporting laboratories and labor and delivery facilities, should adopt the following strategy for the prevention of perinatal GBS disease.

Who. Since no combinations of clinical and demographic information could identify a subset of women whose screening would identify the majority of carriers, the screening programs should be applied universally to all pregnant women.

When. Vaginal and rectal cultures should be performed from 35 to 37 weeks gestation. The samples must be labeled ''GBS detection''.

How. Three different technical methods could be employed. According to CDC recommendations, swabs should be placed in selective broth medium, then subcultured in sheep blood agar plates. The organism suggestive of GBS should be identified by antigen detection or by CAMP test. Susceptibility to eritromycin and clyndamycin should be verified only for penicillin-allergic patients. In alternative, after culture in selective medium, the samples could be plated on GBS (Islam) or Granada agar plates, allowing direct GBS identification by detection of carotenoid pigment production. Strains that do not produce pigment require further GBS identification. Finally, swabs can be directly cultured in Instant Granada medium tubes, with identification within 18 hours.

However, it must be considered that this method does not allow identification of non-pigment producers strains.

Intrapartum prophylaxis should be given to all pregnant women identified as GBS carriers. In addition, prophylaxis should be given to all women with preterm onset of labor (<37 weeks) and unknown GBS colonization status; to all women with GBS isolated from the urine in any concentration during their current pregnancy; to women who have previously given birth to an infant with GBS disease; to all women with unknown GBS colonization status and with prolonged (>18 hours) membrane rupture or intrapartum fever.

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