Cryopreservation of human ovarian tissue

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As survival rates for young cancer patients continue to improve, protection against iatrogenic infertility caused by chemotherapy and/or radiotherapy assumes a higher priority. As things stand, women patients have few options to preserve their fertility while children have none at all.

For centuries it has been thought that a state of 'suspended animation' might be obtained through the application of extreme cold to living organisms. Such phenomena do occur in nature but, ordinarily, freezing is lethal. Improved understanding of the mechanisms involved has led to the development of cryopreservation methods for an impressive range of cells.

Cryopreservation below –130°C allows virtually indefinite storage of cells (liquid nitrogen is the usual holding medium 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 cryoprotectant chemicals (Wilmut, 1972). 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. To achieve this end, the cell must attain a state of equilibrium, in which the cryoprotectant and water concentrations, extracellularly and intracellularly, are equal (Mazur, 1984; Pegg, 1987). Equilibration of cells with cryoprotective agents takes place before cooling but the cryotoxic nature of many solutes requires that the exposure time is short and carried out at a low temperature to minimize the damage.

Formation of intracellular ice and a build up of salt in dehydrated cells are hazards of cryopreservation, although these hazards are minimized by optimal protocols using slow freezing and rapid rewarming. Alternatively, rapid freezing using very high molar strength cryoprotective agents (vitrification) can be an effective strategy for avoiding the formation of ice crystals, and the convenience and speed of this approach are appealing.

Storage of tissue is an enigma, as most tissues are heterogeneous and protocols must strike a compromise between optimal conditions for the different types of cells. In addition, problems can arise when ice forms extracellularly, because it can cleave tissues into fragments or cause extravasation from damaged blood vessels in anastomosed grafts (Ashwood-Smith, 1986). The permeability to water of each cell type present may be different and hence there may be no satisfactory set of cooling and rewarming conditions that suits all of them. In practice, the theoretical optima are not usually known for all cell types and protocols are devised more on the basis of empirical observations than theory.

BACKGROUND

Pioneering attempts to cryopreserve gonadal tissue were described in the 1950s when the primary aim was to restore endocrine function to ovariectomized mice and rats (Parkes and Smith, 1953; Parkes, 1957). Mouse ovaries were cooled to –79°C in a glycerol–saline mixture before thawing and autografting to subcutaneous sites. Graft survival was assessed by the resumption of oestrous cycles as indicated by cornification of vaginal epithelial cells (Parkes and Smith, 1954). In rats, cycles ceased within 2 days of ovariectomy, but the subsequent insertion of fresh or frozen-thawed grafts restored endocrine function within 7–8 days and 2–3 weeks, respectively (Deanesly and Parkes, 1956). More dramatically still, mice that had been ovariectomized and received ovarian grafts conceived and delivered normal pups (Parrott, 1960).

Since these early experiments, significant advances have been made in the field of cry-

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otechnology, most importantly the introduction of controlled rate freezing apparatus and the development of more efficient cryoprotectants. 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 before 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, primordial follicles extracted from mouse ovaries and cryopreserved using dimethyl sulphoxide (DMSO) were able to restore fertility to oophorectomized animals after transplantation in plasma clots to the vacant ovarian bursa (Carroll and Gosden, 1993). The grafts of frozen-thawed cells re-organized into morphologically distinguishable ovaries, and produced signs of oestrogenic activity (Carroll et al, 1990a; Carroll and Gosden, 1993). More recently, significant success has been achieved in producing live offspring by natural mating after transfer of cryopreserved mouse ovaries to the ovarian bursa of ovariectomized recipient females (Gunasena et al, 1997a,b; Sztein et al, 1998). Moreover, the pups born following this process were also fertile having a normal litter size (Gunasena et al, 1997a). Live births after autografting have also been reported for Wistar rats using DMSO or ethylene glycol as cryoprotectants (Aubard et al, 1998).

It remained doubtful whether this technique would prove as successful in human tissue, which has a dense fibrous stroma. In a novel experiment the sheep model was selected for investigation because of its similarity to the human ovary both in size and composition (Gosden et al, 1994a). Fresh ovarian tissue was frozen in DMSO, thawed and autografted at opposite sides to the ovarian pedicle of ovariectomized lambs. The ovaries were not frozen in toto, but after preparation of thin cortical slices to provide optimal conditions for penetration with cryoprotectants during freezing and better perfusion after transplantation. Approximately 3 months later, the animals were mated and two pregnancies were recorded, resulting in the birth of healthy lambs, one originating from ovulation in a fresh graft and the other from a frozen-thawed graft. Meanwhile, other authors gained experience in ovarian auto-grafting in sheep, and did not find any damage to either primordial, primary, secondary or antral follicles after cryopreservation using DMSO (Salle et al, 1998).

EFFECT OF CHEMO/RADIOTHERAPY ON OVARIAN FUNCTION

The ovarian cortex of young women contains several hundred thousand primordial follicles (Knobil and Neill, 1994); even small pieces (1 mm^3) may contain several hundred follicles. Oocytes in primordial follicles are smaller, possess fewer organelles, and have no zona or cortical granules, so they are potentially easier to freeze (Oktay et al, 1998a). Aggressive chemotherapy and/or radiotherapy for the treatment of cancer can severely deplete the follicular store, often compromizing ovarian function (Sanders et al, 1996).

The age of the patient and the type of treatment play a significant role in the risk of ovarian failure (Marcello et al, 1990). This gonadotoxic effect is of particular concern because over recent years improvements in the treatment of a wide range of both solid and haematological malignancies have lead to significant increases in long-term survival rates of patients, especially of younger ages (Boring et al, 1994). Estimates indicate that by the beginning of the new millennium, 1 in 1000 adults will be survivors of childhood malignancy (Birch et al, 1988).

A survey of 38 000 male and female patients, or their partners, who received high dose chemotherapy or total body irradiation with allogeneic or autologous stem cell transplantation found extremely low fecundity rates (129 pregnancies) (Apperley and Reddy, 1995). Even if ovarian failure does not occur immediately, there is a substantial risk of premature menopause, particularly in those aged over 30 years (Whitehead et al, 1983). One mathematical model estimated that a 90% reduction of the germ cell population before the age of 14 years 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 before gonadotoxic therapy, it is wise to safeguard the fecundity of these patients before treatment. Harvesting and storing primordial follicles in bulk before cancer treatment and returning them by transplantation after full remission of the disease, or in vitro growth after freeze-storage offers a potential option of restoring fertility to such patients.

ADVANTAGES OF OVARIAN TISSUE CRYOPRESERVATION

The advantages of successful isolation and cryopreservation of human primordial follicles are many (*Table 1*). Ovarian biopsies can be obtained without delay and the lengthy and expensive hormonal priming required for in-vitro fertilization (IVF) is redundant. Although the prospect of growing human primordial follicles to maturity in vitro is still a long way off, the possibility of in vitro growth of frozen-thawed primordial follicles offers the potential of storing large numbers of female gametes for future development and research, and opens the door for ovarian tissue banking for cancer patients. Even paediatric patients can benefit, where tissue storage may be the only option available for preserving fertility. Indeed, the large numbers of primordial follicles and relatively quiescent state of prepubertal ovaries should increase the chances of success.

RESTORING FERTILITY WITH FROZEN-THAWED OVARIAN TISSUE

In theory, frozen-banked ovarian tissue can be used in a number of ways to restore fertility. Insertion of cryopreserved ovarian tissue at the orthotopic site is the only method by which ‘natural’ fertility can be restored (Nugent et al, 1997). The laparoscopic insertion of cortical biopsies into the ovarian fossa of a surgically resected ovary where they can be secured with sutures and/or tissue glue is one theoretical possibility. Alternatively, cortical slices could be placed on an intact ovary with a preserved vasculature; animal experiments demonstrated that this approach is effective (Gosden, 1990). The benefits of this procedure are twofold: first, endocrine cycles should be restored, thus negating the requirements for hormone replacement therapy in patients with ovarian failure and second, natural fertility may be regained.

TABLE 1.
Advantages and disadvantages of freeze storing ovarian cortical biopsies

Advantages	Ovarian biopsies could be obtained without delay and the lengthy hormonal stimulation of in-vitro fertilization is redundant
	Cortical biopsies are rich in primordial follicles in which the oocytes are small, less differentiated and lack either a spindle or zona pellucida
	The in vitro growth phase of frozen-thawed follicles provides an opportunity for repairing any damage sustained during freezing
	The possibility of in vitro growth or grafting of frozen-thawed tissue offers the potential of storing large number of female gamete for future development and research
	Freeze-storing ovarian tissue offers the opportunity of experimental investigation of ovarian function
Disadvantages	Optimal freezing conditions may differ between cell types
	Frozen-thawed tissue requires grafting or in vitro growth to reach maturity
	In vitro growth of human primordial follicles to maturity requires months in culture and as yet unsuccessful
	Autografting of cryopreserved tissue from cancer patients has the potential risk of re-introducing disease
	Follicle loss as a result of post-grafting ischaemia significantly shortens the life span of the graft

Small pieces of cortical tissue may be more easily grafted at heterotopic sites, potentially under local anaesthesia, allowing oocyte retrieval and IVF. 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 in the recovered tissue (Marconi et al, 1997; Von Eye Corleta et al, 1998). The advantage of these sites is that it is easy to monitor follicle growth for egg retrieval by aspiration and to remove tissue remnants later on.

Preliminary investigations have been carried out to quantify follicle survival in human tissue after heterotopic grafting. Cortical biopsies from consenting patients undergoing laparoscopy were bisected and one half was grafted to the anterior surface of the uterus for ~14weeks while the other half was histologically prepared for a control follicle count. When the grafts were retrieved, histological examination indicated that ~30% of follicles survived in comparison to the numbers recorded in fresh non-grafted tissue (Nugent et al, 1998a). Recently, frozen-thawed human cortical strips were grafted in a pocket in the pelvic wall of a patient. Following gonadotrophin stimulation, a dominant follicle emerged in the grafted tissue which continued to grow following cessation of hormone treatment (Oktay et al, 1999).

DIFFICULTIES IN GRAFTING OVARIAN TISSUE

One concern of autografting frozen-banked tissue is how long the graft can be expected to function. Mathematical modelling suggests that the follicle loss accelerates as the population size falls to <25000 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 auto-grafted with either fresh or frozen-thawed tissue, respectively, maintained fertility for four litters, compared with 100% of sham-operated controls (Gunasena et al, 1997a). 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, while the control females had 13 litters (Candy et al, 1997). 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 post-grafting ischaemia, and not damage of germ cells by freezing and thawing, significantly shortens the fertile period (Aubard et al, 1999).

More encouraging results were recently reported when fresh and frozen ovaries were grafted orthotopically into ovariectomized mice recipients (Candy et al, 2000). Recipients of fresh and frozen ovarian grafts and unoperated controls reproduced continuously for up to 11 months and had a similar number of litters (6.2, 8.4 and 6.3 respectively) with a similar number of pups per litter (5.4, 6.3 and 6.3 respectively). In contrast to previous reports of reduced litter sizes and a shortened reproductive lifespan after orthotopic grafting of fresh and cryopreserved tissue, in the above study the long-term reproductive performance of recipients of fresh and frozen ovaries was similar to untreated control females. This is the first conclusive demonstration that frozen ovarian grafts may continue functioning for the normal reproductive lifespan of a species.

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 (22 months) at the same frequency as in unoperated controls, although elevated follicle-stimulating hormone (FSH) and inhibin concentrations resembled the profiles of older animals (Baird et al, 1999). 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 humans to ultimately determine the functional capacity of grafted, frozen-thawed tissue. Meanwhile, reducing the size of the grafts (Krohn, 1977), or pre-treating the grafts with compounds such as vascular endothelial growth factor or fibroblast growth factor, which stimulate vascularization (Redmer and Reynolds, 1996), may be beneficial. In addition, administration of antioxidants following grafting may also be helpful in reducing the lipid peroxide concentrations, thus improving the follicle survival rates (Nugent et al, 1998b).

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. This presents a significant risk in patients with blood-borne diseases such as leukaemia, or those whose ovaries are involved in disease (breast cancer). Transmission of lymphoma cells by this approach was shown in an AKR mouse model (Shaw et al, 1996). In this model, pieces of ovarian tissue from female mice, which had died from end-stage lymphoma, were transplanted either fresh or after cryopreservation. All these animals were free from lymphoma at the time of transplantation, and all died within 20 days of grafting. Ovaries and surrounding tissues contained large homogeneous populations of lymphoid cells, which could be shown to have arisen from the grafted ovarian tissue by genetic analysis. This was true for animals grafted with fresh and cryopreserved tissue.

To date, there is insufficient information available in human subjects, although preliminary studies in patients with Hodgkin's disease indicates the risks of ovarian relapse are negligible or absent (Meirow et al, 1998). Furthermore, highly sensitive tests, such as fluorescent techniques or polymerase chain reaction (PCR) should enable the testing of ovarian tissue for the presence of malignant cells before cryopreservation (Moomjy and Rosenwaks, 1998).

Transplantation into a different in vivo environment is an alternative option to autografting frozen-banked tissue. Severe combined immunodeficiency (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 compromized, thereby allowing acceptance of foreign grafts (Bosma et al, 1983, 1989). 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 xenografting under the kidney capsules of SCID mice (Gosden et al, 1994b). Similar success was reported with frozen-thawed marmoset 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 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 (Newton et al, 1996). Furthermore, follicles within human tissue grew to antral stages measuring up to 5 mm in diameter after 6weeks 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.

PRACTICAL ISSUES OF OVARIAN TISSUE CRYOPRESERVATION

Small specimens can be obtained from the ovarian cortex using a device known as a 'biopter' (Casmed, Branstead, Surrey, UK), which is adapted for laparoscopic use from a similar instrument used for vulval punch biopsies (Meirow et al, 1999). Since the biopsies are relatively superficial, there is little bleeding and there have been no reported problems with post-operative adhesion formation. Alternatively, a whole ovary can be recovered, which facilitates preparation of tissue slices large enough for orthotopic autografting.

Laparoscopic oophorectomy is now a well-established surgical technique and, although there are a number of ways to perform the procedure, it is important to keep the integrity of the Fallopian tube and mesosalpinx intact when future natural fertility is the objective. The ovarian ligament is divided by bipolar diathermy and scissors, restricting the burn to the ligaments. An EndoGIA (USA Surgical Corporation, Conneticut, USA) gun is carefully placed over the hilus of the ovary, and the Fallopian tube retracted away from the operative field. Once the gun is fired and the ovary removed, a neat row of small staples remains, indicating a potential future orthotopic graft site. Piercing the ovarian capsule with scissors releases the medulla making the ovary easier to remove in a small bag through a 12 mm laparoscopic port.

The importance of preserving uterine function must not be overlooked, since this organ is vulnerable to high-dose irradiation in childhood. Unless surrogacy is considered, there is little point in storing ovaries because replacement hormones can treat menopausal symptoms. Other causes of concern are to whom should such services be offered and what are the chances that the patient will survive long enough to become a functional parent? There are no easy answers to these questions and each patient should be considered on an individual basis. People considering ovarian tissue storage need to be reminded of the experimental nature of the procedure until clinical utility is proven. 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 the 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 banks and freezers (Tedder et al, 1995; Fountain et al, 1997). Patients should be screened before tissue banking and those who test positive for blood-borne viral infections 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 (or her guardian) consents to an upper limit and also to the disposal of tissue in the event of death or mental incapacitation. Recently the British Fertility Society has published its recommendations for good practice on the storage of ovarian tissue to assist professionals involved in gonadal tissue storage (Nugent et al, 2000).

CONCLUSIONS

Human studies have shown that a high proportion of the follicle population within the ovarian tissue survives cryopreservation. To date, there are no safe and practical methods for using this banked material to restore fertility. 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 successful use of frozen-banked tissue for assisted reproduction. In addition, clinical, psychological and ethical factors must be considered and contributions from specialists in all the relevant disciplines will be required for full success. **HM**

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KEY POINTS

- The clinical need to preserve the fertility potential of young women and children with malignant diseases has become more urgent in view of the improved long-term survival rates.
- The storage of ovarian tissue could be considered only if the patient understands the present limitations and experimental nature of the technology.
- The main options being considered for frozen-thawed ovarian tissue are via transplantation or cell culture.
- Autografting, which has the potential advantage of restoring natural fertility, may risk re-introduction of malignant disease.
- Xenotransplantation of human ovarian tissue to immunodefient host animals is a theoretical possibility, but more appropriate as an experimental model rather than as a clinical tool.