Current status of the cryopreservation of human unfertilized oocytes

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Cryopreservation facilitates the long-term storage of oocytes from patients in danger of losing ovarian function and allows greater flexibility in fertility services for other patients. If some of the oocytes collected following ovulation stimulation are stored prior to fertilization, this alleviates many of the ethical concerns associated with embryo preservation. Concerns that cryopreservation could lead to disruption of the spindle and chromosomes, thus leading to genetic abnormalities of the offspring produced, mean that this procedure is not permitted in some countries. The recent spate of human live births from thawed oocytes has prompted the granting of the first licence allowing the use of thawed oocytes in the UK. However, the success rate of this procedure is still low and further research is required to refine these techniques and to develop new ones.

Key words: cryopreservation/cryoprotectant/freezing/oocyte

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Introduction

Recovery of oocytes from low temperatures has proven to be more technically challenging than similar preservation of embryos and spermatozoa. The oocyte has a short life span and unless it fuses with a single spermatozoon to become fertilized, it will degenerate. In order to undergo fertilization and development the oocyte needs to maintain the integrity of several of its unique structural features, many of which are affected by low temperatures, e.g. the zona pellucida, the cortical granules, the microtubular spindle and condensed chromosomes. The mature oocyte (~130 µm in diameter) is also one of the largest mammalian cells. As large single cells, they have a low surface area to volume ratio and, in addition, a low permeability to water; neither factor being conducive to cryopreservation.

Cryopreservation methods

Cryopreservation below ~130°C allows virtually indefinite storage of cells or tissues (liquid nitrogen is the usual holding medium and is at a temperature of ~196°C). It is the process of cooling cells to these temperatures and recovering them from storage that is potentially damaging. The damage caused to cells can be alleviated by the addition of so-called cryoprotectant chemicals. The concentration of cryoprotectant needed is dependent upon the subsequent cooling rate but the concentration should be kept to a minimum in order to reduce the toxic effect that such chemicals may have on cells. Lowering the temperature at which cells are exposed to cryoprotectants will help to reduce toxicity. The rate of cooling employed will dictate the warming rate required. Slow cooling (~1°C/min) to low subzero temperatures (~70°C) requires slow warming whereas slow cooling to intermediate subzero temperatures (~30°C) makes rapid warming feasible. Slow cooling is usually carried out in the presence of reasonably low concentrations of cryoprotectant (~1.5 mol/l). Alternatively, cells can be cooled rapidly in the presence of higher concentrations of cryoprotectant (~4 mol/l) with rapid warming – so-called ultra-rapid cooling techniques. The final commonly applied cooling technique is that of vitrification where very high concentrations of cryoprotectant (~6 mol/l) are employed together with very fast cooling and warming rates in an attempt to avoid ice formation. All of these techniques have been applied to human oocytes with varying degrees of success.

History of mature human oocyte cryopreservation

Following the successful cryopreservation of murine embryos, studies on the preservation of oocytes ensued. Since the first report of live births following the cryopreservation of mammalian mammals. The first successful pregnancy was achieved in 1988 using a technique involving the use of a slow cooling rate and a concentration of cryoprotectant.

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oocytes (Whittingham, 1977), oocytes of several species have been successfully cryopreserved, with live births being reported for mouse, rabbit, bovine and human. Such success has been largely due to the refinement of cryopreservation protocols (Bernard and Fuller, 1996).

The first human live births resulted from a freezing protocol similar to that used first with the mouse. Briefly, oocytes which had been exposed to 1.5 mol/l dimethylsulphoxide (Me2SO; a cryoprotectant) for 15 min on ice, were slowly cooled to −36°C then immersed and stored in liquid nitrogen. Thawing was rapid and dilution of the cryoprotectant was achieved by the addition of phosphate buffered saline. Of 50 oocytes, 38 survived freezing and thawing and at least 75% fertilized following exposure to spermatozoa, with 60% cleaving to 6–8-cell stage. Embryo transfers were performed on seven patients; two pregnancies ensued resulting in one twin (Chen, 1986) and one singleton (Chen, 1988) birth. A further live birth has been reported following oocyte cryopreservation in Me2SO (van Uem et al., 1987) but this time stepwise increments of cryoprotectant concentration were performed at 37°C. The oocytes were incubated in the final concentration of 1.5 mol/l Me2SO for 55 min at 24°C. Slow cooling then ensued to a temperature of −70°C. Thawing was achieved by placing the straws at room temperature and followed by stepwise dilution of the cryoprotectant. Of the 28 oocytes thus treated, seven survived, two embryo transfers were performed and one pregnancy ensued.

Despite these initial successes the technique was not widely adopted due to poor reproducibility of the results. In a contemporary study (Diedrich et al., 1987), 283 human oocytes were cryopreserved following a 10 min equilibration in 1.5 mol/l Me2SO at room temperature. Only 27.4% oocytes were viable following thawing, 58% of those underwent fertilization and two pregnancies ensued, neither of which continued to term. Another study compared three methods of cryopreservation (Al Hasani et al., 1987), these were slow cooling in the presence of 1.5 mol/l Me2SO (method 1), slow cooling in the presence of 1.5 mol/l of the cryoprotectant propane-1,2-diol (PrOH) plus sucrose (method 2) and ultra-rapid cooling in the presence of 3 mol/l Me2SO plus sucrose (method 3). A total of 329 oocytes were frozen, 217 by method 1, 66 by method 2 and 46 by method 3. Of the 144 oocytes thawed by method 1, 40 were intact and 20 fertilized; with method 2, 38 were thawed, 12 were intact and nine fertilized and with method 3, 23 were thawed and one was intact but did not fertilize. A high rate of polyplody was reported with method 2 (44%), the rate being 20% with method 1. Polyplody can be the result of multiple sperm entry, of retention of the polar body or may be due to relocation of the chromosomes. The authors suggested that the high rate of polyplody may have been the result of cortical granules being damaged or by the granules moving back to the centre of the cytoplasm thereby allowing multiple sperm entry. At the binding of sperm cortical granules ordinarily fuse with the oolemma, rupture and release their contents into the perivitelline space thereby forming a block to further sperm entry. The quality of the oocytes cryopreserved in this study may have been an important factor since the best quality oocytes were used for fresh transfer and many of the oocytes frozen were immature on collection and were matured for 24 h pre-freeze.

Zona pellucida damage

Work using the mouse model allowed the realization that cryopreservation induced changes in the zona pellucida and/or caused premature release of the contents of cortical granules (Vincent et al., 1990), thereby inhibiting fertilization. When the zona pellucida was bypassed, either by zona drilling (Carroll et al., 1990) or removing it completely (Wood et al., 1992) rates of fertilization were restored. Dissolution of the zona in α chymotrypsin has been used as a measure of zona hardening (Matson et al., 1997) and found to be more prolonged in mature murine oocytes cryopreserved in the presence of PrOH with slow cooling than in fresh oocytes. Addition of fetal bovine serum or bovine serum albumin to the freezing and dilution medium has been found to protect the zona pellucida (Carroll et al., 1993). Exposure to cryoprotectant has also been reported to have an adverse affect on the human oocyte zona pellucida. One study found 60% morphological survival of a small number of oocytes using one of four methods (Sathananthan et al., 1987). The main features of cryoinjury were cracks in the zona pellucida, disruption of the plasma membrane and extensive disorganization of the ooplasm, the latter being a particular feature of oocytes which had been vitrified. Best preservation of ultrastructure was obtained by slow cooling in Me2SO or PrOH, despite the fact that all of the oocytes slow cooled in Me2SO were the ones which had failed to fertilize. Most abnormalities were observed in vitrified oocytes. Ultrastructural evidence of premature cortical granule release has been found in oocytes exposed to Me2SO or PrOH at room temperature for a total period of 25 min with stepwise increase/decrease in concentration to a maximum of 1.5 mol/l (Schalkoff et al., 1989). One of the seven control oocytes examined in this study also showed exocytosed granules; this was possibly due to oocyte handling. On the other hand, the abundance of cortical granules in 15 human oocytes assessed after thawing in 1.5 mol/l PrOH has been reported (Gook et al., 1993). Whether the release of cortical granule contents or some other mechanism results in zona hardening the technique of intracytoplasmic sperm injection (ICSI) can be used to overcome problems associated with the zona pellucida following cryopreservation. One study took 74 oocytes which had survived cryopreservation using 1.5 mol/l PrOH with sucrose and slow cooling and randomly allocated them to be fertilized using conventional IVF or ICSI (Kazem et al., 1995). Five oocytes fertilized following IVF and 17 fertilized via ICSI. Only one oocyte fertilized by IVF went on to cleave while 16 cleaved following ICSI. The first study to show normal development to hatching blastocyst following ICSI of cryopreserved human oocytes (Gook et al., 1995b) achieved 50% fertilization with either conventional IVF or ICSI following cryopreservation in 1.5 mol/l PrOH plus sucrose and slow cooling followed by fast warming. This approach seems to have overcome the poor fertilization rates after thawing demonstrated in a clinical trial of oocyte cryopreservation (Serafini et al., 1995) but will not overcome the problems of poor cleavage and development rates.

Cytoskeletal damage

Further improvements in oocyte survival post-thaw came with the realization that exposure to low temperatures or Me2SO affects cytoskeletal elements, disruption of which may lead to an
abnormal number of chromosomes (aneuploidy). The microtubular spindle upon which the chromosomes are aligned was found to be sensitive to cooling in murine oocytes but demonstrated the ability to recover if incubated at 37°C for sufficient time (Pickering and Johnson, 1987; Sathananthan et al., 1992). However, disruption of the spindle of the cooled human oocyte was found to be irreversible when freshly collected mature oocytes were cooled to room temperature either for 10 or 30 min and then incubated at 37°C for either 1 or 4 h (Pickering et al., 1990). Following incubation at room temperature for 10 min half of the oocytes had disrupted spindles and after 30 min all spindles were disrupted; abnormalities included reduction in spindle size, spindle disorganization and spindle disappearance sometimes associated with chromosome dispersal. Less than half of the oocytes regained normal spindle morphology following incubation at 37°C although chromosome dispersal was only evident after 30 minute incubation at room temperature. Irreversible disruption of the spindle and dispersal of chromosomes was also found following the movement of oocytes to room temperature for 10–30 min (Almeida and Bolton, 1995). Returning oocytes to 37°C for 1 or 4 h after a 2 min incubation at room temperature did allow restoration of normal spindle structure. The authors found evidence of normal spindle structure together with dispersed chromosomes as well as abnormal spindles and compact chromosomes. They also reported a direct association between temperature induced spindle damage and chromosomal abnormalities in parthenotes developed from oocytes exposed to room temperature. The oocytes used in this study were those which had failed to fertilize 18–20 h post-insemination from patients with male factor infertility. The reduced ability of human oocytes to undergo spindle reformation may be linked to the fact that they contain less foci of pericentriolar material, which form the sites of tubulin organization, than do murine oocytes.

Cryoprotectants also cause disruption of the microtubular spindle (Johnson and Pickering, 1987). However, since Me2SO favours the polymerization of tubulin whilst low temperature causes tubulin depolymerization, the combination of Me2SO and low temperature together has a protective effect. Exposure of human oocytes to Me2SO at 37°C has been shown to result in low fertilization rates (Pickering et al., 1991) whereas exposure to the same cryoprotectant at 4°C gave much improved results (10% versus 75% fertilization, respectively). Human oocytes have been observed to have partially disorganized spindles following cooling to 0°C for 20 or 60 min in the presence or absence of Me2SO (Sathananthan et al., 1988). Chromosomes tended to clump together or were dislocated in the cortical ooplasm in cooled oocytes but widespread scattering of the chromosomes was not observed. Importantly, no stray chromosomes were present in human oocytes exposed to PrOH without cooling or following thawing even though the spindle was absent or abnormal (Gook et al., 1993).

**Variation in cryopreservation techniques**

Many different cryopreservation techniques have been applied to the human oocyte. The use of glycerol, which is generally considered to be less toxic than the previously mentioned cryoprotectants, was investigated in 1991 (Hunter et al., 1991). The effects of equilibration of oocytes in either Me2SO (15 oocytes) or glycerol (13 oocytes) were compared. Following cryopreservation, oocytes were inseminated, but only one oocyte cleaved to the two cell stage following cryopreservation in Me2SO and no cell divisions were recorded following cryopreservation in glycerol or exposure to glycerol without cooling.

The use of rapid cooling methods has also been explored by several investigators. Conventional slow cooling with glycerol or Me2SO, several vitrification techniques and two rapid cooling techniques have been compared (Feichtinger et al., 1988). Fertilization of fresh oocytes was obtained following slow cooling in Me2SO (one out of four fertilized) and following vitrification in the so-called VS1 solution, which consists of 2.62 mol/l Me2SO, 2.62 mol/l acamid, 1.3 mol/l PrOH and 6% polyethylene glycol, and was used successfully for the vitrification of murine embryos (Rall and Fahy, 1985). Variable results have been obtained following cryopreservation of murine oocytes using this mixture (Kola et al., 1988; Kono et al., 1991) with malformed fetuses being reported following exposure to the vitrification solution with and without cooling. The human oocytes were exposed to VS1 for a shorter time period than used for the original embryo study and sucrose was used to aid dilution of the cryoprotectant. This resulted in seven out of 21 fertilized oocytes but of these, three were multi-pronucleate. Two of five cleaved and were transferred but no pregnancy occurred. It should be noted that the acamid component of VS1 is a possible human carcinogen. Another study involved rapidly cooling groups of oocytes in 2.8–4.2 mol/l Me2SO with 0.25–0.625 mol/l sucrose (Pensis et al., 1989). After 15 min exposure to 3.5 mol/l Me2SO + 0.5 mol/l sucrose at 4°C followed by plunging into liquid nitrogen for storage, warming and dilution in 0.2 mol/l sucrose, 80% of oocytes were recovered. However, the number of oocytes treated was not stated and the appearance of the spindle and chromosomes was not assessed nor their ability to fertilize. Fresh oocytes were reported to survive freeze–thawing better than oocytes which had failed to fertilize. A total of 20 randomly allocated fresh mature oocytes were vitrified following 15 s exposure to 90% VS1 at room temperature (Hunter et al., 1995). In all, 13 oocytes survived and nine fertilized although further development was inhibited. The authors also vitrified seven oocytes but did not attempt to fertilize them in order to check for spontaneous activation, evidence of which there was none.

Although all the human live births achieved up to this point had used Me2SO as cryoprotectant, attention switched to slow cooling in the presence of PrOH following the use of PrOH for embryo freezing. Cryopreservation of murine oocytes using PrOH has been reported to cause high rates of parthenogenetic activation (Van der Elst et al., 1992) and has recently been shown to increase intracellular calcium concentration (Litkouhi et al., 1999). In two studies where small numbers of human oocytes were used to compare the effects of Me2SO and PrOH (Al Hasani et al., 1987; Todorow et al., 1989), both reported higher rates of survival for human oocytes cryopreserved in PrOH compared with Me2SO, but as stated earlier the rate of polyploidy was high in the PrOH treated group. Significantly better survival of human oocytes (64%) than murine oocytes (4%) has been shown following cryopreservation by a slow freeze, rapid thaw method using PrOH (Gook et al., 1993). These results represent quite a considerable improvement in survival rates compared with earlier studies. In 1994 (Gook et al., 1994) the karyotypes of four human oocytes which had been cryopreserved in PrOH and which had
undergone normal fertilization were examined and were all found to contain two sets of 23 chromosomes. Absent or abnormal spindles were observed in all of the oocytes cryopreserved which survived but failed to fertilize but no stray chromosomes were observed. In a follow-up study (Gook et al., 1995a), of 22 fresh oocytes cryopreserved using the PrOH slow freeze–rapid thaw protocol, 27% showed activation whilst oocytes exposed to cryoprotectant without freezing and untreated oocytes showed no activation. Four of the cryopreserved oocytes were observed to have extruded the second polar body and formed a single pronucleus, none of the oocytes exhibited two or more pronuclei and hence would not have been mistaken for a fertilized oocyte.

A number of live births have recently been reported using the method of slow cool–rapid thaw in the presence of PrOH and sucrose. In 1997 (Porcu et al., 1997), the first report of a human live birth following oocyte cryopreservation and ICSI was presented. Four of the twelve mature oocytes survived freeze–thawing, two of which fertilized and one cleaved. This embryo was transferred at the 4-cell stage and amniocentesis revealed a normal 46XX karyotype. A series of studies using oocytes derived from failed-to-fertilize IVF or ICSI programmes (Tucker et al., 1996), fresh oocytes donated for cryopreservation (Tucker et al., 1996, 1998a) or oocytes donated by IVF patients (Tucker et al., 1998a,b) were undertaken. In all, 55% of aged oocytes survived the freeze–thaw. It was possible that some of these were activated in that no pronucleus formation was seen, but cleavage occurred, alternatively this could be the result of delayed fertilization from the initial insemination. Of the fresh oocytes cryopreserved, 24% survived and five pregnancies were initiated, two miscarried at <8 weeks, one miscarried at 10 weeks and one delivered twins at 39 weeks with one ongoing at the time of the report at 37 weeks. With the 45 oocytes from IVF patients the survival was poor, 2.2% for mature oocytes and 44% for sibling immature oocytes. Three of the immature oocytes matured to metaphase II and ICSI was performed. Two transfers were performed and one live birth resulted from the transfer of two immature cryopreserved oocytes (Tucker et al., 1998b). In this study, only a proportion of the oocytes collected were frozen and these were the poorer quality oocytes although atretic oocytes were not frozen. In other studies (Tucker et al., 1996, 1998a,b), the protocol for cryopreservation of oocytes varied from 6–20 min exposure to PrOH followed by 2–10 min exposure to 1.5 mol/l PrOH plus sucrose and in three instances oocytes were cooled to room temperature for 10 min prior to addition of cryoprotectant. The authors justify such changes to protocols on observations of the oocyte shrink/swell response on addition of cryoprotectant but admit that their rationale for changes was somewhat arbitrary. In another study, 10 fresh oocytes were donated by one patient and cryopreserved (Polak de Fried et al., 1998). Three oocytes survived the freezing and two fertilized following ICSI and were replaced. One healthy boy was delivered. A triplet pregnancy has been reported (Young et al., 1998) following freezing of 10 donated oocytes. Eight of the oocytes survived and went on to fertilize. Of these five cleaved (two 2-cell embryos and three 4-cell embryos) and were transferred. A triplet pregnancy was confirmed by ultrasound at 8 weeks, but at 10 weeks the pregnancy was interrupted. Two delivered pregnancies have been reported (Vidali et al., 1998) from 471 thawed oocytes. Porcu et al. recently presented the results of their oocyte freezing programme in which they recruited patients <38 years old with tubal infertility, no previous IVF failure and with at least 10 oocytes retrieved — all of which would be cryopreserved (Porcu et al., 1999). Of 1769 oocytes frozen, 1502 had been thawed. They reported a 54% survival rate, 57% fertilization and 91% cleavage. They have achieved 16 pregnancies resulting in 11 births (seven singleton and two twin pregnancies).

A modification to the slow cooling PrOH freezing method has resulted in the birth of two sets of twins with four pregnancies ongoing (Yang et al., 1999). The fertilization rate using ICSI was reported to be similar to that of sibling fresh oocytes obtained from the same cycle. Yang et al. exposed the oocytes to the cryoprotectant at 37°C for 5 min rather than at room temperature which they found to give improved results (Yang et al., 1998).

Some success has recently been reported following vitrification of human oocytes. Cha et al. (1999) reported a pregnancy from oocytes were treated with 5.5 mol/l of the cryoprotectant ethylene glycol plus 1.0 mol/l sucrose. A total of 14 oocytes were stored, seven immature and seven mature. All survived thawing but the immature oocytes did not mature in culture. Of the mature oocytes, six fertilized and all cleaved. The oocytes were transferred at the 2–8-cell stage and a live birth was reported. A further birth has been reported from 17 thawed oocytes, again following short exposure to high concentrations of ethylene glycol and cooling in an open pulled straw (Kuleshova et al., 1999).

**Cryopreservation of immature oocytes**

Since one of the main problems in the cryopreservation of mature oocytes arises from the sensitivity of the microtubular spindle to low temperatures and cryoprotectants, one way of circumventing these problems would be to preserve oocytes at the immature germinal vesicle stage at which no microtubular spindle is present. The use of immature oocytes would mean that patients could receive less hormonal stimulation to produce the oocytes or oocytes may even be retrieved without any stimulation. Fully grown, meiotically competent immature oocytes can be retrieved transvaginally (Trinson et al., 1994) although the procedure does need to be modified from that used for retrieval of mature oocytes.

Cryopreservation of immature human oocytes from patients having received hormonal stimulation has resulted in recovery and maturation to metaphase II, but both the initial survival of oocytes (10 out of 25) and rates of maturation (two out of 10) were low (Mandelbaum et al., 1988). Immature human oocytes obtained from unstimulated ovaries which had been rapidly cooled in the presence of 3.5 mol/l M2SO plus 0.5 mol/l sucrose were found to be capable of meiotic maturation after thawing but a premature and apparently partial condensation of the chromosomes was observed in nearly half of the oocytes treated whereas such a phenomenon was never seen in murine oocytes (Van Blerkom and Davis, 1994). Neither ploidy after maturation nor ability to fertilize were determined in the human oocytes. Toth et al. found that oocytes obtained from non-stimulated patients gave variable results (Toth et al., 1994b). They compared oocytes following slow or rapid cooling in 1.5 mol/l PrOH and of 1333 oocytes cooled slowly, 15% survived and 58% matured whereas with rapid cooling 43% of the 95 oocytes survived and 27%
matured. Oocytes from stimulated patients showed no difference in in-vitro maturation (>70%) and fertilization rates (>50%) between fresh and frozen-thawed immature human oocytes. However, blastocyst development was lower for cryopreserved oocytes (3.3%) than for fresh oocytes (11.5%). Further development was not assessed (Toth et al., 1994a). A subsequent study achieved high survival rates post-thaw of immature oocytes from stimulated ovaries which had been cooled slowly in 1.5 mol/l PrOH (Baka et al., 1995). Of the 98 oocytes thawed, 63% survived and 68% matured in culture. Of these 81% had a normal spindle and 83% had chromosomes arranged on the metaphase plate. Six oocytes had an abnormal spindle with chromosomes dispersed throughout the spindle. When oocytes from unstimulated ovaries were cryopreserved using 1.5 mol/l PrOH and sucrose (Son et al., 1996) 54 of the 98 oocytes survived, 59% matured, 43% fertilized and 17% cleaved. The rates of maturation, fertilization and cleavage were significantly lower than non-frozen controls. In another study using the same cryopreservation method (Park et al., 1997) the spindle and chromosome arrangement of cryopreserved immature human oocytes was studied. The oocytes were obtained from follicles 2–5 mm in size in unstimulated ovaries and were cryopreserved with their cumulus intact. Oocytes had abnormal chromosome arrangement (aneuploidy or polyploidy) in 77.8% of cases, and 70% of oocytes exhibited an abnormal spindle. These figures were significantly different from those of untreated controls and from oocytes exposed to the cryoprotectant without freezing at 48h post-cryopreservation. Significantly less cryopreserved oocytes matured to metaphase II than control oocytes.

The disadvantages of immature oocyte cryopreservation include the fact that an additional maturation procedure is required. Although in-vitro maturation of oocytes is routinely successful in some animal species, this is not the case for human oocytes. Only a few successful pregnancies from immature oocytes have been achieved thus far (Cha et al., 1991; Trounson et al., 1994 Tucker et al., 1998b). Also immature oocytes may need to be preserved with their cumulus cells intact since these cells are likely to be necessary for maturation to occur, in that case the cryopreservation protocol would need to be a compromise between the best protocol for the oocyte and that for the cumulus cells.

Cryopreservation of ovarian tissue

It has been suggested that preservation of the female gamete may be best achieved by storing pieces of ovarian tissue which contains numerous small oocytes within follicles. Oocytes in the early stage primordial follicles are smaller, possess fewer organelles and have no zona or cortical granules hence they are potentially easier to freeze (Oktay et al., 1998). The presence of intact human follicles in frozen–thawed cortical tissue has been demonstrated by histological studies (Hovatta et al., 1996). Follicles at similar developmental stages were found before and after freezing with no signs of tissue necrosis. Some evidence of stromal tissue damage has been reported by others after thawing (Goek et al., 1999). Viability of frozen–thawed human tissue has been shown by grafting tissue under the kidney capsule of severe combined immunodeficiency (SCID) mice (Newton et al., 1996). Frozen cortical ovarian slices have been used to restore fertility to sheep resulting in the birth of a lamb (Gosden et al., 1994). The sheep ovary resembles that of humans in terms of both size and composition. Recently, cortical strips of human ovary which had been frozen were thawed and replaced in a pocket in the pelvic wall of a patient. Following stimulation with hormones a dominant follicle emerged in the grafted tissue which continued to grow following cessation of hormone treatment (Oktay et al., 1999). Grafting of frozen–thawed tissue from cancer patients should be applied with caution since metastatic cells present in the blood supply may survive the freeze–thaw process and re-implant, a situation which has been shown to occur in mice (Shaw et al., 1996).

The use of immunodeficient animals in which to mature thawed follicles has been suggested but obviously raises ethical concerns (Kim et al., 1999). An alternative option would be to culture follicles in vitro to produce mature oocytes. The development in vitro of mouse oocytes from primordial follicles which were subsequently fertilized and, on embryo transfer, resulted in one live birth, has been demonstrated (Eppig and O’Brien, 1996). In this system the ovaries of new-born mice were grown in organ culture for 8 days and the developing oocyte–granulosa cell complexes isolated and cultured for an additional 14 days. With human ovarian tissue the culture period would need to be considerably extended and the conditions needed for maturation are likely to be more complex. Mechanical isolation and growth of preantral and small antral follicles has recently been achieved (Abir et al., 1997). Isolation of primordial follicles from fresh and frozen thawed tissue is possible (Oktay et al., 1997). Such follicles should be more abundant than later stage follicles but will require long periods of growth in vitro as well as the need to be switched into the growing phase. Primordial and primary follicles within human ovarian tissue have been shown to survive for up to 21 days when cultured as slices following freeze–thawing (Hovatta et al., 1997).

Conclusions

Results from cryopreserved oocytes have been, and still are, somewhat variable. The percentage of live births from thawed oocytes varying from 1–10. One of the main determining factors in oocyte survival is the quality of the oocytes cryopreserved. The initial births were achieved with fresh good quality oocytes and the encouraging recent results (Porcu et al., 1999) have been achieved with fresh oocytes from a selected group of patients. Where oocytes of varying stages of maturity have been collected and allowed to mature pre-cryopreservation; where the best oocytes have been used for fresh embryo transfer and even where excessively large numbers of oocytes have been retrieved (Goek et al., 1994), survival has been generally poor. It is understandable that the poorest quality oocytes are the ones that are donated for research and this unfortunately hampers results. It has also been suggested (Tucker et al., 1998a) that it may be unrealistic to expect >70% survival of oocytes since, unlike embryos, oocytes have not been pre-selected by the process of fertilization.

The coupling of oocyte cryopreservation and the ICSI technique has appeared to facilitate the recent spate of live births. If cryopreservation does undermine the structure of the zona pellucida and/or cortical granules then ICSI will overcome
problems of sperm penetration and polyspermy but in so doing bypasses one of the main selection processes in determining the fate of the thawed oocyte. Having overcome this barrier the fertilized oocyte still needs to divide and maintain a normal complement of chromosomes. Despite the reports of spindle disruption, reports of scattering of chromosomes are less common particularly when PrOH is used as the cryoprotectant. However, polyplody and activation are more common following cryopreservation in PrOH. Monitoring oocytes following cryopreservation for normal cleavage post-fertilization and performing ploidy checks should elucidate where these processes have occurred. Of the 28 human live births reported, no reports have mentioned any malformations.

Although the PrOH slow freeze–rapid thaw protocol has yielded numerous live births there is no evidence that this is indeed the best protocol for preservation of human oocytes. Further studies to develop and refine techniques for human oocyte cryopreservation are clearly required. Due to the scarcity of human oocytes for research, progress is likely to be slow and systematic studies to compare different methods will be difficult. Recent studies have revealed alternative methods for cryopreservation of oocyte and produced live births. Success has been achieved in vitrifying human oocytes using the cryoprotectant favoured for mammalian embryo freezing, ethylene glycol. Such rapid methods would facilitate reduction in the time oocytes are exposed to potentially damaging high subzero temperatures but also require higher cryoprotectant concentrations than those used for slow cooling methods. Vitrification should not be ruled out because of disappointing results in the past with potentially toxic combinations of cryoprotectants. Another area for potential improvement is an increase in the temperature at which ice formation is induced prior to slow cooling. An increase from –8°C to –6°C to –4.5°C improved survival of immature and failed-to-fertilize human oocytes following cooling in PrOH from 32 to 56 to 93% respectively (Träd et al., 1999).

When developing new methods of cryopreservation, initial studies to determine morphological survival and normality of spindle and chromosomes in a pilot study are advisable prior to fertilization studies. Having determined protocols, they should be strictly adhered to since slight changes in temperature or time of exposure to cryoprotectant can change survival. Care must also be taken to ensure that the storage temperature does not exceed –130°C.

Useful data can be generated by using mammalian oocytes as a model. However, results must be extrapolated to the human situation with a degree of caution since there are differences between the oocytes of different species. Human oocytes are almost double the size of murine oocytes resulting in a difference in the surface area to volume ratio. Taking this into account, the oocyte membrane permeability characteristics which determine the osmotic response of oocytes during cryoprotectant exposure and cooling, do show subtle differences for human and mouse oocytes (Paynter et al., 1999). Nevertheless, interesting data is being produced using murine oocytes. The replacement of sodium with choline in the freezing medium has improved survival of murine oocytes and should be explored with human oocytes (Stachek et al., 1998).

The use of immature oocytes is still in its infancy (Mandelbaum et al., 1998) and protocols for the recruitment of good quality oocytes need to be improved. Oocytes from unstimulated ovaries have given consistently poor results. Cryopreservation protocols may need to be designed specifically for immature oocytes since the osmotic response of these oocytes varies from that of mature oocytes plus immature oocytes may need to be preserved with their surrounding cumulus cells intact since these cells are beneficial in the maturation process. Protocols for the in-vitro maturation of oocytes also need to be improved.

The techniques for freezing of ovarian tissue and/or isolated follicles need to be optimized (Newton et al., 1998). Techniques for in-vitro maturation of follicles are likely to require many years of study before mature fertilizable oocytes can be produced from early stage follicles. Even relatively late stage immature oocytes cannot be reliably matured in vitro at present. Freezing of ovarian tissue may be the only option for preservation of fertility for young cancer patients and it is possible that the techniques required for production of viable oocytes will be available when the tissue is required.

Currently the option most likely to result in pregnancy for adult cancer patients with impaired fertility who are unable to preserve embryos is that of mature oocyte freezing. Ovarian stimulation will help to increase the number of oocytes retrieved but, even so, the number of oocytes recovered is likely to be sufficient for only one or two attempts at IVF post-thaw. Whilst the technique is not without risk, recent results are very encouraging. However, further research is necessary to improve the currently poor success rates (Gook and Edgar, 1999). The area where progress is likely to proceed fastest, although its application may be limited in some countries, is that of oocyte donation. Better quality oocytes can be expected to be retrieved from donors and results from such oocytes may be available more rapidly since the storage period is likely to be less than that for oocytes stored for patients’ own use.

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