Future developments in assisted reproduction in humans*

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The advent of human *in vitro* fertilization (IVF) over 30 years ago has made the oocyte and preimplantation embryo uniquely accessible. This accessibility has given rise to new micromanipulation techniques, such as intracytoplasmic sperm injection for treatment of male infertility, as well as embryo biopsy for preimplantation diagnosis of both genetic disease and aneuploidy, a major cause of early embryo demise and miscarriage. In the UK, average pregnancy rates after IVF and embryo transfer are < 25%, even after transfer of several embryos. Unfortunately, a third of these pregnancies involve multiple gestations. Research is currently focusing on methods to improve IVF success rates while reducing twin and triplet pregnancies and their associated increased morbidity and mortality. One approach is to develop screening methods to identify the most viable embryos, so that transfer of fewer healthy embryos will result in a higher proportion of singleton pregnancies. Screening methods include optimizing culture conditions for prolonged culture and selection of viable blastocysts for transfer, or embryo biopsy and aneuploidy screening. Assisted reproduction is also increasingly important in other branches of medicine: survival rates for cancer sufferers are improving continually and there is now a significant need for approaches to preserve fertility after sterilizing chemo- and radiotherapy treatment. Techniques for cryopreserving male and female gametes or gonadal tissue are being developed, although systems to grow and mature these gametes are in their infancy. Finally, there are also concerns regarding the safety of these new assisted reproductive technologies.

It is estimated that about one million babies have been born world-wide as a result of *in vitro* fertilization (IVF) and embryo transfer (K. Nygren, personal communication). The last three decades have witnessed the development of revolutionary techniques to overcome infertility, starting with the successful fertilization of human oocytes *in vitro* (Edwards *et al.*, 1969) and followed nearly 10 years later by the birth of the first 'test tube' baby after transfer of an embryo fertilized *in vitro* (Steptoe and Edwards, 1978). The accessibility of viable gametes and embryos *in vitro* has led to a number of new developments in assisted reproduction, including cryopreservation and storage of embryos for later transfer (Mohr and Trounson, 1985), fertilization of oocytes with a single injected spermatozoon to alleviate severe male infertility (Palermo *et al.*, 1992) and diagnosis of genetic defects before implantation (Handyside *et al.*, 1990).

However, despite the fact that IVF is now a well-established treatment for infertility, success rates remain low, with only about 23% of women who undergo treatment becoming pregnant (HFEA, 2000). A large proportion of embryo loss appears to occur during preimplantation stages with only 50% of embryos cultured *in vitro* reaching the blastocyst stage by day 6 (Hardy, 1993; Hardy *et al.*, 2001), and < 15% of transferred embryos developing into a baby (HFEA, 2000) (Fig. 1). Several embryos are generally transferred to improve pregnancy rates, but this has resulted in an unacceptably high rate of multiple gestation (27% of pregnancies (HFEA, 2000)). Concerns persist regarding the safety of intracytoplasmic sperm injection (ICSI). Furthermore, there are still no satisfactory approaches, apart from gamete donation, to restore fertility for men and women who undergo sterilizing cancer treatments during childhood and early adulthood or for women who suffer a premature menopause. Life expectancy is increasing and it is predicted that many women will live for 40 or more years beyond the menopause, with age-related sub-fertility beginning during their late thirties.

Efforts are being made to improve implantation rates after

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Fig. 1. Decreasing survival of human embryos after IVF and embryo transfer. (Data from Dawson *et al.*, 1995; Hardy *et al.*, 2000; HFEA, 2000.)

Fig. 2. Strategies for improving success rates after IVF.

IVF by improving culture conditions, optimizing gamete quality and developing new techniques of selecting viable embryos for transfer (Fig. 2). These efforts should lead to the transfer of fewer embryos and fewer multiple gestations without compromising pregnancy rates. The decline in IVF success rates with age is thought to result mainly from an increased incidence of age-related aneuploidy in the oocyte (Eichenlaub-Ritter, 1998). Screening of embryos for

aneuploidy using techniques developed for the preimplantation diagnosis of genetic disease is a promising approach for improving pregnancy rates for older women. In addition, efforts are being made to cryopreserve and store viable gametes, either in isolation or within gonadal tissue. The combination of improved cryopreservation and storage techniques with either autotransplantation of gonadal tissue or *in vitro* maturation of gametes will provide means by which men and women undergoing cancer treatment can preserve some degree of fertility.

Improving success of IVF

Embryo culture

Over the past decade there has been considerable interest in optimizing culture media for supporting human embryos after IVF and before embryo transfer (Fig. 2). Improvements in blastocyst formation and number of cells have been achieved by reducing glucose concentrations (Conaghan *et al.*, 1993), adding amino acids (Devreker *et al.*, 1998) and supplementing media with growth factors (Lighten *et al.*, 1998; Martin *et al.*, 1998; Sjoblom *et al.*, 1999; Spanos *et al.*, 2000). There have been no large prospective randomized studies on the effects of culture media on outcome after IVF, apart from one which reported that although glucose-free medium improved embryo quality, pregnancy rates were not increased (Coates *et al.*, 1999).

During preimplantation human development, the nutritional requirements of the embryo change from a predominant need for pyruvate during early cleavage to a high uptake of both pyruvate and glucose at the blastocyst stage (Hardy *et al.*, 1989). The changing nutritional requirements of the embryo coupled with the changing *in vivo* environment as it moves from the Fallopian tube to the uterus have led to the development of two-step or sequential culture media for the culture of blastocysts (Gardner *et al.*, 1998). These new culture media have allowed the development of new embryo selection techniques.

Embryo selection: blastocyst transfer

One approach to increasing pregnancy rates is to improve the selection of viable embryos. Currently embryo transfer in most units is carried out on day 2 or 3 after IVF, at the two- to eight-cell stage. Embryos are selected on the basis of morphology and rate of development, and the fastest developing embryos of the best morphology are selected for transfer. Although there is some correlation between morphology and blastocyst formation (Hardy, 1999) and implantation (Giorgetti *et al.*, 1995), selection on the basis of morphology and rate of development remains an unsatisfactory and imprecise method of selecting viable embryos. It is impossible to identify visually with certainty which embryos at early cleavage stages will subsequently arrest. One approach is to culture embryos to the blastocyst

stage and transfer them on day 5 or 6, allowing the identification of developing embryos (Fig. 2) and the synchronous transfer of blastocysts, rather than cleavage stage embryos, to the uterus. It had been hoped also that, during extended culture, the chromosomally abnormal embryos so common at early cleavage stages (Jamieson *et al.*, 1994; Munné *et al.*, 1995) would arrest and fail to complete preimplantation development. However, although few human blastocysts have total chromosomal abnormality, a large number of blastocysts have various proportions of abnormal cells and are mosaic (Evsikov and Verlinsky, 1998; Ruangvutitert *et al.*, 2000).

Results of blastocyst transfer have been variable, with some centres reporting implantation and pregnancy rates in excess of 45 (Gardner *et al.*, 1998) and 65% (Milki *et al.*, 2000). However, a large prospective randomized study of 1299 unselected patients reported pregnancy rates of 25 and 28% on day 3 and day 5, respectively, for all embryos transferred. Blastocysts transferred on day 5 had an implantation rate of 26% per embryo, compared with 18% for eight-cell embryos transferred on day 3 (Huisman *et al.*, 2000).

The observation of large offspring syndrome in domestic species (Young *et al.*, 1998) and the effects of culture on gene expression (Ho *et al.*, 1995; Niemann and Wrenzycki, 2000) and imprinting (Doherty *et al.*, 2000; Khosla *et al.*, 2001) in rodent and bovine embryos has increased concerns about the possible deleterious effects of extended culture on fetal and adult health in humans (Leese *et al.*, 1998). However, a recent retrospective study found no effect of human blastocyst culture on birth weight, but did find an effect on the sex of the offspring, with an increase in the proportion of males (Menezo *et al.*, 1999). This finding may be the result of the faster development of male human embryos (Ray *et al.*, 1995) skewing the sex ratio of blastocysts on day 5, when the transfers took place.

Embryo screening: preimplantation genetic diagnosis (PGD)

Preimplantation genetic diagnosis (PGD) allows the diagnosis of a genetic disorder in an embryo before its implantation in the uterus. PGD is of benefit to couples at risk of passing on a genetic disease to their offspring. Conventional prenatal diagnosis is carried out between week 12 and week 16 of pregnancy by chorionic villus sampling or amniocentesis. If the fetus is affected, couples have to consider terminating the pregnancy. An alternative approach is to remove one or two blastomeres from an eight-cell stage embryo after IVF (Fig. 3), analyse the blastomeres using fluorescent *in situ* hybridization (FISH) or PCR, and identify affected embryos. As only unaffected embryos are transferred back to the uterus after PGD, termination can be avoided. The first births arising from the application of this technique occurred in 1990 (Handyside *et al.*, 1990) and there are currently over 150 unaffected children born after PGD (ESHRE PGD Consortium, 2000).

Fig. 3. (a) Photomicrograph of intracytoplasmic sperm injection (ICSI). An unfertilized human oocyte is immobilized with a holding pipette (right) and injected with a single spermatozoon. (b) Photomicrograph of embryo biopsy. An eight-cell human embryo is held by gentle suction with a holding pipette (right). A single cell is aspirated through a hole in the zona pellucida for analysis. (Photographs courtesy of Ben Lavender, Wolfson Family Clinic, Hammersmith Hospital.)

FISH allows the detection of specific chromosomes in an intact interphase or metaphase nucleus and can be used to identify the sex of an embryo (which is important for X-chromosome-linked diseases) (Delhanty *et al.*, 1994; Staessen *et al.*, 1999), and detect chromosomal aneuploidies, such as Down syndrome, and major chromosomal aberrations such as translocations (Scriven *et al.*, 1998; Conn *et al.*, 1999). Single gene defects can be diagnosed by amplifying specific gene sequences using PCR (Handyside and Delhanty, 1997). Despite the exquisite sensitivity of single-cell PCR protocols used for PGD, accurate and reliable diagnosis can be hampered by amplification failure, contamination and allele drop out, and therefore new PCR strategies are continually being evaluated.

A large number of oocytes need to be retrieved for a successful PGD cycle, as not all will fertilize, cleave, undergo successful biopsy and be identified as suitable for transfer (Fig. 4). Most embryos (96%) survive biopsy. Pregnancy rates after transfer of biopsied embryos (16% per treatment cycle; ESHRE PGD Consortium, 2000) are less than those after conventional IVF (23% per cycle; HFEA, 2000).

Fig. 4. Percentage of oocytes retrieved for preimplantation genetic diagnosis that are at metaphase II, undergo fertilization, cleave, have an informative diagnosis made and are suitable for transfer. COC: cumulus–oocyte complex. (Data from European Society for Human Reproduction and Embryology, 2000.)

Since this technique was first used clinically (Handyside *et al.*, 1990), closer examination of human embryos has shown that intercellular variability within the embryo is common, with both normal and abnormal cells in close proximity. About 60% of day 4 human embryos have one or more binucleate cells (Hardy *et al.*, 1993), and about 40% of embryos have chromosomally abnormal cells, which are haploid, polyploid or aneuploid (Munné *et al.*, 1994; Harper *et al.*, 1995). This mosaicism has serious implications for PGD, with the risk that the cell removed by embryo biopsy may not be representative of the rest of the embryo (Handyside and Delhanty, 1997). Many groups take the extra precaution of removing two cells from an eight-cell embryo to confirm the diagnosis. However, it is important to remember that > 50% of PGD cases are carried out for sex-linked diseases, and are diagnosed on the basis of sex (ESHRE PGD Consortium, 2000). In these cases, mosaicism is unlikely to result in misdiagnosis, as an embryo is either male or female. In general, FISH is carried out using a mixture of probes, and two X signals or one Y and one X signal (in addition to confirmation of ploidy with an autosomal signal) must be observed to diagnose sex accurately. Unfortunately, four misdiagnoses by PCR of monogenic disorders have been reported (3.4%; ESHRE PGD Consortium, 2000).

The possibility of diagnosing defects by analysing the gene product (mRNA) rather than the gene itself is being investigated. This investigation involves developing highly sensitive RT–PCR techniques to analyse RNA from single blastomeres. Ultimately, it is hoped that a sample of cytoplasm, rather than whole blastomeres, will provide enough mRNA to identify affected embryos (Steuerwald *et al.*, 2000). However, persistence of maternal oocyte RNA beyond the time when the embryonic genome is switched on may confound the diagnosis, and this technique is still in its infancy (Taylor *et al.*, 1997).

Embryo screening for aneuploidies

The incidence of chromosomal abnormalities in spontaneous abortions up to 7 weeks is > 60%, significantly greater than the 3–9% found in induced abortions of a similar age (Boué *et al.*, 1985). This finding indicates that embryo aneuploidy is a major contributor to implantation failure and hence low IVF success rates. Around 20% of morphologically normal human embryos developing *in vitro* have gross chromosomal abnormalities (Jamieson *et al.*, 1994) and this contributes to preimplantation embryo arrest, as about 70% of arrested embryos are abnormal (Munné *et al.*, 1995). Furthermore, the incidence of

aneuploid embryos increases with increasing maternal age (Munné *et al.*, 1995). In an attempt to improve implantation rates, there is growing interest in the possibility of screening embryos before transfer, particularly those from older patients and women with recurrent IVF failures or recurrent miscarriage. The use of embryo biopsy in conjunction with FISH has resulted in improved pregnancy rates (Gianaroli *et al.*, 1999a; Munné *et al.*, 1999).

Although FISH has the great advantage of providing information about interphase nuclei, only a limited number of chromosomes can be analysed at any one time. The most effective way of obtaining complete chromosome information from any cell is by karyotyping nuclei in metaphase. Until recently, conventional methods of obtaining metaphase spreads from single blastomeres were technically demanding, resulting in a small proportion of analysable metaphases. However, fusion of the blastomere with an intact or enucleated mammalian oocyte can produce readable metaphases in > 90% of cases (Willadsen *et al.*, 1999). With the advent of spectral imaging, analysis of all 23 chromosomes present in a single cell or polar body can be achieved (Marquez *et al.*, 1998). This technology should allow both numerical and structural chromosomal abnormalities to be detected without the need to produce patient-specific probes. An alternative approach of whole genome amplification and comparative genomic hybridization has been applied successfully to single blastomeres (Wells and Delhanty, 2000), and allows the detection of chromosome copy number and structural aberrations for all the chromosomes and identification of aneuploid embryos.

As with PGD, a serious problem with the above approaches is how representative removed cells are of the embryo as a whole. Approximately 40% of human embryos are mosaic (Munné *et al.*, 1994, 1995; Harper *et al.*, 1995), containing a mixture of normal diploid blastomeres and abnormal aneuploid blastomeres. The fact that in these cases a proportion, rather than all, of blastomeres have abnormal chromosomal complements indicates that these abnormalities are the result of non-disjunction during embryogenesis, rather than during meiosis I (Handyside and Delhanty, 1997). This non-disjunction may be the result of the failure of the cell cycle checkpoint mechanisms (Hunt, 1998) that ensure the division of each blastomere into two genetically identical daughter cells. If cell cycle checkpoint genes are expressed embryonically, the initial cleavage divisions may be more prone to error, as the embryonic genome does not become activated until the four- to eight-cell stage in humans (Braude *et al.*, 1988; Tesarik *et al.*, 1988), which could explain the high incidence of aneuploidy during early cleavage. More research is required regarding the fate of these cells as it is not known when or how they are excluded from development.

Improving success: oocyte quality

Van Blerkom *et al.* (1998) have suggested that the developmental competence of mouse and human early

embryos is related to the metabolic capacity of the mitochondria. It is thought that mitochondrial replication does not begin until after implantation, and that paternal contribution is minimal, and therefore the preimplantation embryo is completely reliant on maternally inherited mitochondria in the oocyte (Van Blerkom *et al.*, 1998). Deletions and mutations in oocyte mtDNA may lead to mitochondrial dysfunction, influencing energy production and apoptosis in oocytes and early embryos, resulting in aberrant chromosomal segregation or developmental arrest (Van Blerkom *et al.*, 1998).

Cytoplasmic transfer from metaphase II donor oocytes to mature recipient oocytes from women with recurrent IVF failure has been carried out in an attempt to restore normal growth in developmentally compromised oocytes and embryos (Cohen *et al.*, 1998). The first birth after transfer of anucleate donor oocyte cytoplasm into recipient eggs was reported by Cohen *et al.* (1997) and there have been over 30 births worldwide since then (Barritt *et al.*, 2001). Concerns regarding the safety of this technique have been raised after the report that 2 of 17 fetuses in one series had an abnormal 45,XO karyotype (Barritt *et al.*, 2001). Furthermore, the developing embryo would be a recipient of three genomes, the nuclear complements from the mother and father and some or all of the mitochondrial complement from the donor. In addition, donor oocytes may carry mtDNA rearrangements and should be screened (St John and Barratt, 1997). Certainly, more experimental work is required before nuclear and cytoplasmic transfer can be used routinely in clinical practice.

Nuclear transfer has also been proposed for the treatment of mitochondrial disease (Tesarík *et al.*, 2000) whereby a karyoplast containing metaphase II chromosomes from women with repeated failures of embryo development due to defective oocyte cytoplasm is fused to enucleated donor oocytes.

Preservation and restoration of male fertility

Intracytoplasmic sperm injection (ICSI)

The use of small numbers of spermatozoa during IVF results in fertilization failure. In these cases, it is possible to inject a single spermatozoon into the ooplasm of a mature metaphase II oocyte, a procedure known as intracytoplasmic sperm injection (ICSI) (Fig. 3). The first successful pregnancies were in the early 1990s (Palermo *et al.*, 1992). Since then, the technique has become a widely accepted treatment for couples with severe male factor infertility.

If no spermatozoa are present in the ejaculate, it is possible to aspirate spermatozoa from the epididymis (microsurgical epididymal sperm aspiration, MESA) or directly from the testis (testicular sperm extraction, TESE). In conjunction with ICSI, MESA and TESE have allowed men with obstructive and non-obstructive azoospermia (that is, congenital absence of the vas deferens or defects in sperm maturation) to father children (Silber *et al.*, 1994, 1995).

ICSI of ejaculated, epididymal and testicular spermatozoa yields similar fertilization (approximately 60%), cleavage and ongoing pregnancy (approximately 20%) rates (Silber *et al.*, 1995; Tarlatzis and Bili, 2000).

In cases in which mature spermatozoa cannot be retrieved, injection of immature spermatids (Fishel *et al.*, 1995; Tesarik *et al.*, 1995) and spermatocytes (Sofikitis *et al.*, 1998) has resulted in pregnancies. Success rates after ICSI with round spermatids are low, probably because a high proportion of germ cells from men with severe testicular failure are apoptotic and have damaged DNA (Tesarik *et al.*, 1998). *In vitro* maturation of germ cells would allow the identification of undamaged cells for ICSI (for a review, see Tesarik and Mendoza, 1999) and may also be necessary for successful fertilization.

ICSI – risks and dangers

Although ICSI is now used widely as a treatment for male infertility, concerns about its safety and the long-term effects on offspring remain. ICSI was developed directly in humans. Basic research in other species, such as rhesus monkeys, is only now being undertaken and has already highlighted abnormalities of nuclear remodelling and sperm decondensation after ICSI (Hewitson *et al.*, 2000). ICSI bypasses many potential control points in the fertilization process such as sperm binding to and penetration of the zona pellucida and sperm fusion with the oolemma, allowing fertilization by spermatozoa with poor motility or abnormal morphology.

After ICSI, there is a slightly higher frequency of sex chromosome aberrations (Bonduelle *et al.*, 1998a; Tarlatzis and Bili, 2000). The procedure may disturb the maternal meiotic spindle or the condensation of paternal chromosomes, leading to chromosome non-disjunction. The higher prevalence of abnormal karyotypes in infertile men (Meschede *et al.*, 1998) raises concerns about the danger of transmitting traits responsible for male infertility. There is also debate about evidence of postnatal developmental delays in children conceived using ICSI (Bowen *et al.*, 1998; Bonduelle *et al.*, 1998b).

The increasing use of immature spermatozoa, spermatids and spermatocytes for ICSI, with reports of two cases of congenital abnormalities (Zech *et al.*, 2000), emphasizes the importance of careful investigations into gene expression and genomic imprinting during spermatogenesis, especially in infertile patients.

Sperm cryopreservation

Human sperm cryopreservation has a well-established history and is used routinely for donor and IVF programmes. However, cryopreservation destroys a major proportion (approximately 50%) of spermatozoa in a typical semen sample and decreases sperm transport and survival, so more frozen–thawed than fresh spermatozoa are required for successful insemination (Holt, 2000). Therefore, sperm cryopreservation for men with low numbers of spermatozoa

and poor sperm quality is an unsatisfactory option, although the development of ICSI has overcome this problem to a large extent.

Recent studies have shown that similar fertilization rates (approximately 60%) and pregnancy rates are obtained after ICSI with frozen–thawed epididymal (Tournaye *et al.*, 1999) and testicular (Habermann *et al.*, 2000) spermatozoa. Human spermatids from testicular biopsies have been cryopreserved, thawed and used for ICSI with the subsequent birth of a healthy baby (Gianaroli *et al.*, 1999b).

In vitro maturation and transplantation of human spermatozoa

Chemotherapy or radiotherapy can result in long-term or permanent azoospermia. Currently, men who undergo such treatments are offered the chance to cryopreserve and store spermatozoa. In prepubertal boys awaiting cancer treatment, it is not possible to obtain an ejaculated sperm sample, and cryopreservation of a testicular biopsy may be the only method of preserving fertility (Hovatta, 2001). After recovery, autologous germ cell transplantation back to the adult testis will hopefully result in reinitiation of spermatogenesis and may be a useful clinical approach to preserving fertility after cancer treatment. Heterogeneous mouse testicular cells have been transplanted successfully into genetically sterile mice (Brinster and Zimmermann, 1994) and it is even possible to mature spermatozoa from one species in the testis of another species (Clouthier *et al.*, 1996). Mouse germ cells have been transplanted successfully after culture (Brinster and Nagano, 1998) and cryopreservation (Avarbock *et al.*, 1996). Finally, testicular cells, including germ cells, have been injected successfully into the primate testis *in vivo*, with the result that transplanted germ cells are present 4 weeks after transfer (Schlatt *et al.*, 1999).

Preservation and restoration of female fertility

Embryo cryopreservation

The increased incidence of multiple pregnancy after IVF has led many centres to limit the number of embryos transferred. Embryo cryopreservation (Fig. 5) allows supernumerary embryos produced after IVF to be stored, thawed and transferred during later cycles, resulting in live birth rates of about 12% (HFEA, 2000). In a different context, long-term survival rates for young cancer patients have improved significantly, resulting in a need to preserve their fertility (Donnez and Bassil, 1998). Although it is possible to store embryos before cancer treatment, this involves a potentially dangerous delay of approximately 6 weeks (Newton, 1998). Furthermore, there are a number of situations in which embryo cryopreservation is not appropriate, for example, it is not an option for children and may be unacceptable for young women without a partner. For these women, an alternative is cyopreservation of oocytes or ovarian tissue (Fig. 5).

Oocyte cryopreservation

Chen (1986) reported the first pregnancy after cryopreservation of mature oocytes. However, the success rates using this approach have remained very low. Survival of oocytes after thawing is poor and cryopreserved oocytes show zona hardening because of premature cortical granule release (Trounson and Kirby, 1989). Although zona hardening can be overcome with ICSI or zona drilling, there is worrying evidence of increased aneuploidy in frozen-thawed oocytes. Mature oocytes are arrested in metaphase of the second meiotic division (MII), and increased aneuploidy is thought to be the result of depolymerization and reassembly of the meiotic spindle and associated chromosome loss during cooling and warming (Pickering *et al.*, 1990).

An alternative approach to oocyte preservation is to cryopreserve immature oocytes before they resume meiosis, at the germinal vesicle (GV) stage (Fig. 5). At this stage, the chromosomes are still enclosed within a nuclear membrane, making them less prone to chromosome loss. However, oocytes need to be matured *in vitro* after thawing. Human fully grown GV oocytes do not survive cryopreservation well. Less than 40% of oocytes survive thawing and only approximately 20% of surviving oocytes mature to metaphase II *in vitro* (Mandelbaum *et al.*, 1988), with low rates of blastocyst formation (Toth *et al.*, 1994). These low rates may be the result of the difficulty of optimizing cryopreservation for both small densely packed cumulus cells and a single large oocyte with a large amount of cytoplasm.

Cryopreservation and transplantation of ovarian tissue

Cryopreservation of immature oocytes at earlier stages of growth has also been investigated. Cryopreservation of ovarian tissue began in the 1950s, when frozen-thawed ovaries were autografted back to subcutaneous sites to restore endocrine function in ovariectomized rats (Parkes and Smith, 1952). Subsequent studies in sheep (which have a similar ovarian morphology to humans) renewed interest in orthotopic autografting as a way to restore natural fertility (Gosden *et al.*, 1994). Since the ovary is too large to freeze in its entirety, ovarian cortical tissue has to be stored as thin slices or pieces (Fig. 5).

Ovarian cortex is populated mainly by primordial follicles which have several characteristics that make them less prone to cryoinjury than are fully grown oocytes. Oocytes in primordial follicles are small, arrested in prophase of meiosis I, lack a zona pellucida and peripheral cortical granules and contain only small amounts of cold-sensitive lipid (Shaw *et al.*, 2000). A histological study showed morphologically normal follicles in frozen-thawed human ovarian cortical biopsies (Hovatta *et al.*, 1996). Although it is possible to recover good numbers of viable primordial follicles (approximately 70%) from cryopreserved ovarian tissue after thawing (Oktay *et al.*, 1997), current culture protocols are inadequate to maintain sustained growth *in vitro* to a mature stage at which fertilization can take place.

Fig. 5. Stages at which oocytes and embryos can be cryopreserved.

However, the long-term survival of frozen-thawed human ovarian tissue has been demonstrated after grafting of the cortical tissue under the kidney capsule of immunodeficient mice, resulting in follicular development to the antral stage (Oktay *et al.*, 1998). In theory, these oocytes could then be aspirated, matured *in vitro* and fertilized. However, moral and ethical concerns arise with the use of such an approach to treat human infertility, although it may be applied successfully to the preservation of endangered species, transgenic animals and rare breeds (Newton, 1998).

Alternatively, cryopreserved ovarian tissue can be autografted back to the donor to restore fertility. There has been some success with this technique in sheep, with pregnancies after grafting of frozen-thawed ovarian tissue (Gosden *et al.*, 1994), and maintenance of oestrous cycles for nearly 2 years (Baird *et al.*, 1999). Ovarian function has been partially restored in a 29-year-old woman after transplantation of her cryopreserved ovarian tissue (Oktay and Karlikaya, 2000). A major concern with autografting cryopreserved tissue from cancer patients is the risk of reintroduction of malignant cells (Shaw and Trounson, 1997).

Growth of human follicles and oocytes *in vitro*

Techniques to grow and mature oocytes *in vitro* before IVF would avoid transplantation and would have the added

Fig. 6. Strategies for growing and maturing human oocytes *in vitro*. COC: cumulus–oocyte complex; ECM: extracellular matrix. (Adapted from Hardy *et al.*, 2000.)

advantage of reducing exposure of a woman to exogenous gonadotrophins during superovulation treatment (with the associated risk of ovarian hyperstimulation syndrome). This approach may ultimately be useful in conventional IVF. Oocyte donation also involves hormonal stimulation and oocyte retrieval, making the process arduous for donors and resulting in a shortage of donor eggs. More women may participate in donation programmes if ovarian tissue rather than oocytes could be donated. Importantly, *in vitro* growth (IVG) of human follicles will provide a unique opportunity for examining the mechanisms that regulate early folliculogenesis.

Although the culture of ovarian follicles to various states of maturity is well established in species such as mice, rats, sheep and cows, growth of human follicles has met with limited success. In mice, isolated secondary follicles can be cultured to preovulatory stages (Nayudu and Osborn, 1992), artificially ovulated and their oocytes fertilized *in vitro*. This procedure has resulted in a few viable offspring after embryo transfer (Spears *et al.*, 1994). One mouse has been produced by IVF after the growth and maturation of primordial follicles *in vitro* (Eppig and O'Brien, 1996). In domestic species, embryos are produced commercially from *in vitro* matured oocytes from antral follicles, but success rates are low. Cow, pig and sheep preantral follicles have also been grown *in vitro* to various stages of maturity

(for a review see Telfer *et al.*, 1999) and oocytes from pig preantral follicles have been grown and fertilized, giving rise to blastocysts (Wu *et al.*, 2001).

Techniques to grow human follicles *in vitro* are currently being developed and have been, in general, adapted from those used successfully in other species. Preantral follicles have been isolated enzymatically (Roy and Treacy, 1993) and mechanically (Abir *et al.*, 1997) and grown for several weeks, and some follicles have undergone antrum formation (Fig. 6). Long-term culture of primordial follicles from fetal ovaries has resulted in follicle growth, oocyte maturation and even first polar body expulsion (Zhang *et al.*, 1995). In contrast, primordial follicles isolated from adult ovaries initiated growth, but did not survive beyond 24 h *in vitro*, and sustained high rates of oocyte loss (Abir *et al.*, 1999).

One of the most promising techniques for preantral follicle culture is that of the tissue-slice culture system designed for the growth of human primordial, primary and secondary follicles to large preantral and occasionally early antral stages (Hovatta *et al.*, 1997, 1999; Wright *et al.*, 1999). Follicles are cultured within small pieces of ovarian cortex (obtained via laparoscopy), which has the advantage of maintaining all the normal ovarian intercellular contacts and support (Fig. 6). Most primordial follicles initiate growth in human ovarian cortex (Wright *et al.*, 1999), although

Fig. 7. Percentage of cumulus–oocyte complexes (COCs) retrieved after *in vitro* maturation, fertilization and cleavage of oocytes aspirated from small follicles (2–16 mm in diameter). (Data from Hardy *et al.*, 2000.)

many follicles undergo atresia during culture. Studies using this approach have shown that supplementation of the culture medium with FSH significantly reduces atresia, and stimulates follicle development, demonstrating that follicles are responsive to FSH before antrum formation (Wright *et al.*, 1999).

In vitro maturation of oocytes

Resumption of meiosis in fully grown oocytes, germinal vesicle breakdown, extrusion of the first polar body and acquisition of the ability to be fertilized may all occur during *in vitro* maturation (IVM). Immature oocytes can be aspirated from small follicles of > 2 mm in diameter. The oocytes, surrounded by their cumulus cells, can then be matured *in vitro* (Fig. 6). In future, it may be possible to obtain oocytes that have been grown from preantral follicles *in vitro*.

The first human birth after IVM was achieved in 1983 (Veeck *et al.*, 1983). A few cases have been reported subsequently, including births after the aspiration and IVM of oocytes retrieved from women who have natural or partially stimulated cycles (Mikkelsen *et al.*, 1999) and women with polycystic ovarian disease (Trownson *et al.*, 1994).

When oocytes are removed from small antral follicles and placed in culture, approximately 60% will have

undergone nuclear maturation within 48 h. After exposure to spermatozoa, about 40% of oocytes will undergo normal fertilization, exhibiting two pronuclei and extruding the second polar body (Fig. 7). Between 20 and 25% of fertilized oocytes will undergo cleavage (Cha and Chian, 1998), but pregnancy rates after the transfer of such embryos have been extremely low (1–2%) and most embryos arrest between the four- and eight-cell stages. However, more encouraging results have been reported by Chian *et al.* (1999).

A number of factors may influence the fertilization, cleavage, blastocyst and pregnancy rates achieved after IVM, including the hormonal environment *in vivo* or *in vitro* and the composition of the culture medium (van de Sandt *et al.*, 1990). Further research in these areas will lead to greater understanding of oocyte maturation and should result in improved implantation and pregnancy rates.

Future of IVG and IVM in humans

A three-stage system encompassing initiation of growth in cortex pieces, follicle isolation and further *in vitro* growth followed by oocyte retrieval and IVM is envisaged for the production of oocytes competent to be fertilized. However, many problems need to be overcome before such a system can be established. For example, human stromal tissue is dense, and mechanical isolation has proved time-consuming

and delivers a low yield of follicles, whereas enzymatic isolation can damage follicle integrity (Abir *et al.*, 1999; Hovatta *et al.*, 1999). Human follicles also have a long maturation time (about 6 months *in vivo*) and are large when fully grown compared with mouse follicles (20 and 0.6 mm, respectively) (Gougeon, 1996). The development of systems that can support human follicle growth physically and nutritionally is a challenge.

Although the long-term goal of maturing human oocytes *in vitro* is close to being achieved, careful scientific studies should be carried out before such oocytes are used clinically. Most follicles *in vivo* are destined to undergo atresia, and only about 400 of the 2×10^6 follicles with which women are endowed are ovulated. *In vitro* growth and maturation rescues early follicles that would otherwise undergo atresia. However, this technique may override natural selection mechanisms, allowing damaged oocytes to survive. If these oocytes were to be fertilized, abnormal embryos might be produced. In domestic species, *in vitro* culture of embryos, particularly after IVM, leads to a high proportion of fetuses with large offspring syndrome (for a review, see Young *et al.*, 1998) which have phenotypic abnormalities. In addition, a mouse derived from an *in vitro* matured primordial follicle (Eppig and O'Brien, 1996) developed liver and neurological defects and became excessively obese. *In vitro* culture conditions may also be deleterious to oocytes, especially during IVM when they are undergoing meiosis I, as chromosomal abnormalities often arise at this time. However, the development of the culture conditions necessary to support normal folliculogenesis and oocyte maturation should yield considerable information about these processes as well as providing a much needed clinical breakthrough.

Conclusions

New approaches to preserving fertility in both men and women are being investigated, and will most likely be clinically available within the next decade or so. The continuing low pregnancy rates after IVF and embryo transfer imply that it may not be possible to improve upon natural conception rates. However, developing better embryo selection techniques to identify and choose viable and chromosomally normal embryos offer the best hopes of improving upon natural conception rates while simultaneously reducing the physical, psychological and economic burden of multiple gestations.

Novel techniques for alleviating human infertility are being developed continually. However, in the last decade, there has been a strong drive to put these into clinical practice, and attempt to achieve pregnancies and live births before fundamental basic research in other species has been carried out. The oldest IVF child is only in her early twenties, the oldest child produced from a frozen embryo is a teenager and the oldest ICSI child is < 10 years old. It is not known whether there will be adverse effects of new techniques such as ICSI on reproductive performance and

health in later years or, perhaps more importantly, on the reproductive performance and health of future generations.

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