Human Reproduction Update, Vol.8, No.1 pp. 1–9, 2002

Alternative approaches in IVF*

Bart C.J.M.Fauser[1,7], Philippe Bouchard[2], Herjan J.T.Coelingh Bennink[3], John A.Collins[4], Paul Devroey[5], Johannes L.H.Evers[6] and Andre van Steirteghem[5]

[1]Division of Reproductive Medicine, Erasmus University Medical Center Rotterdam, The Netherlands, [2]Service d'Endocrinology, Hôpital St Antoine, Paris, France, [3]NV Organon, Research and Development Unit, Oss, The Netherlands, [4]McMaster, Hamilton, Canada, [5]Center of Reproductive Medicine, Free University, Brussels, Belgium and [6]Department of Obstetrics and Gynaecology, University of Maastricht, The Netherlands

[7]To whom correspondence should be addressed. E-mail; fauser@gyna.azr.nl

Various new developments in clinical and basic science which may impact on IVF in the near or distant future will be discussed in this review. These key areas include the regulation of early follicle development and the extended in-vitro culture of oocytes and embryos. Moreover, alternative compounds and ovarian stimulation protocols will be discussed, along with highlights in the development of the cryopreservation of excess oocytes or embryos. Finally, the health economics of IVF is addressed.

Keywords: cryopreservation/embryo culture/follicle development/IVF health economics/ovarian stimulation

TABLE OF CONTENTS

Introduction

In-vitro fertilization (IVF) as practised today is complex, time-consuming and expensive, and generates much stress, side effects and chances for complications. Success rates have improved over the years, with around 25% pregnancies per cycle in recent years. Moreover, awareness is growing throughout the world that the rate of multiple pregnancies—especially triplet and higher-order pregnancies—following IVF can no longer be accepted (Fauser *et al*., 1999). This overview will discuss several recent developments in clinical and basic science which may impact on IVF in the near or distant future. Key issues are the development of simplified and less vigorous ovarian stimulation regimens, and reducing the incidence of multiple pregnancies following IVF. Certainly, these developments will ultimately improve the balance between success and complications and the overall cost-effectiveness of IVF.

Areas covered in this overview include studies concerning early pre-antral follicle development and extended in-vitro culture of oocytes. Recent observations concerning the development of new compounds and regimens for ovarian stimulation are also discussed, as well as the current status of embryo cryopreservation. Finally, the health economics of IVF will be explained.

Early follicle development

The primary goal of IVF treatment is the birth of a healthy baby. Until recently, the major focus of clinical researchers has been the manipulation of ovarian function by exogenous FSH in an attempt to generate as many follicles as possible. Improved understanding of mechanisms regulating early follicle development may give rise to more refined and individualized stimulation protocols. Perhaps in the distant future, ovarian stimulation can be replaced completely by the in-vitro maturation of oocytes. In addition, totally new approaches for the control or enhancement of fertility are on the horizon for future applications.

The major functional unit in the ovary is the follicle, which consists of an oocyte surrounded by one or more layers of somatic cells. The destiny of each follicle is determined by endocrine and intra-ovarian factors. During normal folliculogenesis a tight interaction exists between the oocyte and surrounding granulosa

*This review represents an overview based on lectures presented during a Round Table Conference organized by B.C.J.M.Fauser, entitled 'Alternative approaches in IVF', Lisbon, Portugal, September 2000, and sponsored by NV Organon. Invited lecturers included A.G.Byskov, A.J.Hsueh, M.M.Matzuk, D.T.Baird, I.Boime, B.Tarlatzis, P.Barri, K.Diedrich, B.A.Lessey, J.A.Collins, J.Smitz, B.Behr, M.Plachot, M.H.E.C.Pieters, L.Hamberger and J.Shaw.

cells. Several distinctly different steps can be identified: (i) the initial recruitment of resting primordial follicles involved in depletion and eventually exhaustion of the stock of follicles; (ii) early development of pre-antral follicles, which seems to be under the control of intra-ovarian autocrine and paracrine factors; and (iii) the late advanced growth of antral follicles and cyclic recruitment controlled by FSH, resulting in a limited number of follicles which continue to grow during the follicular phase of each menstrual cycle (see also Figure 1). These processes are regulated differently, and hence will be discussed separately.

Initial recruitment of primordial follicles

In the human, female oogonia proliferate during early embryonic life before differentiating into primary oocytes. The pool of resting primordial follicles reaches its maximum size around 20 weeks of fetal life. Follicles can remain at this inactive stage for many decades. Initial recruitment is the term indicating primordial follicles leaving this resting pool. It is unknown as yet whether this initiation of growth is due to reduced inhibitory influences or to direct stimulation by local intra-ovarian factors. The potential role of the oocyte itself should also be taken into consideration. Initial recruitment is a continuous process, and in the human around 1000 primordial follicles start growing every month. The morphological characteristics of follicle recruitment include an increased size of the oocyte and a transition of surrounding granulosa cells to a cuboid shape. In humans, it is not known at this stage whether primordial follicles can directly undergo atresia.

Early follicle development

Once recruited, the follicle develops through the primary and secondary stages before an antrum is formed. Specific oocyte or granulosa cell-derived factors are involved in the development of pre-antral follicles, such as c-kit (a tyrosine kinase receptor), its ligand, stem cell factor (for review, see McNatty *et al*., 1999) and Wilms tumour 1. The involvement of androgens and the insulin-like growth factor family in early follicle development has recently been described in the monkey model (Vendola *et al*., 1999). Although some observations suggest that the process of early follicle development is sensitive to FSH, FSH knock-out studies (Layman and McDonough, 2000) as well as humans presenting with defective FSH receptors or after hypophysectomy have shown convincingly that the early stages of follicle development remain unaffected.

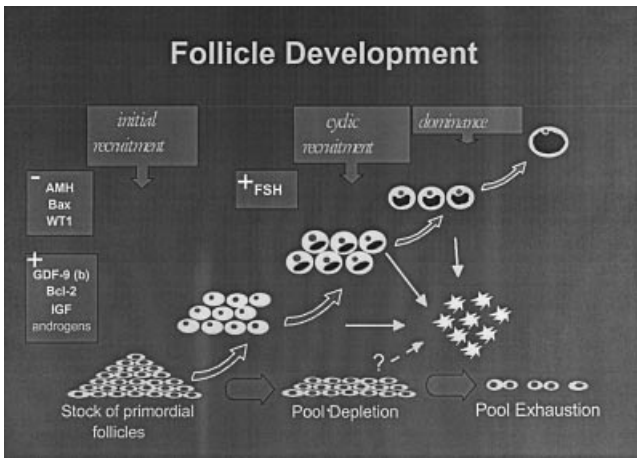

Figure 1. Schematic representation of factors involved in early and late follicle development. Adapted from McGee and Hsueh (2000).

Growth differentiation factor-9 (GDF-9) is a member of the transforming growth factor β (TGF-β) super family with highest homology to bone morphogenic protein-15 (BMP-15). This factor is exclusively expressed in oocytes from pre-antral and early antral follicles, and is absent from primordial follicles. GDF-9-deficient female mutant mice were infertile due to defective follicle development. The ovaries were small, and no follicles beyond the one-layer granulosa cell stage could be observed. Primordial follicle numbers were clearly increased in these animals compared with controls (Dong *et al*., 1996). In the absence of GDF-9, follicles do not reach the secondary stage and are incapable of forming multiple layers of surrounding theca cells (Matzuk, 2000). GDF-9 also stimulates growth of pre-antral follicles in culture (Hsueh *et al*., 2000). BMP-15/GDF-9 double knock-outs also present with clearly abnormal folliculogenesis (Yan *et al*., 2001). Intriguingly, Inverdale ewes which are heterozygous for a naturally occurring X-linked mutation of the BMP-15 gene have increased ovulation rates, while ewes that are homozygous for this mutation develop primary ovarian failure sheep (Galloway *et al*., 2000). Much attention is currently focused towards another related factor, GDF-9B. These and other observations demonstrate for the first time that oocyte-derived factors are required for somatic cell differentiation and interaction with endocrine factors such as FSH.

Anti-Müllerian hormone (AMH), yet another member of the TGF-β superfamily of growth and differentiation factors, is involved in male sexual differentiation. However, mRNA for AMH (and its type II receptor) has recently been shown to be expressed in ovarian granulosa cells of pre-antral and small antral follicles, suggesting a functional role. More importantly, ovaries obtained from fertile female AMH knock-out mice had increased numbers of pre-antral and small antral follicles, but reduced numbers of primordial follicles, suggesting a direct or indirect role for AMH in follicle pool depletion (Durlinger *et al*., 1999). It appears likely that more oocyte or granulosa cell-derived factors important for early folliculogenesis will be identified in the near future. These factors may represent a new avenue for the in-vitro maturation of oocytes and for the assessment and possibly also the manipulation of ovarian ageing.

Specific genes encoding for proteins involved in the initiation, progression and completion of programmed cell death (apoptosis) have been identified in recent years. Some proteins (e.g. Bcl-2) delay or inhibit apoptosis, whereas others (e.g. Bax) induce apoptosis. Out of the 500 000 follicles present in the human ovary at the beginning of reproductive life, only 400 or so reach the pre-ovulatory stage and ovulate. Hence, follicle atresia rather than differentiation is the fate of the great majority of female germ cells. The magnitude of demise of follicles at early stages of development may affect follicle pool depletion (the number of follicles entering the growth phase) and resulting reproductive life span. Indeed, several studies have established a correlation between mRNA encoding for Bax and granulosa cell apoptosis. In addition, reduced follicle loss could be observed both in mutant mice lacking *Bax*, or in transgenic mice where *Bcl-2* was overexpressed in the ovary, suggesting that the life span may be extended by reducing apoptosis (Morita *et al*., 1999; Perez *et al*., 1999).

Late follicle development

FSH acts as a survival factor for antral follicles, most of which will undergo atresia, with only a few being rescued by the intercycle rise in serum FSH level (also referred to as cyclic recruitment) (Fauser and van Heusden, 1997). FSH is essential for final granulosa cell differentiation allowing for estrogen biosynthesis.

Several distinct differences between initial and cyclic recruitment can be identified (McGee and Hsueh, 2000), including: (i) differences in developmental stages of the follicle (primordial versus large antral); (ii) oocyte status (starting to grow versus completion of growth); (iii) regulatory factors involved (locally derived growth factors versus FSH); and (iv) timing (continuous throughout reproductive life versus cyclic related to the onset of a menstrual cycle) (Otsuka *et al*., 2001).

Normally, only a single follicle reaches the pre-ovulatory Graafian stage in each cycle releasing the oocyte during ovulation. Other follicles from the recruited cohort enter atresia, due to insufficient FSH support. Only the dominant follicle continues to grow despite decreasing FSH levels, because of its increased FSH sensitivity along with the induction of LH receptors on granulosa cells. Indeed, it has been shown recently that LH can drive dominant follicle growth in the late follicular phase (Sullivan *et al*., 1999).

Oocyte/embryo culture

Culture of immature oocytes

The use of elaborate media or co-culture can result in an enhanced development of human immature oocytes *in vitro* (Cha *et al.*, 2000). However, although the resulting fertilization rates for IVF or ICSI reach 45% and 75% respectively, implantation and clinical pregnancy rates remain low. This may—among several other reasons—be due to abnormal cytoskeletal organization leading to mitotic spindle deficiency and subsequently to nuclear disorganization or chromosomal abnormalities in embryos (Nogueira *et al*., 2000). Conclusions are difficult to draw due to the small size of the studied series published so far, as well as the existing differences in culture media employed, timing of oocyte retrieval and stimulation regimes used. In addition, many unsolved issues such as the presence of steroids in culture medium, the significance of recombinant LH, the role of epidermal growth factor (Smitz *et al*., 1998) and meiosis-activating sterol (Byskov *et al*., 1995) as well as the importance of metalloproteinases and the activin/inhibin system are currently under investigation.

The in-vitro maturation of oocytes may induce permanent changes in the expression of imprinted genes, and this may result in abnormal fetal development (Young *et al*., 2001). Moreover, the maturation systems used for human oocytes are mainly based on animal experiments. For these reasons, the in-vitro maturation of oocytes from small antral follicles must be regarded as an experimental procedure which should not be applied clinically unless its safety has been proven (Sinclair *et al*., 2000).

In-vitro blastocyst development

In human IVF, 80–90% of day 2–3 embryos selected for transfer fail to survive implantation. Consequently, attempts to minimize the chance of a twin gestation by replacing only one cleaving embryo result in a decreased pregnancy rate per IVF cycle. By moving the embryo transfer to the blastocyst stage (besides achieving an improved embryo–endometrial synchrony), only embryos with an activated embryonic genome and high developmental potential are given the chance to implant (Jones and Trounson, 1999). In this way, a 50% implantation rate per blastocyst (double that obtained from cleavage-stage embryos) may be achieved, resulting in high pregnancy rates following transfer (Behr, 1999).

By using sequential serum-free media, more than 50% of embryos may develop into blastocysts. It should be noted, however, that blastocyst transfer may coincide with disadvantages such as the need for additional patient and staff education, the requirement for more laboratory incubator space, as well as with a 5–7% risk of no development of embryos suitable for transfer at an early stage (Gardner *et al*., 1998). Furthermore, a 5% monozygotic twinning rate following blastocyst transfer has been reported (Costa *et al*., 2001; Sheiner *et al*., 2001).

At present, patients who seem to benefit the most from blastocyst transfer are those who are at risk of high-order multiple gestation, as well as those with multiple IVF failures. Among the still unresolved issues, the value of blastocyst cryopreservation as well as the effectiveness of a single blastocyst transfer following pre-implantation genetic diagnosis for aneuploidy screening must be addressed urgently. Altogether, the need for a randomized controlled trial comparing the efficacy of a single day 5–6 blastocyst versus a day-3 embryo replacement in minimizing the occurrence of a twin pregnancy is imperative. Moreover, follow-up studies should be performed on the health of children conceived after blastocyst transfer.

Assessment of oocyte and embryo quality

Assessment of embryo quality has barely changed since the beginning of IVF. The focus has been on blastomere number, size and shape of the blastomeres, and the presence or absence of extracellular fragments. However, as implantation and clinical pregnancy rates in IVF are still far from optimal, the predictive value of currently accepted morphological criteria applied to day 2–3 embryos has been questioned. In fact, embryos considered to be unsuitable for embryo transfer on days 2–3 of culture are able not only to develop to blastocysts *in vitro* (Rijnders and Jansen, 1998), but also to implant following transfer to the uterus. Conversely, many of the morphologically optimal embryos on days 2–3 of culture may fail to develop to blastocyst stage, while a significant proportion of them are chromosomally abnormal (Plachot *et al*., 1987; Munné and Cohen, 1998). It appears that the developmental history of the embryo may also be used as a marker predicting chances of implantation (van Royen *et al*., 2001). A close relationship exists between blastocyst quality and success of transfer (Gardner *et al*., 2000). The blastocyst grading system is based on the degree of expansion of the blastocyst cavity, the inner cell mass (potential embryonic cells) and the trophectoderm (potential placental cells). A fully expanded blastocyst with numerous tightly packed inner cells and many cohesive cells within the trophectoderm is thought to be optimal for implantation. It should be noted, that the development of an embryo to the blastocyst stage is not a confirmation of chromosomal normality, as at least 10% of blastocysts are chromosomally abnormal (unpublished data).

The evaluation of oocyte quality has attracted less attention in conventional IVF, as the precise assessment of nuclear maturity is not easy to perform (due to the presence of surrounding cumulus cells). Attempts to grade cumulus–oocyte complexes morphologically show only weak correlation with the real maturity of oocytes. Metaphase II oocytes can only be assessed after the removal of cumulus cells by hyaluronidase, as is performed in ICSI cycles. IIIt is generally accepted that good quality human metaphase II oocytes should have a clear moderately granular cytoplasm, a small perivitelline space and a clear, colourless zona pellucida. However, oocytes deviating from the ideal morphotype may have a normal capacity for fertilization and embryo development following ICSI. The so-called ideal oocytes may represent only 34% of all those retrieved.

It is important to define the developmental potential of embryos originating from morphologically abnormal oocytes by conducting large studies which relate oocyte morphology to the outcome of ICSI. Moreover, the evaluation of embryo morphology needs to be readdressed, as—depending on maternal age—between 20 and 50% of morphologically normal embryos appear to be chromosomally aberrant (Gianaroli *et al*., 2001; Sandalinas *et al*., 2001).

Ovarian stimulation protocols

The key issues related to ovarian stimulation and drugs used for that purpose remain maximal efficacy with minimal chances for ovarian hyperstimulation syndrome (OHSS) and the avoidance of multiple gestations. Clearly, agreement exists that multiple pregnancy can be largely avoided by replacing only a single embryo. In Europe there is a clear trend to limit replacement to two embryos, thereby restricting the problem of multiple pregnancies to twins. The major problem related to single embryo transfer remains judgement of the quality of embryos. It should be noted that a pregnancy rate per cycle of approximately 10% is accepted by the scientific community for in-vivo ovulation induction, whereas the same rate for IVF procedures is considered too low. Simplification of IVF procedures by using GnRH antagonists may increase the acceptability of a relatively low pregnancy rate following single embryo transfer.

The single most important issue related to ovarian stimulation remains the avoidance of life-threatening OHSS. Less vigorous stimulation of the ovaries (referred to as soft protocols or minimal hyperstimulation) (Fauser *et al*., 1999) may help to achieve this goal. The price to pay is an increased percentage of cycles with low or no response. Furthermore, OHSS is only partially related to the dose of the drugs used to stimulate the ovary. The individual sensitivity of the patient to FSH stimulation may be more important (Fauser and van Heusden, 1997). The introduction of GnRH antagonists may also reduce the risk of OHSS. Because of the competitive mechanism of action of antagonists, HCG for final oocyte maturation may be replaced by a single injection of a GnRH agonist. Data supporting the significant reduction in the risk of developing OHSS by replacing HCG with a GnRH agonist have not yet gained sufficient attention (Itskovitz *et al*., 1991).

Drugs and protocols for ovarian stimulation

Few prospective randomized studies have been performed concerning drugs and protocols for ovarian stimulation. Even some of those studies have problems, including: (i) too few patients treated to reach sufficient statistical power; (ii) the use of surrogate endpoints such as the number of oocytes and embryos rather than the single most relevant clinical endpoint (viable pregnancies resulting in live birth); and (iii) the application of rigid regimens in highly selected patient groups. It is rather surprising that definite conclusions are frequently drawn from inadequate studies. Although less satisfying for the individual investigator, fewer but larger studies in unselected patient populations focusing on viable pregnancy rates are needed.

New treatment protocols for IVF have become possible due to the recent introduction of two comparable GnRH antagonist compounds, cetrorelix and ganirelix. Both multiple and single-dose regimens are effective in preventing a premature LH rise (Bouchard and Fauser, 2000). In contrast to the widely used GnRH agonist long protocol, the antagonist is started several days after the initiation of exogenous FSH. These regimens are much shorter and not complicated by chances of cyst formation due to the GnRH agonist 'flare' effect. Furthermore, the use of antagonists reduces the required total dose of FSH by approximately 20%. Surprisingly, initial clinical studies applying GnRH antagonists showed a somewhat lower vital pregnancy rate (Bouchard and Fauser, 2000), but a learning curve for the clinician may be the most likely explanation for this finding. However, the possibility of a relative overdose of the antagonist—especially in low bodyweight women—should also be considered. Several studies have suggested that extremely low late follicular phase LH levels are detrimental for IVF outcome (Westergaard *et al*., 2000; Filicori and Cognigni, 2001). Since the GnRH antagonists have become available, discussion concerning the use of a natural unstimulated cycle for IVF has re-emerged. It is possible to manipulate the LH surge during the development of a spontaneously growing single follicle by delaying this surge with a GnRH antagonist. The disadvantage remains the availability of one oocyte only, resulting in relatively low pregnancy rates. Moreover, since the introduction of GnRH antagonists the possible use of clomiphene citrate has again been suggested.

The debate concerning the comparison of clinical outcome following the use of recombinant versus urinary FSH continues. There is no question that recombinant FSH is more pure and is synthesized more consistently (resulting in reduced batch-to-batch variability), without any risk of contamination. Recombinant FSH products have been shown by prospective studies to result in a small but significantly higher viable pregnancy rate compared with different urinary FSH preparations (Daya and Gunby, 1999), though the difference is only ~5%. The counter-argument used by those favouring the use of urinary FSH relates to the difference in price, although this economic argument should be judged in the light of the total cost of the treatment per cycle. New gonadotrophin drugs will become available, such as short- and long-acting FSH that have half-lives of about 0.5 and 4 days respectively. Long-acting FSH may also be achieved with chimeric molecules such as FSH-CTP (where the carboxy-terminal part of HCG, which is responsible for its long half-life, is combined with the FSH-β chain) (Bouloux *et al*., 2001).

Standard FSH regimens in combination with GnRH agonists include the long, short and ultra-short protocols. In general, the long protocol, starting with GnRH agonist treatment in the luteal phase of the preceding cycle, is preferred due to a small but

significant increase in vital pregnancy rate as shown by meta-analysis (Daya, 2000).

Fixed, incremental and decremental FSH dose regimens are used without clear evidence of major differences in either outcome or side effects. The starting dose of FSH appears to be higher in the USA (225–300 IU/day) compared with elsewhere (150–225 IU/day). The higher starting dose results in significantly more oocytes and OHSS, and a lower chance of cancellation due to absent or low response. Although kinetic data of FSH show an inverse relationship between serum FSH concentrations and body weight, it is rather surprising that body weight is not used to titrate the starting dose. During recent years a debate has continued on less vigorous ovarian stimulation, described as minimal stimulation or soft/mild protocols. In principle, this can be achieved by either using a lower daily dose of FSH with a more careful dose increase, and/or by starting FSH treatment later in the cycle in case short or ultra-short GnRH agonist protocols or GnRH antagonist are applied. Such mild protocols are expected to decrease the incidence of—but not eliminate—OHSS because the individual ovarian sensitivity is at least as important as the FSH dose. The disadvantages of soft protocols will be a lower number of oocytes and a higher incidence of cancelled cycles.

The induction of oocyte maturation

A very important unsolved problem related to ovarian stimulation for IVF is the quality of the oocyte. As oocyte quality is a decisive factor for embryo quality and the resultant pregnancy rate, there is an urgent need to develop new methods for better judgement of oocyte and/or embryo quality, as discussed earlier. The extension of in-vitro embryo culture up to the blastocyst stage will eliminate some inferior embryos with genetic or other developmental abnormalities, but certainly not all. Improved embryo or oocyte selection seems to be crucial in order to embark upon single embryo transfer and less vigorous ovarian stimulation. To resume meiosis and induce luteinization in IVF protocols, urinary HCG at a dose of 5000–10 000 IU has been the standard treatment for many years. New developments in this area are the availability of recombinant LH and HCG. When exogenous LH is used to mimic the LH surge, two injections with a 1-day interval may be required (Loumaye *et al*., 1999) due to the short half-life of LH (10–20 min). GnRH antagonists provide another new opportunity to replace HCG since the mode of action of the antagonists is competitive. Instead of GnRH agonists down-regulating and internalizing receptors, they can be used to replace HCG by using the initial gonadotrophin stimulatory 'flare' effect. Recent evidence suggests that the use of a GnRH agonist in this way may avoid OHSS in case too many follicles are developing, combined with an unchanged reproductive potential of the oocytes obtained. An alternative solution to solve the problem of imminent OHSS due to abundant follicular development is to switch to a (high-dose) GnRH antagonist (de Jong *et al*., 1998), though the latter approach implicates loss of the cycle for IVF.

Cryopreservation

Oocyte cryopreservation

The cryopreservation of supernumerary embryos increases the chance of obtaining a pregnancy from a single IVF cycle. Frozen embryo storage can give rise to ethical and legal problems which could be bypassed in case oocytes might be cryopreserved in a reliable manner. Gamete cryopreservation can also be important in the case of cancer therapy to preserve the chance of reproduction. While human embryo cryopreservation is now routine, it remains a research procedure for oocytes (Mandelbaum *et al*., 1998).

The problems associated with cryopreservation of the mature oocyte are related to its large volume, and to the fact that it is a very specialized cell. The freezing process must not only maintain the cellular viability, but the potential for fertilization and subsequent embryo development must also be preserved. Several anomalies have been reported following cryopreservation, including abnormalities of the zona pellucida, induction of parthenogenetic activation and, more importantly, meiotic spindle anomalies. The efficiency of oocyte cryopreservation expressed as the number of children born per frozen–thawed oocyte is <1%. Whilst anomalies of the zona pellucida might be overcome through the use of ICSI, spindle anomalies could be overcome by freezing at the GV stage. Nevertheless, optimal culture conditions for the maturation of GV oocytes have not yet been established. Meiotic spindle anomalies might also be avoided by using vitrification techniques (Le Gal and Massip, 1999). By doing so, the problem of chilling sensitivity of the meiotic spindle after slow cooling of oocytes can be avoided. The safety and efficacy of vitrification techniques is yet to be determined, however (Chen *et al*., 2000).

Cryopreservation of ovarian tissue

One of the chief attractions of storing ovarian tissue is that some of the problems associated with cryopreservation of mature or immature oocytes are circumvented. Primordial follicles contain an oocyte that is much smaller than the mature cell, and they appear to be more tolerant to freezing and thawing. The small size of primordial follicles, the absence of zona pellucida, the low number of support cells, its low metabolic rate, the arrest at prophase I of meiosis, the small amounts of chilling-sensitive lipids present and the lack of cortical granules make the primordial follicle less sensitive to cryoinjury than a mature oocyte (Shaw *et al*., 2000).

Very small pieces of ovarian tissue must be used for optimal effectiveness. However, the problem with this approach is that the restoration of fertility depends on the re-implantation of ovarian tissue either to the same person or to an immunologically compatible recipient. When the ovary has been removed for reasons of cancer, the risk of re-implanting cancerous cells is present (Shaw *et al*., 1996). An alternative is that early-stage follicles could be cultured through the stages where it is possible to mature and fertilize the oocyte *in vitro*. At present, however, ovarian tissue cryopreservation, the re-implantation, and the culture of early-stage follicles are still at the experimental stage

Cryopreservation of human embryos

There are three main reasons to cryopreserve embryos for thawing and transfer at a later date. The first reason is that the current use of ovarian stimulation regimens yields a high number of oocytes which can be fertilized. In order to reduce the number of multiple

births, most IVF clinics transfer (maximally) two to three good-quality embryos, with supernumerary embryos being frozen, stored and used in subsequent cycles. As a result, more than one transfer from a single stimulation cycle can be performed, thereby increasing the overall chance of pregnancy. The second reason is the availability of embryo cryopreservation, which allows embryo transfer to be delayed for those women who are at risk of developing severe OHSS. The final reason is that donated embryos can be frozen to eliminate the need for synchronization of the donor and recipient cycles; moreover, the donated frozen embryos can be held in quarantine until screening for transmissible viruses has been completed. The importance of human embryo cryopreservation will vary from centre to centre, and will also depend on the embryo cryopreservation criteria that each centre will develop.

It would be highly desirable, as has been suggested previously (Jones *et al*., 1995), to establish a method that would allow not only a correct evaluation of the cryopreservation of human embryos *per se*, but also an evaluation of the role of embryo cryopreservation in enhancing the total reproductive potential of a single treatment cycle and the possibility of comparing the cryopreservation of embryos between different centres. The embryologist/clinician requires three main types of information concerning the different variables of freezing and thawing. First, the percentage of patients with a fresh transfer who also have material available for cryopreservation (cycle cryopreservation rate), and the percentages of embryos assigned to fresh transfer (embryo transfer rate) and to cryopreservation (embryo cryopreservation rate) is required. Second, the percentage of embryos that survive the freezing–thawing process and the pregnancy potential of the surviving embryo is needed. As a result, the best way to report results of freeze–thawing human embryos would be as the number of children born per frozen and thawed embryo. Third, the contribution of the results of cryopreservation to the total pregnancy potential of the oocyte collection cycle would be required.

A formula has been proposed (Testart, 1988) to compare the success rate of IVF according to various parameters, including the policy for embryo cryopreservation. This formula would enable all IVF teams to present their data in an identical manner. Taken together, we feel that these suggestions (Testart, 1988; Jones *et al*., 1995) should become common practice when reporting cryopreservation data, and in this way over- or underestimations of the importance of human embryo cryopreservation could be avoided. Moreover, the exact role of human embryo cryopreservation in the entire infertility treatment could be more correctly estimated.

There are two main strategies for cryopreserving human embryos. The first strategy involves selecting embryos for fresh transfer and for cryopreservation before morphology becomes a substantial factor. Freezing is then performed at the two pronuclear (2PN) stage. There are several advantages when freezing 2PN embryos: (i) it is more acceptable for some persons with ethical problems; (ii) the cell is at interphase of mitotic division; (iii) there are no anucleate fragments present (this is known to be negatively correlated with cryopreservation outcome); and (iv) 2PN freezing is a good alternative in case of suboptimal culture conditions. In some countries, freezing at the 2PN stage is required by law. The disadvantages of 2PN embryo freezing are that, despite optimized evaluation techniques for 2PN embryos, it is difficult to choose the embryos for fresh transfer. As a result, the fresh cycle is not optimized. Another disadvantage is that a situation may arise in which the zygotes left in culture for fresh transfer do not cleave further.

The second strategy involves optimizing fresh transfer, thereby allowing the morphologically best embryos to be transferred fresh. Embryos can be frozen at the 2- to 8-cell stage and at the blastocyst stage. The advantage of 2- to 8-cell stage freezing is that embryos can be selected for transfer, and that the cleavage potential of remaining embryos can be established before freezing. A good selection is possible for embryo transfer in the collection cycle and for freezing of supernumerary embryos since the potential of embryos to cleave is ascertained. Consequently, it is often necessary to freeze a cohort of embryos at different developmental stages and of different morphological qualities. As a result, it is more difficult to keep the embryos 100% intact after thawing, and damaged blastomeres coexist with undamaged ones on a regular basis.

An even better selection for embryos to be frozen involves freezing at the blastocyst stage. Blastocysts can lose some trophectoderm cells due to freezing without viability loss (Veiga *et al.*, 1997). There is also a better synchronization of embryonic stage and uterine endometrial development. Recent improvements in culture media make it possible to obtain high rates of blastocysts without co-culture. Transfer of fresh blastocysts seems to produce high implantation rates; hence blastocyst freezing may become the preferred method for preserving human embryos.

When comparing 2PN, multicellular and blastocyst freezing it is difficult to provide conclusive data on the eventual superiority of one of the protocols. It is both clear and important that the cycle cryopreservation rate and the embryo cryopreservation rate will differ depending on whether embryos are frozen at the zygote, multicellular or blastocyst stage. The stage of cryopreservation will also influence the number of embryos that are frozen, as well as the implantation rate per embryo transferred (which varies from 6 to 13%) (Veeck *et al*., 1993; Kaufman *et al*., 1995; Mandelbaum *et al*., 1998; A.van Steirteghem, unpublished data). The reasons for the different cryobiological behaviour between blastocysts obtained using different culture systems need to be clarified (Ménézo *et al*., 2000). Nowadays, fresh blastocyst transfer leading to the transfer of one viable blastocyst or transfer of one top-quality 8-cell embryo to avoid multiple pregnancies at all costs is considered by some patients. Before such a strategy is offered to the patients it must be proven that freezing supernumerary embryos or blastocysts is a reliable and safe procedure.

Follow-up of children after cryopreservation

In the total cohort of children (aged up to 18 months) conceived from cryopreserved embryos, no differences were found in major pathological features compared with children conceived from fresh embryos or with those conceived spontaneously (Wennerholm *et al*., 1997, 1998). Minor handicaps, behavioural disturbances, learning difficulties and dysfunction of attention cannot be ruled out at this age. It has also been shown that cryopreservation of mouse embryos induced some long-term effects (Dulioust *et al*., 1995), although the methodology of this study was criticised. As a result, long-term follow-up studies are needed in order to prove the safety of freezing–thawing processes in IVF.

Health economics of IVF

Health economics comprises more than a simple comparison of costs and effects, although these might be the two factors most easily taken into account. In a economic analysis, differentiation should be made between direct costs (consultations, personnel, equipment, drugs, complications, monitoring frequency) and indirect costs (travel, time off work, lost wages, long-term complications such as OHSS and multiple pregnancies, and obstetric and neonatal care). The effects, in the case of IVF, consist predominantly of outcome (pregnancy or live birth). Apart from these, patient characteristics, the patient's appreciation of a pregnancy and preferences for treatment and emotional distress are important but difficult to weigh (Ryan, 1999). It is even more difficult to include eventual savings and benefits to patients and society into the equation. Well-defined, objective and clinically relevant endpoints (such as singleton live births per started treatment, or per patient) should be employed and the background treatment-independent pregnancy chance should be taken into account.

Direct costs

Direct procedure costs per IVF cycle vary considerably from country to country, and can be high (e.g. $1000 in Ireland, $2500 in Japan, $4000 in Lebanon and $8000 in the USA). No evidence has been found that the success rate of IVF correlates with the cost. In the USA, the cost per delivery has been calculated as $66 667 for the first treatment cycle and $114 286 for the sixth cycle (Neumann *et al*., 1994). In Scandinavia (direct cost per procedure $4000), the cost per delivery is $15 540 (Granberg *et al*., 1998). The calculated direct costs of infertility care in the UK are listed in Table I (Philips *et al*., 2000). It has been shown that if only 6% of all infertile couples required IVF, then in a group of 100 couples the cost of infertility diagnosis would be $76 993, the cost of IVF $71 820, and the combined costs of all other infertility treatments would be $127 991 (Collins *et al*., 1997). Hence, the cost of IVF treatment in six couples among a group of 100 infertile couples is equal to the costs of infertility diagnosis in the entire group.

A full economic evaluation requires a randomized comparison of alternative interventions with respect to both costs and outcomes. Only two such studies have been published. In the first study (Karande *et al*., 1999), IVF was compared with standard therapy and the incremental cost calculated. The procedure cost was $13 255 for IVF and $9557 for standard therapy, and the pregnancy rates were 34.8 and 56.0% per couple respectively. Hence, this study clearly favours the current standard infertility algorithm over IVF, the marginal cost being $17 300 less per additional pregnancy with standard care compared with IVF. In the second study, which was conducted in the Netherlands (Goverde *et al*., 2000), IVF was compared with intra-uterine insemination (IUI) in a spontaneous cycle, and with IUI in a cycle with controlled ovarian stimulation (75 IU FSH). These authors reported procedure costs of $1650, $446 and $298 for IVF, IUI + FSH and IUI respectively. The generalizability of this study has been criticised because the low IVF pregnancy rate (33/270; 12.2%) per cycle, and the high IUI pregnancy rate in spontaneous (i.e. unstimulated) cycles (25/338; 7.4%) appear atypical. The marginal cost per additional pregnancy by IVF over IUI in a spontaneous cycle was $42 972 per couple. This study, with an IVF success rate which is at least realistic in some areas of the spectrum of severity of infertility problems, illustrates that the marginal benefits of IVF over IUI are expensive. The study also shows that IVF in the real world involves patients' choice: although they were offered six cycles of either IUI or IVF free of charge, the patients elected to have only four and three cycles respectively, which impacted on the pregnancy rate per couple.

Table I. Cost of infertility services in the United Kingdom (US$)

Procedure	Cost per cycle	Cost per delivery
Clomiphene	3	18
Surgery	1163	11 634
HMG/IUI	327	1636
Donor insemination	186	2478
IVF/ICSI	1454	4691
IVF	1202	5008

Adapted from Appendix for UK expected treatment costs provided by Philips *et al*. (2000).
IUI = intra-uterine insemination.

Indirect costs

An important part of the indirect costs of IVF is determined by multiple pregnancies. The projected delivery and neonatal costs of multiple pregnancy deliveries in the USA for the year 2000 are $51 715 for twins, $149 598 for triplets and $247 482 for quadruplets, compared with $9000 for singletons. This would lead to $639 million due to multiple pregnancy care compared with $470 million for the combined direct costs of all IVF and ICSI procedures in the USA (Collins and Graves, 2000). If a policy to transfer only two embryos were to be adopted, the total costs would decrease from $1110 million to $863 million ($470 million direct IVF/ICSI costs, $393 million additional multiple pregnancy care costs) and to $470 million if elective single embryo transfer were to be adopted as a general policy. These authors thus demonstrated that the neonatal cost of IVF-associated multiple pregnancy is 36% more than the direct cost of the IVF procedure. The childhood healthcare and educational costs of pre-term birth add to the multiple pregnancy costs. Preventing multiple pregnancies would save enough money to pay for all IVF procedures in the USA.

The relationship between IVF supply and demand depends in part on a hypothetical estimate of the demand or need for IVF cycles. If standard treatment could provide 40% of all infertility couples with a pregnancy, and if 5% would need IVF/ICSI for bilaterally occluded tubes and another 5% for severe oligozoospermia, then 50% would qualify for IVF because of persistent infertility. Furthermore, if 10% of the population consisted of married or cohabiting women aged 18–44 years, and if 10% of these (i.e. 1% of the population) had a fertility problem, then 10 000 women per 1 million population would need infertility diagnosis and treatment. Assuming that only half of them would in fact seek infertility care, this would mean 5000 infertile couples per million population. Of these couples, 2500 would qualify for IVF because of persistent infertility and 500 because of severe tubal or male factor infertility. If only half of these were to accept IVF, the assumed need for IVF resources would be at least 1500 procedures per annum per million population.

Table II. IVF availability: cycles performed per annum per million population

No. of cycles			
<100	100 to 499	500 to 999	>1000
Austria, Egypt, Italy, Kazachstan Latin America, Poland, Russia, Spain Thailand, Turkey	Belgium, Canada, Czech Rep., Germany Greece, Hong Kong, Hungary, Ireland Japan, Korea, Singapore, Slovenia Switzerland, Taiwan, UK, USA	Australia/NZ, Denmark, Finland, France Iceland, Netherlands, Norway, Sweden	Israel

The availability of IVF in cycles per million population per year in 35 countries reporting sufficiently detailed figures to calculate IVF availability is shown in Table II. It is clear that supply and demand of IVF services are not in balance in the vast majority of these countries. There are more IVF centres in countries with higher-quality national health programmes, and in countries with more public spending on healthcare. In many countries the IVF cost per cycle represents a substantial portion of the Gross Domestic Product per capita. Thus, each cycle of IVF represents a large portion of average family income per annum. The average couple would have to spend 10–20% of their annual family income on one or more IVF attempts.

Recommendations

Many challenges to make significant improvements in IVF have appeared to emerge. However, caution should be shown with the premature clinical introduction of new strategies before their efficacy and safety have been established convincingly. The following statements may summarize different topics covered in the current review:

1. The improved understanding of mechanisms regulating early follicle development may give rise to refined, individualized ovarian stimulation protocols. Eventually, ovarian stimulation may be replaced by the in-vitro maturation of oocytes. However, series published to date are extremely small, and the safety of this procedure remains to be established.

2. Implantation rates per embryo transferred and resulting clinical pregnancy rates after IVF are still far from optimal. The evaluation of embryo quality may improve with extended embryo culture, but further studies in unselected patient groups are urgently needed.

3. Subtle ovarian stimulation protocols along with a reduction in the number of embryos transferred also seem crucial steps for the improvement of efficacy and safety of IVF. Moreover, the exact role of embryo cryopreservation in enhancing the total reproductive potential of a single treatment cycle should be assessed in a more objective and reproducible manner.

4. Caution should be shown in the interpretation of clinical studies employing surrogate endpoint parameters such as the number of oocytes retrieved or implantation rates per embryo transferred. IVF outcome should be expressed as (singleton) live births per started treatment, and the study group involved should be representative and clearly described. There is a great need for randomized controlled trials to compare outcome and costs of standard IVF with alternative interventions in a full economic evaluation.

5. In developed nations, IVF utilization is far below the estimated need among infertile couples, principally because of the high cost. The major cost components are the price of ovarian stimulation drugs, cycle monitoring and multiple pregnancy deliveries. More conservative ovarian stimulation drugs and restrained embryo transfer protocols would reduce the overall cost of IVF and facilitate the extension of this substantial health benefit to more infertile couples in all countries.

References

Behr, B. (1999) Blastocyst culture and transfer. *Hum. Reprod.*, **14**, 5–6.

Bouchard, B. and Fauser, B.C. (2000) Gonadotropin-releasing hormone antagonist: new tools vs. old habits. *Fertil. Steril.*, **73**, 18–20.

Bouloux, P.M., Handelsman, D.J., Jockenhovel, F. *et al.* (2001) First human exposure to FSH-CTP in hypogonadotrophic hypogonadal males. *Hum. Reprod.*, **16**, 1592–1597.

Byskov, A.G., Andersen, C.Y., Nordholm, L. *et al.* (1995) Chemical structure of sterols that activate oocyte meiosis. *Nature*, **374**, 559–562.

Cha, K.Y., Han, S.Y., Chung, H.M. *et al.* (2000) Pregnancies and deliveries after *in vitro* maturation culture followed by *in vitro* fertilization and embryo transfer without stimulation in women with polycystic ovary syndrome. *Fertil. Steril.*, **73**, 978–983.

Chen, S.U., Lein, Y.R., Chao, K.H. *et al.* (2000) Cryopreservation of mature human oocytes by vitrification with ethylene glycol in straws. *Fertil. Steril.*, **74**, 804–808.

Collins, J.A. and Graves, G. (2000) The economic consequences of multiple gestation pregnancy in assisted conception cycles. *Hum. Fertil.*, **3**, 275–283.

Collins, J.A., Feeny, D. and Gunby, J. (1997) The cost of infertility diagnosis and treatment in Canada in 1995. *Hum. Reprod.*, **12**, 951–958.

Da Costa, A.L., Abdelmassih, S., de Oliviera, F.G. *et al.* (2001) Monozygotic twins and transfer at the blastocyst stage after ICSI. *Hum. Reprod.*, **16**, 333–336.

Daya, S. (2000) GnRH agonist protocols for pituitary desensitization in *in vitro* fertilization and gamete intrafallopian transfer cycles. *Cochrane Database Syst. Rev.*, CD001299

Daya, S. and Gunby, B. (1999) Recombinant versus urinary FSH for ovarian stimulation in assisted reproduction. *Hum. Reprod.*, **14**, 2207–2215.

De Jong, D., Macklon, N.S., Mannaerts, B.M., Coelingh Bennink, H.J. and Fauser, B.C. (1998) High dose GnRH antagonist may prevent OHSS caused by ovarian stimulation for IVF. *Hum. Reprod.*, **13**, 573–575.

Dong, J., Albertini, D.F., Nishimori, K. *et al.* (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, **383**, 531–353.

Dulioust, E., Toyama, K., Busnel, M. *et al.* (1995) Long-term effects of embryo freezing in mice. *Proc. Natl Acad. Sci. USA*, **92**, 589–593.

Durlinger, A.L., Kramer, P., Karels, B. *et al.* (1999) Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse. *Endocrinology*, **140**, 5789–5796.

Fauser, B.C. and van Heusden, A.M. (1997) Manipulation of human ovarian function: physiological concepts and clinical consequences. *Endocr. Rev.*, **18**, 71–106.

Fauser, B.C., Devroey, P., Yen, S.S. *et al.* (1999) Minimal ovarian stimulation for IVF: appraisal of potential benefits and drawbacks. *Hum. Reprod.*, **11**, 2681–2686.

Filicori, M. and Cognigni, G.E. (2001) Clinical review 126: roles and novel regimens of luteinizing hormone and follicle-stimulating hormone in ovulation induction. *J. Clin. Endocrinol. Metab.*, **86**, 1437–1441.

Galloway, S.M., McNatty, K.P., Cambridge, L.M. *et al*. (2000) Mutation in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genet.*, **25**, 279–283.

Gardner, D.K., Schoolcraft, W.B., Wagley, L. *et al.* (1998) A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum. Reprod.*, **13**, 3434–3440.

Gardner, D.K., Lane, M., Stevens, J. *et al.* (2000) Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil. Steril.*, **73**, 1155–1158.

Gianaroli, L., Magli, M.C. and Ferraretti, A.P. (2001) The *in vivo* and *in vitro* efficiency and efficacy of PGD for aneuploidy. *Mol. Cell. Endocrinol*., **183** (Suppl.), S13–S18.

Goverde, A.J., McDonnell, J., Vermeiden, J. *et al.* (2000) Intrauterine insemination or in-vitro fertilisation in idiopathic subfertility and male subfertility: a randomised trial and cost-effectiveness analysis. *Lancet*, **1**, 13–18.

Granberg, M., Wikland, M. and Hamberger L. (1998) Financing of IVF/ET in the Nordic countries. *Acta Obstet. Gynecol. Scand.*, **77**, 63–67.

Hsueh, A.J., McGee, E.A., Hayashi, M. *et al.* (2000) Hormonal regulation of early follicle development in the rat ovary. *Mol. Cell. Endocrinol.*, **163**, 95–100.

Itskovitz, J., Boldes, R., Levron, J., Yohanan, E., Kahana, L. and Brandes, J. (1991) Induction of preovulatory LH surge and prevention of ovarian hyperstimulation syndrome by GnRH agonist. *Fertil. Steril*., **56**, 213–220.

Jones, G.M. and Trounson, A.O. (1999) Blastocyst stage transfer: pitfalls and benefits. The benefits of extended culture. *Hum. Reprod.*, **14**, 1405–1408.

Jones, H., Veeck, L. and Muascher, S. (1995) Cryopreservation: the problem of evaluation. *Hum. Reprod.*, **10**, 2136–2138.

Karande, V.C., Korn, A., Morris, R.N., Rao, R., Balin, M., Rinehart, J., Dohn, K. and Gleicher, N. (1999) Prospective randomized trial comparing the outcome and cost of *in vitro* fertilization with that of a traditional treatment algorithm as first-line therapy for couples with infertility. *Fertil. Steril.*, **71**, 468–475.

Kaufman, R., Ménézo, Y., Hazout, A. *et al.* (1995) Cocultured blastocyst cryopreservation: experience of more than 500 transfer cycles. *Fertil. Steril.*, **64**, 1125–1129.

Layman, L.C. and McDonough, P.G. (2000) Mutations of follicle-stimulating hormone-beta and its receptors in human and mouse: genotype/phenotype. *Mol. Cell. Endocrinol*., **161**, 9–17.

Le Gal, F. and Massip, A. (1999) Cryopreservation of cattle oocytes: effects of meiotic stage, cycloheximide treatment, and vitrification procedure. *Cryobiology*, **38**, 290–300.

Loumaye, E., Piazzi, A. and Engrand, P. (1999) The use of rec LH, GnRH agonist or hCG to trigger ovulation. In: Shoham, Z., Collins, C.M. and Jones, H.S. (eds), *Female Infertility Therapy Current Practice*. Martin Dunitz, London, pp. 125–135.

Mandelbaum, J., Belaisch-Allart, J., Junca, A. *et al*. (1998) Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. *Hum. Reprod.*, **13**, 161–177.

Matzuk, M.M. (2000) Revelations of ovarian follicle biology from gene knock out mice. *Mol. Cell. Endocrinol*., **163**, 61–66.

McGee, E.A. and Hsueh, A.J. (2000) Initial and cyclic recruitment of ovarian follicles. *Endocr. Rev.*, **21**, 200–214.

McNatty, K.P., Heath, D.A., Lundy, T. *et al*. (1999) Control of ovarian follicular development. *J. Reprod. Fertil*., **54**, 3–16.

Ménézo, Y., Veiga, A. and Pouly, J. (2000) Assisted reproductive technology (ART) in humans: facts and uncertainties. *Theriogenology*, **53**, 599–610.

Morita, Y., Perez, G.I., Maravei, D.V. *et al.* (1999) Targeted expression of Bcl-2 in mouse oocytes inhibits ovarian follicle atresia and prevents spontaneous and chemotherapy-induced oocyte apoptosis *in vitro*. *Mol. Endocrinol.*, **13**, 841–850.

Munné, S. and Cohen, J. (1998) Chromosome abnormalities in human embryos. *Hum. Reprod. Update*, **4**, 842–855.

Neumann, P.J., Gharib, S.D. and Weinstein, M.C. (1994) The cost of a successful delivery with *in vitro* fertilization, *N. Engl. J. Med.*, **331**, 239–243.

Nogueira, D., Staessen, C., Van de Velde, H. *et al.* (2000) Nuclear status and cytogenetics of embryos derived from in vitro-matured oocytes. *Fertil. Steril.*, **74**, 295–298.

Otsuka, F., Moore, R.K. and Shimasaki, S. (2001) Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary. *J. Biol. Chem.*, **276**, 32889–32895.

Perez, G.I., Robles, R., Knudson, C.M. *et al*. (1999) Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. *Nature Genet.*, **21**, 200–203.

Philips, Z., Barraza-Llorens, M. and Posnett, J. (2000) Evaluation of the relative cost-effectiveness of treatments for infertility in the UK. *Hum. Reprod.*, **15**, 95–106.

Plachot, M., de Grouchy, J., Junca, A.M. *et al.* (1987) From oocyte to embryo: a model, deduced from *in vitro* fertilization, for natural selection against chromosome abnormalities. *Ann. Genet.*, **30**, 22–32.

Rijnders, P.M. and Jansen, C.A. (1998) The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. *Hum. Reprod.*, **13**, 2869–2873.

Ryan, M. (1999) Using conjoint analysis to take account of patient preferences and go beyond health outcomes: an application to *in vitro* fertilization. *Social Sci. Med.*, **48**, 535–546.

Sandalinas, M., Sadowy, S., Alikani, M., Calderon, G., Cohen, J. and Munné, S. (2001) Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. *Hum. Reprod*., **16**, 1954–1958.

Shaw, J.M., Bowles, J., Koopman, P., Wood, E.G. and Trounson, A.O. (1996) Fresh and cryopreserved ovarian tissue samples from donors with lymphoma transmit the cancer to graft recipients. *Hum. Reprod*., **11**, 1668–1673.

Shaw, J., Oranratnachai, A. and Trounson, A. (2000) Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology*, **53**, 59–72.

Sheiner, E., Har-Vardi, I. and Potashnik, G. (2001) The potential association between blastocyst transfer and monozygotic twinning. *Fertil. Steril*., **75**, 217–218.

Sinclair, K.D., Young, L.E., Wilmut, I. and McEvoy, T.G. (2000) In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. *Hum. Reprod*., **15** (Suppl. 5), 68–86.

Smitz, J., Cortvrindt, R. and Hu, Y. (1998) Epidermal growth factor combined with recombinant human chorionic gonadotrophin improves meiotic progression in mouse follicle-enclosed oocyte culture. *Hum. Reprod.*, **13**, 664–669.

Sullivan, M.W., Stewart, E., Krasnow, J.S. *et al*. (1999) Ovarian response in women to recombinant follicle stimulating hormone and luteinizing hormone: a role for LH in the final stages of follicular maturation. *J. Clin. Endocrinol. Metab*., **84**, 228–232.

Testart, J. (1988) Results of in-vitro fertilisation with embryo cryopreservation and a recommendation for uniform reporting. *Fertil. Steril.*, **49**, 156–158.

Van Royen, E., Mangelschot, K., De Neubourg, D., Laureys, I., Ryckaert, G. and Gerris, J. (2001) Calculating the implantation potential of day 3 embryos in women younger than 38 years of age: a new model. *Hum. Reprod.*, **16**, 326–332.

Veeck, L., Amundsen, C., Brothman, L. *et al.* (1993) Significantly enhanced pregnancy rates through cryopreservation and thaw of pronuclear stage oocytes. *Fertil. Steril.*, **59**, 1202–1207.

Veiga, A., Sandalinas, M., Benkhalifa, M. *et al.* (1997) Laser blastocyst biopsy for preimplantation diagnosis in the human. *Zygote*, **5**, 351–354.

Vendola, K., Zhou, J., Wang, J., Famuyiwa, O.A., Bievre, M. and Bondy, C.A. (1999) Androgens promote insulin-like growth factor I expression and initiation of follicle development in the primate ovary. *Biol. Reprod*., **61**, 353–357.

Wennerholm, U., Hamberger, L. Nilsson, L. *et al.* (1997) Obstetric and perinatal outcome of children conceived from cryopreserved embryos. *Hum. Reprod.*, **12**, 1819–1825.

Wennerholm, U., Albertsom-Wikland, K., Bergh, C. *et al.* (1998) Postnatal growth and health of children born after cryopreservation as embryos. *Lancet*, **351**, 1085–1090.

Westergaard, L.G., Laursen, S.B. and Yding Andersen, C. (2000) Increased risk of early pregnancy loss by profound suppression of luteinizing hormone during ovarian stimulation in normogonadotrophic women undergoing assisted reproduction. *Hum. Reprod.*, **15**, 1003–1008.

Yan, C., Wang, P., DeMayo, J. *et al*. (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol. Endocrinol*., **15**, 854–866.

Young, C.E., Fernandez, K., McEvoy, T.G. *et al*. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genet*., **27**, 153–154.

Received on June 18, 2001; accepted on October 12 ,2001