The cryopreservation of ovarian tissue as a strategy for preserving the fertility of cancer patients

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The cryopreservation of ovarian tissue is a promising new method for conserving the fecundity of young cancer patients from the sterilizing effects of chemotherapy and/or radiotherapy. In murine and ovine studies the orthotopic insertion of frozen–thawed ovarian grafts into sterilized hosts has resulted in the birth of healthy offspring. Examination of human ovarian tissue after cryopreservation has shown that substantial numbers of morphologically normal and viable primordial follicles survive freeze–thawing. To date however, there is no efficient procedure for using the frozen-banked tissue to restore fertility to patients with ovarian failure. Autografting at an orthotopic or heterotopic site has the greatest potential for success but there is concern that the technique may reintroduce malignant cells to patients in remission from disease. Follicle isolation from the thawed tissue and growth to maturity in vitro is a preferable option but at present the technique is in its infancy. This review presents past and present research in the field of ovarian tissue cryopreservation and explores the possible strategies by which frozen-banked tissue from cancer patients could be used to restore fertility.

Key words: cryopreservation/human/ovary

Introduction
The human ovarian cortex is populated by a finite store of primordial germ cells, the numbers of which decline throughout life as a result of ovulation and atresia. At birth ~10^6 follicles are present and by puberty only 25% remain (Baker, 1971; Faddy et al., 1992). The rate of follicle loss accelerates at ~37.5 years of age until ~1000 follicles remain, cyclical endocrine function then ceases and the female becomes menopausal (Faddy et al., 1992). The precise timing of the menopause is determined by both the size of the original germ cell store and the rate of depletion during life. In the Western world the menopause occurs at a median age of 51 years (Faddy et al., 1992).

Aggressive chemotherapy and/or radiotherapy for the treatment of cancer can severely deplete the follicular store often compromising ovarian function (Lushbaugh and Cassaret, 1976; Damewood and Grochow, 1986; Apperley and Reddy, 1995; Sanders et al., 1996). This gonadotoxic effect is of particular concern because over recent years improvements in the treatment of cancer have led to significant increases in the long-term survival rates of patients, especially of younger ages (Boring et al., 1994). The age of the patient plays a significant role in the risk of ovarian failure after treatment (Horning et al., 1981; Chapman, 1982; Fisher and Cheung, 1984; Marcello et al., 1990). Following particularly aggressive treatment regimes patients >40 years of age are more likely to experience permanent ovarian failure, whilst in younger patients amenorrhoea is often temporary. Even with the retention of ovarian function a severe depletion of the follicle store may
lead to premature menopause. One mathematical model estimated that a 90% reduction of the germ cell population before the age of 14 could result in permanent ovarian failure by 27 years of age (Faddy et al., 1992). Since accurate predictions of subsequent ovarian function or fertile lifespan cannot be made prior to gonadotoxic therapy it is wise to attempt to safeguard the fecundity of these patients before treatment. At present the three methods for storing fecundity are: (i) cryopreservation of embryos, (ii) oocytes and (iii) ovarian tissue.

Embryo cryopreservation

During routine in-vitro fertilization (IVF) procedures it is virtually impossible to control the numbers of mature oocytes which develop in response to ovarian stimulation. There is a trend towards a higher pregnancy rate when more embryos are transferred to the uterus, but the risks of multiple pregnancy have led many countries to impose legal limits on the number of transfers per cycle. The frozen-banking of excess embryos allows additional transfers to be performed without expensive and time-consuming gonadotrophic stimulation. In humans the first live birth following frozen embryo transfer was reported in 1985 (Mohr and Trounson, 1985) and today the success rate following the procedure is high (Karlström et al., 1997; Marcus et al., 1997).

Cancer patients can freeze-bank embryos following follicle aspiration and IVF prior to, or during, a suitable break in treatment (Brown et al., 1996). Unfortunately, only limited numbers of oocytes are collected and the technique is not universally applicable to cancer patients, being inappropriate for prepubertal girls and often unacceptable to single women who do not wish to use donor spermatozoa. Furthermore, a window of ~6 weeks may be required for ovulation induction which could present an unacceptable delay in cancer treatment. In addition, if ovarian stimulation is carried out, malignant growth of oestrogen-sensitive tumours such as carcinoma of the breast may, at least in theory, be enhanced. Patients who have already undergone a round of cancer treatment face further problems. Normal ovarian function is required for successful outcome of oocyte collection, thus a poor result may be expected in patients whose follicle store has been depleted (Toner et al., 1993). It should also be borne in mind that oocytes collected between courses of chemotherapy have grown to maturity in a toxic environment and, although there is no evidence in humans to suggest that these cells carry genetic abnormalities, it would be imprudent to ignore potential risks to the progeny (Becker and Schoneich, 1982).

In addition to practical problems, the frozen-storage of human embryos raises many ethical and legal issues (Trounson and Dawson, 1996). In particular, if cancer treatment fails and the mother dies ‘orphan embryos’ are created, the disposal of which raises moral and ethical dilemmas. In view of these problems a preferable alternative may be the storage of oocytes prior to insemination.

Mature oocyte cryopreservation

The cryopreservation of mature oocytes is, in theory, an attractive alternative to embryo storage for cancer patients. In addition, the procedure would facilitate egg donation programmes, eliminating the problem of donor/recipient synchronization and allowing the creation of frozen oocyte banks. Although the technique has proved successful in animal studies (Whittingham, 1977; Al-Hasani et al., 1989; Fuku et al., 1992) human oocyte freezing, using a slow cooling protocol, has proved frustrating and rarely successful. Post-thaw survival and fertilization rates are highly variable and often poor, ranging from 25 to 69% and from 32 to 75% respectively (Trounson, 1986; Al-Hasani et al., 1987; Van Uem et al., 1987; Mandelbaum et al., 1988; Gook et al., 1993; Gook et al., 1995) and only a limited number of pregnancies and live births have been reported (Chen, 1986; Van Uem et al., 1987; Porcu et al., 1997).

Mature oocytes are arrested at metaphase of the second meiotic division when the cell is a highly organized unit with a zona pellucida, meiotic spindle apparatus and cortical granules. Damage to any of these structures may have a detrimental effect on post-thaw viability. A problem commonly encountered following cryopreservation is a failure of the spermatozoon to penetrate the egg, resulting in a drop in fertilization rates (Trounson and Kirby, 1989; Wood et al., 1992; Carroll et al., 1993). An unexplained hardening of the zona pellucida seems to be the cause, since zona drilling reverses the effect (Carroll et al., 1990). In clinical practice this does not present a significant problem since intracytoplasmic sperm injection (ICSI) can be used to inject the male gamete directly into the oocyte, thereby eliminating the requirement for ‘natural’ fertilization (Gook et al., 1995; Porcu et al., 1997).

A more alarming observation is the detrimental effect of cryopreservation on the spindle apparatus (Pickering and Johnson, 1987; Johnson and Pickering, 1987; Van der Elst et al., 1988; Pickering et al., 1990; Baka et al., 1995). In metaphase II oocytes the chromosomes are aligned along a delicate spindle of microtubules which are sensitive to cooling and cryoprotective solutions. Microtubules play a major role in the separation of sister chromatids during the
second meiotic division and any disruption of the structure may result in non-disjunction and an increased risk of polyplody or aneuploidy (Al-Hasani et al., 1987; Glenister et al., 1987). Recent studies of human and rodent oocytes, however, have suggested that this concern may be unjustified since cryopreservation has not been found to significantly increase the risk of chromosomal abnormalities (Gook et al., 1994; Bos-Mikich and Whittingham, 1995). Nevertheless, until the risk can be eliminated oocyte cryopreservation cannot be offered as a safe and reliable means of preserving fecundity.

Immature oocyte cryopreservation

Of all the follicles which enter the growing pool each cycle, only one will usually ovulate, while the remainder undergo atretic degeneration. Immature oocytes, aspirated from the growing follicles, are less differentiated than metaphase II oocytes and have a disassembled microtubule apparatus and decondensed chromatin housed within a nuclear membrane. These features may make the cells more tolerant of freeze–thawing damage than metaphase II oocytes. The disadvantage of immature oocyte cryopreservation is that after thawing the cells must be matured in vitro before fertilization (Gosden et al., 1998). In-vitro maturation (IVM) has proved highly successful in animals and ~50% of cattle blastocysts generated by IVM/IVF form fetuses after transfer to recipients (Trounson et al., 1994). Furthermore, frozen–thawed murine and bovine immature oocytes matured and fertilized in vitro formed blastocysts, which when transferred to recipients produced live young (Candy et al., 1994; Suzuki et al., 1996).

Human IVM has, in contrast, proved more problematic and although a limited number of pregnancies have been achieved, the clinical success rate is low (Cha et al., 1991). Only 37–59% of cryopreserved immature oocytes survive freeze–thawing, of which 20–83% undergo successful maturation to metaphase II (Mandelbaum et al., 1988; Toth et al., 1994a; Toth et al., 1994b; Son et al., 1996). The rate of blastocyst formation is also compromised by freeze–thawing prior to IVM (3 versus 11.5% in fresh controls) (Toth et al., 1994b). To date, embryos produced from frozen–thawed immature oocytes have not been transferred to patients, thus their developmental potential is unknown and it remains to be seen whether they are capable of normal growth to full term. Nevertheless, improvements in both follicular aspiration techniques and culture conditions could, with time, make IVM/IVF as successful as conventional IVF and cryopreservation of immature oocytes may then be a viable option for preserving fertility. At present a more reliable technique is required.

The cryopreservation of ovarian tissue

Freeze-banking slices of ovarian cortex is a promising alternative for the preservation of fecundity (Wood et al., 1997; Oktay et al., 1998a). Cortical biopsies can be retrieved from the patient at any stage of the menstrual cycle by laparoscopic technique, or during oophoropexy prior to cancer treatment. The ovarian cortex houses a vast supply of primordial follicles which, being small and undifferentiated, are unlikely to be damaged by the freezing procedure. Autografting frozen–thawed tissue to patients with ovarian failure should restore endocrine function, thereby eliminating the requirement for hormone replacement therapy, and if the graft is replaced at the orthotopic site ‘natural’ fertility may be restored. In addition, the use of banked ovarian tissue to restore fertility evokes no serious immunological, moral or legal problems.

The first attempts at ovarian cryopreservation were carried out in the 1950s when the primary aim was to restore endocrine function to ovariec-tomized mice and rats (Parkes and Smith, 1953; Deanesly, 1954; Parkes, 1956, 1957). Ovaries were cooled to ~79°C in glycerol/saline solution before thawing and autografting to subcutaneous sites. Craft survival was assessed by the resumption of oestrous cycles as indicated by cornification of vaginal epithelial cells (Parkes and Smith, 1953). In rats, cycles ceased within 2 days of ovariec-tomy, but the subsequent insertion of fresh or frozen–thawed grafts restored endocrine function within 7–8 days and 2–3 weeks, respectively (Parkes and Smith, 1953; Deanesly and Parkes, 1956; Parkes, 1957). The success of subcutaneous grafting encouraged attempts to restore fertility to ovariec-tomized mice by the insertion of cryopreserved tissue at the orthotopic site. The procedure resulted in the birth of normal, viable offspring although the success rate was low (Parrott, 1960). Interest in the field subsequently diminished because there seemed few applications for the technology.

Improvement in the prognosis of many young cancer patients has initiated renewed interest in ovarian tissue banking as a means of preserving fecundity. Over recent years significant advances have been made in the field of cryobiology, most importantly the introduction of controlled rate freezing apparatus and the development of more efficient cryoprotective agents. A corresponding improvement in the success of ovarian tissue cryopreservation has been demonstrated. Ovariectomized mice, autografted with frozen–thawed or fresh tissue, demonstrated post-operative oestrous cycles in 75 and 80% of animals, respectively (Harp et al., 1994) and attempts to restore fertility to the murine hosts reported pregnancies in
73–86% of the recipients of cryopreserved tissue (Cox et al., 1996; Gunasena et al., 1997).

It remained doubtful that the technique would prove as successful with human tissue which has a more dense and fibrous stroma. The ovine model was selected for study because of similarity in the structure of the ovaries to those of the human (Gosden et al., 1994a). Fresh ovarian tissue and tissue which had been frozen in dimethyl sulphoxide to –196°C and thawed was autografted to the ovarian pedicle of ovarietomized lambs, at opposite sides. Approximately 3 months later, the animals were mated and two pregnancies were recorded, resulting in the birth of healthy lambs, one originating from an ovulation in a fresh graft and the other from a frozen–thawed graft.

The encouraging results reported from ovine studies justified an extension of the work to human tissue. Ovarian cortical biopsies were obtained from consenting patients and frozen in dimethyl sulphoxide or propylene glycol. Histological examination of the thawed tissue showed the presence of morphologically normal follicles although survival rates were not quantified and long-term survival in vivo cannot be predicted by this method (Hovatta et al., 1996). In addition, organ cultures of the frozen–thawed tissue for 4–21 days indicated that many of the follicles survived and demonstrated follicle growth over this time (Hovatta et al., 1997). The normality of frozen–thawed follicles was confirmed by studies in which enzymatically isolated human primordial follicles were stained for viability or analysed for ultrastructural damage using electron microscopy (Oktay et al., 1997). A comparison of follicles isolated from fresh and cryopreserved biopsies confirmed that there was no significant difference in viability (71.6% versus 71.5%) as assessed by these two techniques.

The cryopreservation and autografting of human ovarian tissue will cause a substantial reduction in the size of the follicle population within the graft and clinical trials are necessary to conduct definitive investigations into the extent of this loss. Human experiments are not feasible at such an early stage in research but an alternative in-vivo environment is provided by mice with severe combined immunodeficiency (SCID) (Bosma et al., 1983, 1989). SCID mice carry a mutation on chromosome 16 which causes impairment in T and B lymphocyte differentiation and normal immune function mediated by these cells is severely compromised, thereby allowing the acceptance of foreign tissue grafts. A proportion of the primordial germ cell population, together with growing follicles up to the antral stage of development, were observed in ovine and feline tissue recovered 9 months after grafting under the kidney capsules of SCID mice (Gosden et al., 1994b).

Similar success was reported with frozen–thawed marsot tissue grafted into a different immunodeficient model, the nude mouse. At autopsy grafts contained follicles at all stages of development and in comparable numbers to those found in freshly grafted control tissue (Candy et al., 1995). The immunodeficient model was subsequently used to investigate follicle survival in human tissue. Ovarian biopsies were frozen–thawed in different freezing media and xenografted under the kidney capsule of SCID mice for 18 days. On retrieval, follicle survival within the xenografts was found to range from 44 to 84% in the best cryoprotective media (Figure 1) (Newton et al., 1996).

Successful cryopreservation procedures require optimization of the cooling and warming rate and the inclusion of molar concentrations of cryoprotective agents to the freezing medium. The colligative properties of these solutes provide cryoprotection by depressing the freezing point, thus reducing the extent of ice formation at a given temperature and minimizing the build up of extracellular salt concentration and the risk of intracellular ice formation (Ashwood-Smith, 1986). Equilibration of cells with cryoprotective agent takes place prior to cooling but the cytotoxic nature of many solutes requires that the exposure time is short and carried out at a low temperature to minimize damage. The most ideal solutes are therefore those which penetrate cells rapidly.

The extent of follicle survival after freeze–thawing is, at least in part, determined by the type of cryoprotective agent included in the freezing medium. In human ovarian tissue, follicle survival rates after freeze–thawing and xenografting into immunodeficient mice were significantly higher in
tissue equilibrated with dimethyl sulphoxide, propylene glycol or ethylene glycol prior to cooling, than in tissue equilibrated in glycerol (Newton et al., 1996). Poor follicle survival was also reported in murine ovarian tissue after cryopreservation in glycerol (Candy et al., 1997a). One explanation for these observations is that glycerol is relatively viscous in comparison to other cryoprotective agents and may not permeate tissue as efficiently, therefore offering lower protection against freezing injury (Ashwood-Smith, 1986). In support of this theory, the number of morphologically normal follicles surviving cryopreservation was found to increase in murine tissue when the duration of equilibration with glycerol was extended from 5 to 60 min (Candy et al., 1997a). If the length of the incubation period with dimethyl sulphoxide, ethylene glycol or propylene glycol was extended, a similar trend was not observed. This probably reflects the fact that at room temperature these cryoprotective agents reach sufficient concentration within the murine tissue during the 5 min equilibration period.

To optimize the duration of the equilibration process for human ovarian tissue, the permeation rates of the different cryoprotective agents have been investigated. Cortical biopsies were exposed to different solutes for various periods of time at temperatures between 0 and 37°C (Thomas et al., 1997; Newton et al., 1998) and nuclear magnetic resonance spectroscopy was used to measure the relative rates of cryoprotectant uptake over time. At 4°C, a 30 min incubation in 1.5 M dimethyl sulphoxide or ethylene glycol was found to be the most efficient equilibration procedure for ovarian tissue prior to cooling (Figure 2) (Newton et al., 1998).

Strategies for restoring fertility with frozen–thawed tissue

In theory, frozen-banked ovarian tissue can be used in a number of ways to restore fertility (Figure 3). Insertion of cryopreserved ovarian tissue at the orthotopic site is the only method by which ‘natural’ fertility can be restored (Gosden and Aubard, 1996; Nugent et al., 1997). The laparoscopic insertion of cortical biopsies into a surgically resected ovary where they can be secured with sutures and/or tissue glue is one theoretical possibility. The benefits of this procedure are twofold: firstly, endocrine cycles should be restored, thus negating the requirements for hormone replacement therapy in patients with ovarian failure; secondly, natural fertility may be regained.

Grafting the thawed tissue at a heterotopic site with a rich vascular bed may be an alternative option. Endocrine cycles should still be restored but ovarian stimulation, follicle aspiration and IVF will be needed to conceive. Heterotopic implants of ovarian tissue could be supported at many locations in the body provided they are easily visualized, accessible and do not have a hepatic circulation which may destroy steroid hormones from the graft, reducing pituitary negative feedback, leading to graft hyperstimulation (Biskind and Biskind, 1949). Previous heterotopic sites have included the subcutaneous tissue of the left axilla and the abdominal wall; in both cases follicles were found

![Figure 2. The permeation rates of the cryoprotective agents (CPA): dimethyl sulphoxide (DMSO), ethylene glycol (EG), propylene glycol (PROH) and glycerol (GLY) through human ovarian tissue at 4°C. The permeation rates of DMSO and EG were equal but significantly greater than those of PROH and GLY which did not differ (P = 0.02) (Newton et al., 1998).](image-url)
Figure 3. The theoretical options for the restoration of fertility to cancer patients using frozen-thawed ovarian tissue.

In the recovered tissue (Leporrier et al., 1987; Marconi et al., 1997). Preliminary investigations have been carried out to quantify follicle survival in human ovarian tissue after heterotopic grafting. Cortical biopsies were used from consenting patients undergoing laparoscopy for assessment of suitability for reversal of sterilization. Samples were bisected and one half was grafted to the anterior surface of the uterus for ~14 weeks while the other half was histologically prepared for a control follicle count. The grafts were retrieved during reversal of sterilization, and histological examination indicated that ~30% of follicles survived in comparison to the numbers recorded in fresh non-grafted tissue (Nugent et al., 1998).

One concern of autografting frozen-banked tissue is how long the graft can be expected to function. Mathematical modelling suggests that follicle loss accelerates as the population size falls to <25,000 and that ovarian function is lost when the number is depleted to <1000 (Faddy et al., 1992). Even if a substantial proportion of follicles survive after frozen-thawed tissue is grafted into patients with ovarian failure, the total follicle population will still be low, such that ovarian function may only be regained for a short time. Murine studies have supported this theory: only 10 and 11% of ovariectomized animals orthotopically autografted with either fresh or frozen-thawed tissue, respectively, maintained fertility for four litters, compared to 100% of sham-operated controls (Gunasena et al., 1997).

In a similar study, the average number of litters from mice receiving fresh and frozen-thawed orthotopic grafts was 6.2 and 8.4, respectively, whilst the control females had 13 litters (Candy et al., 1997b). The results from both studies suggest that, although cryopreservation per se has little effect on the functional lifespan of the graft, follicle loss as a result of grafting significantly shortens the fertile period. More encouraging results were reported in ovine studies in which eight sheep were ovariectomized and grafted bilaterally with frozen-thawed ovarian tissue. Hormonal monitoring indicated that cyclicity continued throughout the study period (22 months) at the same frequency as in unoperated controls, although elevated follicle stimulating hormone (FSH) concentrations indicated ovarian failure was imminent (Baird et al., 1996). Ovine ovarian tissue is more comparable to the human, thus the results of the latter study are probably more clinically relevant. Similar studies are required in the human to ultimately determine the functional capacity of grafted, frozen-thawed tissue.

A second major concern with autografting cryopreserved tissue from cancer patients is whether the graft will contain malignant cells and thus reintroduce disease to patients in remission (Gosden et al., 1997; Shaw and Trounson, 1997). This presents a significant risk in patients with blood-borne diseases such as leukaemia, or those whose ovaries are involved in disease. These fears have been justified by animal studies in which fresh and frozen-thawed murine ovarian tissue, taken from donors with lymphoma and grafted into healthy animals, transmitted malignant disease resulting in mortality in 100 and 85% of hosts, respectively (Shaw et al., 1996). Nevertheless in many cancers, such as Wilms’ tumour and Hodgkin’s disease, metastases do not invade the ovary and frozen-banked tissue from these patients should carry low risk of inducing relapse. Even so, tissue should not be autografted until an efficient and reliable method of screening banked material for malignant cells is developed.

Transplantation into a different in-vivo environment is an alternative option to autografting frozen-banked tissue. Ovine, feline and primate ovarian material grafted beneath the renal capsules of immunodeficient mice was found to revascularize and follicle growth was recorded (Gosden et al., 1994b; Candy et al., 1995). Furthermore, follicles within human tissue grew to antral stages measuring up to 5 mm in diameter after 6 weeks of exogenous FSH stimulation in the renal capsules of hypogonadal SCID mice (Oktay et al., 1998b). In theory, these antral follicles could be aspirated and the oocytes harvested, matured and fertilized in vitro.
Ethical and moral complications mean that the procedure is unfeasible for human clinical application, but could prove useful for endangered species, transgenic animals and rare breeds (see later discussion).

Follicle isolation from ovarian tissue, for in-vivo or in-vitro growth to maturity, allows malignant cells to be ‘cleaned’ from the banked material of cancer patients. In animal studies, murine preantral follicles separated from the surrounding stroma by manual dissection have been suspended in collagen gel and cultured in vitro for 5 days prior to transfer to the kidney capsule of ovariectomized mice for in-vivo growth to maturity (Torrance et al., 1989; Telfer et al., 1990). The grafts restored endocrine function and oocytes harvested from Graafian follicles were successfully fertilized and underwent embryonic development. In addition, both fresh and frozen–thawed primordial follicles, isolated from the stroma, suspended in plasma clots and transferred to the ovaries of sterile hosts restored oestrogenic activity and fertility (Gosden, 1990; Carroll and Gosden, 1993).

In-vitro growth of isolated follicles to maturity has the advantage of avoiding surgical grafting procedures. Using a variety of isolation techniques and culture conditions, immature follicles have demonstrated extensive growth in vitro (Hartshorne, 1997). Murine tissue, teased apart with fine needles, yields many preantral follicles which can be grown to maturity in vitro (Qvist et al., 1990; Nayudu and Osborn, 1992; Cortvrindt et al., 1996a). Mature oocytes, ovulated from these follicles in response to luteinizing hormone, have been fertilized and normal embryonic development was reported (Cortvrindt et al., 1996a) which resulted in the birth of healthy pups following embryo transfer to pseudopregnant females (Spears et al., 1994). Similar developmental potential was also reported if the isolated murine follicles were cryopreserved prior to culture (Cortvrindt et al., 1996b).

Although manual dissection has proved successful, the isolation method is laborious, time consuming and difficult in species with a fibrous stroma such as the human. Enzymatic isolation is more efficient but has been suggested to damage the thecal layer and the basement membrane (Hirao et al., 1994; Hartshorne, 1997). Preantral follicles isolated from human, pig, hamster and mouse ovarian tissue by enzymatic digestion formed antral cavities in culture (Roy and Greenwald, 1989; Eppig and Schroeder, 1989; Roy and Tracey, 1993; Hirao et al., 1994; Abir et al., 1997) and the oocytes harvested from the murine follicles produced viable offspring following fertilization and embryo transfer to pseudopregnant females (Eppig and Schroeder, 1989). Frozen–thawed follicles of large animal species also maintain the ability to grow in culture. Preantral follicles enzymatically isolated from cryopreserved ovine tissue developed to antral stages up to 1 mm in diameter and maintained a three-dimensional structure during 30 days of in-vitro culture in serum-free medium (Figure 4). Oocytes were still viable at the end of in-vitro growth and their diameter was found to have increased significantly during the culture period (H.Newton, unpublished data).

The proportion of preantral follicles within the ovary is relatively small compared with the vast supply of primordial germ cells available (Gosden, 1995). To maximize the oocyte harvests from small ovarian biopsies, in-vitro cul-

Figure 4. (A) A preantral follicle isolated from frozen–thawed ovine tissue on day 0 of culture, bar = 80 µm. (B) After 17 days of in-vitro culture in serum-free medium the follicle has formed an antral cavity, increased in diameter to ~700 µm and maintained a three-dimensional structure. bar = 120 µm.
ture techniques must support growth of the primordial follicle population to maturity. In humans, the factors that initiate follicle growth are unknown and several months may be required for growth to antral sizes (Gougeon, 1986, 1996). Current in-vitro growth protocols are inadequate to sustain this duration of culture. In mice, an alternative two-step strategy has proved successful: for 8 days ovaries were placed in organ culture, during which time early follicular growth was initiated; the developing granulosa-oocyte complexes were then isolated and cultured in vitro for a further 14 days. Mature oocytes were subsequently collected, fertilized and embryos created were transferred to pseudopregnant females resulting in the birth of a viable pup (Eppig and O’Brien, 1996). Although the success rate was low, this strategy is likely to present the best option for growing human primordial germ cells, especially since stromal interactions may be important in early development. The initial stages of the technique have proved successful in large animal species where primordial follicle growth has been initiated in organ cultures of fetal bovine and primate tissue (Wandji et al., 1996, 1997).

**Practical aspects of ovarian tissue banking**

The potential benefits of freeze-banking ovarian tissue have stimulated much media interest and the first clinical cases have recently been reported in the scientific literature (Bahadur and Steele, 1996). Demand for the procedure is expected to increase, thus it is important to set guidelines determining which patients should be offered ovarian cryopreservation. These patients should be provided with counselling in which the experimental nature of the technique is explained; it should also be stressed that, although a substantial proportion of the follicle population is conserved by cryopreservation, no safe and efficient way is currently available for restoring fertility using the banked tissue. The patients are likely to be storing material for many years, thus it is important that they realize that the chances of a successful outcome are uncertain.

Issues concerning safe long-term banking of tissue have recently been highlighted by the reports that hepatitis B and microbial contamination can be transmitted within liquid nitrogen tanks and freezers (Tedder et al., 1995; Fountain et al., 1997). Patients should be screened prior to tissue banking and those who test positive for blood-borne viral infections should have their tissue stored in separate tanks. The possibility of storing material from paediatric patients raises the question of how long banked tissue should be stored. Currently, no time limit has been set for storing immature germ cells, but it is important to make sure the patient consents to an upper limit and also to the disposal of tissue in the event of death or mental incapacitation. An upper age limit should also be set for the use of banked tissue. Some women may view the procedure as a way of delaying childbirth, thus allowing them to pursue a career. Ethical and moral dilemmas, raised by helping older women to reproduce, especially since offspring may be orphaned at a relatively early age, suggest that the procedure should only be offered to patients with sound medical justification.

**Other applications for ovarian tissue cryopreservation**

Populations of many endangered species are currently maintained by captive breeding programmes. The small number of individuals involved in these projects can result in inbreeding depression and lowered species viability after a number of generations. If ovarian tissue from fertile donors or recently deceased animals were frozen-banked, immature follicles could be isolated for growth and maturation in vitro (Johnston et al., 1991) or in vivo in immunodeficient animals. Mature oocytes harvested could be fertilized and sporadically introduced into the breeding population to maintain genetic diversity in a rapidly dwindling gene pool (Ballou, 1992). Frozen spermatozoa and ovarian tissue would also allow the creation of a ‘frozen zoo’ whereby the gametes of many endangered species could be preserved indefinitely. If captive breeding programmes failed and species became extinct, these samples could be thawed and the embryos subsequently created could be transferred to surrogate mothers from closely related species. Harvesting mature oocytes from ovarian tissue for IVF could also be used to increase the population sizes of many important transgenic animals or rare animal breeds.

In summary, human studies have shown that a high proportion of the follicle population within ovarian tissue survives cryopreservation. To date there is no safe procedure for using this banked material to restore fertility and many of the techniques discussed are still in their infancy. Nevertheless, the field of reproductive biology is expanding rapidly and it is likely that the next few years will see significant advances in the technology, allowing the successful use of frozen-banked tissue for assisted reproduction.

**Acknowledgements**

The author gratefully acknowledges the support of the Leukaemia Research Fund.
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Received on February 20, 1998; accepted on June 1, 1998