Low temperature storage and grafting of human ovarian tissue

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Ovarian tissue storage at low temperatures is a promising new method for protecting young cancer patients from the sterilizing effects of chemotherapy and/or radiotherapy. Tissue can be stored and returned to the body in due course as a thin cortical graft since the primordial follicles are distributed peripherally and are relatively resistant to ischaemia. Slices of tissue donated by healthy patients were trimmed to a uniform size and preserved by slow freezing to liquid nitrogen temperatures for up to 2 months in one of the following cryoprotectants: dimethylsulphoxide, ethylene glycol, glycerol, propylene glycol. Their viability was assessed by counting follicles in histological sections 18 days after grafting under the kidney capsules of severe combined immunodeficiency (SCID) mice, and the results were expressed as percentages of the numbers in comparable pieces of ungrafted tissue. While only 10% of the total number of follicles was found in the glycerol group compared to controls, significantly higher percentages (44–84%) survived cryopreservation in the other media. The tissues were sterile when frozen and thawed without a cryoprotectant. These results suggest that if comparable results could be achieved by autografting, a patient's fertility should be safeguarded from cytotoxic treatment.
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Introduction

As long-term survival rates for young people with malignant diseases steadily improve, the long-term effects of chemotherapy and radiation on the gonads assume greater significance and are stimulating fresh efforts to preserve fertility (Ash, 1980; Apperley and Reddy, 1995). Sometimes there is only a temporary lull in spermatogenesis or menstrual rhythmicity, but when treatment is particularly aggressive or the patient is >30 years of age the risks of permanent sterility rise. A man's semen can be cryopreserved so that his partner can have

artificial insemination and women patients too have options for safeguarding fertility, but none of them are rosy. Oophoropexy is sometimes helpful in avoiding exposure to the radiation field (Ray *et al.*, 1970; Hunter *et al.*, 1980), but it gives no protection against alkylating agents and the ovaries may absorb scattered rays. What is more, conception may still require clinical assistance even if this manoeuvre is successful.

Cryopreservation of germ cells is an attractive concept but only a handful of clinical pregnancies have been established so far with human oocytes, even in healthy women (e.g. Chen, 1986). What is more, there is cause for concern that cooling may create more chromosome anomalies (Vincent and Johnson, 1992), and so clinical practice has been suspended pending further studies and information. Freeze-storage of embryos is much more successful and is occasionally used before a woman undergoes myelo-ablative treatment (Atkinson *et al.*, 1994), but the procedure is costly, carries no guarantee of success and may create 'orphan embryos'. Egg donation too is potentially available for sterilized women, but it is more desirable to preserve natural fertility whenever possible.

If ovarian tissue could be cryopreserved before patients receive cancer treatment and then returned as a graft when they are in full remission, the natural state might be restored and conception achieved *in vivo* with their own germ cells. The feasibility was first shown many years ago in laboratory animals and was recently demonstrated in farm animals, whose ovaries more closely resemble those of humans (Gosden *et al*, 1994a). Autografting may be contraindicated, however, if cancer treatment harms the reproductive tract or the ovaries are involved in the disease, and it is better reserved for conditions such as Hodgkin's disease and Wilm's tumour in which these risks are slim. The risks of transmitting disease in grafted tissue carrying malignant cells could, of course, be minimized or eliminated by growing the oocytes *in vitro* from the primordial stage, but this goal is still a distant prospect in humans, though the potential has recently been demonstrated by experiments in mice (Eppig and O'Brien, 1996).

In this study we investigated methods for storing human ovarian tissue at liquid nitrogen temperature by comparing four cryoprotectants. The viability of primordial follicles was assessed after grafting frozen–thawed tissue slices into immunologically tolerant animals. The results suggest that germ cell banking is a practicable proposition.

Materials and methods

Biopsies of ovarian cortex <2 mm thick were obtained from eight consenting patients aged 17–31 years who were undergoing either elective Caesarean section or routine gynaecological surgery. The

study was approved by the local ethics committees in Leeds, UK. Uniformly smooth areas of cortex lacking prominent follicles or luteal tissue were selected for biopsy. The tissues were transferred to dishes containing sterile Leibovitz L-15 medium and cut into 1 mm³ cubes with the ovarian epithelium on one surface. They were assigned randomly to five groups of equal size. One group was fixed immediately in 4% paraformaldehyde for histological examination and served as a control so that the initial numbers of small follicles could be estimated.

To aliquots of Leibovitz medium containing 10% fetal calf serum one of the following substances was added to make a final concentration of 1.5 M: ethylene glycol, dimethylsulphoxide (DMSO), propylene glycol, glycerol. These cryoprotective media were pipetted into separate 1 ml cryogenic vials at 4°C to which the remaining tissues were transferred. The vials were gently rolled (1 Hz) for 30 min to promote rapid equilibration. Additional specimens were frozen in medium without cryoprotectant. The vials were cooled in a programmable freezer as follows (Kryo 10, Planar Instruments): (i) cooled at 2°C/min to −9°C; (ii) seeded manually; (iii) cooled at 0.3°C/min to −40°C; (iv) cooled to −140°C at the higher rate of 10°C/min; (v) plunged into liquid nitrogen and stored at this temperature for up to 2 months. The vials were thawed individually by agitating in water at room temperature (~100°C/min). The cryoprotectant was quickly removed from the tissue by repeated washing in saline and fresh Leibovitz medium.

The tissues were grafted into immunodeficient female mice carrying the SCID mutation (Bosma *et al.*, 1983) which were housed in positive pressure isolators and provided with sterile food and water. Mice aged 8–12 weeks were anaesthetized with a halothane–oxygen mixture and their kidneys were exposed in turn via a single mid-line skin incision and two lateral incisions in the body wall. Two ovarian grafts from different treatment groups were inserted at opposite poles of the kidney immediately under the capsule. After closing the wounds the animals were returned to the isolators where they all made a full recovery. Four additional animals were grafted with fresh ovarian tissue from a 17-year-old patient to estimate the fraction of follicles lost by the grafting procedure alone.

The animals were killed 18 days post-operatively to recover the grafts. Each graft was trimmed free of most adhering renal tissue, fixed in paraformaldehyde and prepared as 4 μm serial histological sections stained with haematoxylin and eosin. Control tissue was prepared similarly, and all the slides were coded to avoid observer bias. Every section was examined at a magnification of ×100 under the microscope and the single distinct nucleolus within the oocyte was used as a marker for counting. The data were tested for statistical significance by analysis of variance, the *t*-test using a Bonferroni adjustment of significance and χ^2 test.

Results

At autopsy, 100 out of 102 grafts could be identified and recovered from the original implant sites. The grafts were distinguishable from surrounding host kidney tissue by their pale coloration. Histological examination confirmed that they were vascularized, except in occasional fibrotic foci. The number of xenografts which still contained at least one primordial follicle ranged from 84 to 96% after freezing and thawing in DMSO or propylene glycol or ethylene glycol (Figure 1). These survival rates were all significantly higher than those for the group cryopreserved with glycerol (35%, $P < 0.0001$). A total of 27 (96%) out of the 28 fresh grafts still contained follicles, which is comparable to the best results
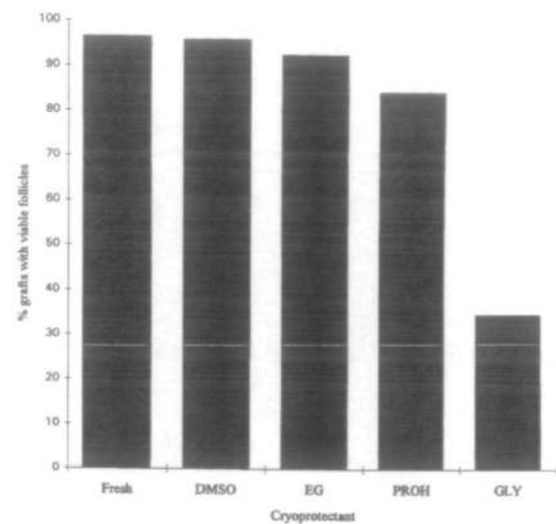

Figure 1. The percentage of ovarian xenografts with viable follicles after freezing and thawing in different cryoprotective agents. $n = 22$–28 grafts per group. DMSO = dimethylsulphoxide; EG = ethylene glycol; PROH = propylene glycol; GLY = glycerol.
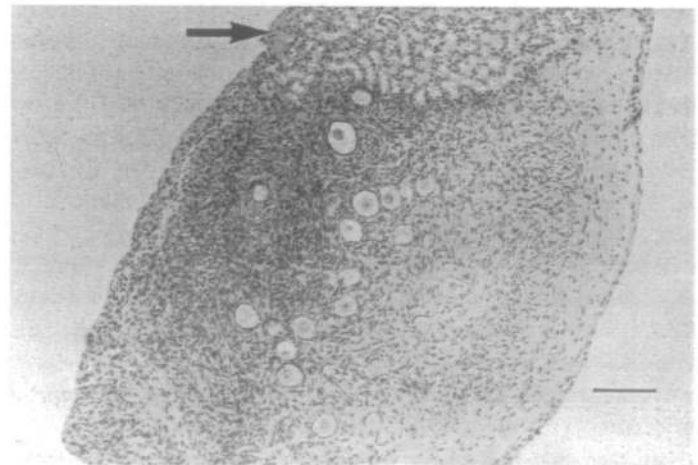

Figure 2. The histological appearance of human ovarian tissue after freezing and thawing in ethylene glycol, 18 days after grafting into SCID mice. Healthy-looking primordial follicles embedded in stroma and a portion of the host kidney tissue can be seen (arrow). Bar = 67 μm; haematoxylin and eosin stain.

obtained with cryopreservation. In every case where tissue was frozen without a cryoprotectant the graft was minute, sterile and composed of only traces of fibrous tissue.

Cryopreserved grafts consisted mainly of healthy stromal cells with virtually no signs of active necrosis (Figure 2). Primordial follicles were widely dispersed throughout the stroma; they measured 30–40 μm in diameter, with a normal oocyte and a single layer of pregranulosa cells. Follicles at more advanced stages of development were rare and none had more than three granulosa cell layers.

The numbers of follicles per graft were highly variable in all groups (Figure 3a). Grafts which were frozen–thawed in DMSO, ethylene glycol and propylene glycol contained on average 16–31 follicles per graft; these numbers were not
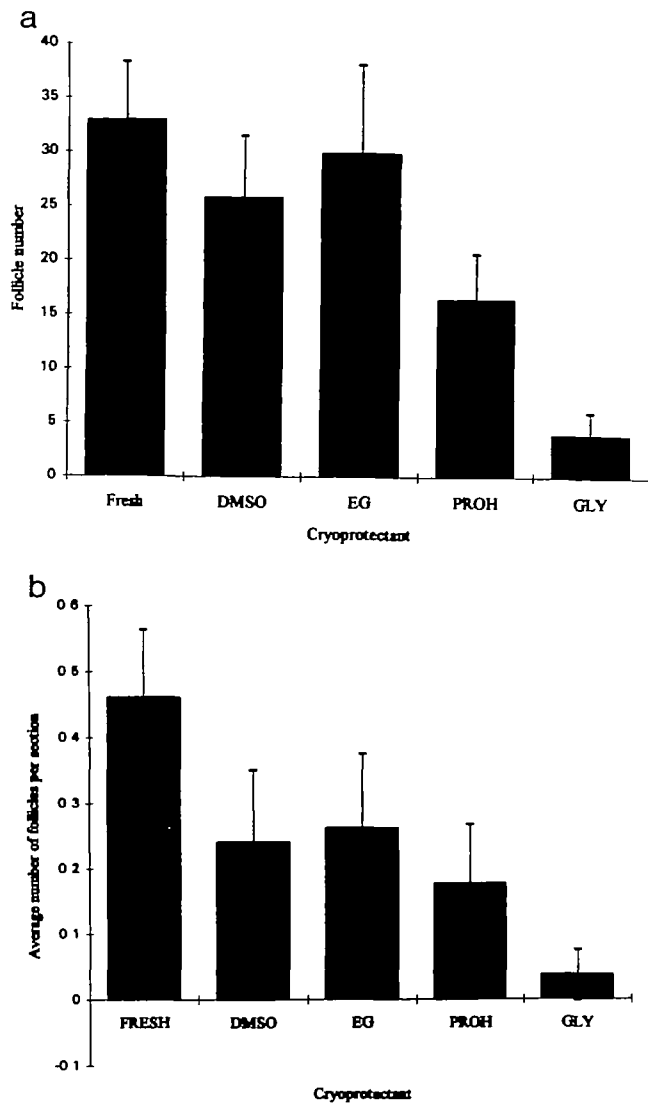
Figure 3. (a) A comparison of the number of follicles in human ovarian tissue frozen–thawed in different cryoprotective solutions and xenografted into SCID mice. n = 23–28 grafts per group (b) A comparison of the density of follicles per section of human ovarian tissue frozen–thawed in different cryoprotective solutions and xenografted into SCID mice n = 23–28 grafts per group. DMSO = dimethylsulphoxide; EG = ethylene glycol; PROH = propylene glycol, GLY = glycerol

Table I. Percentage follicle survival in human ovarian tissue after freeze–thawing in different cryoprotectants and grafting into SCID mice

	Fresh tissue (control)	Dimethyl sulphoxide	Ethylene glycol	Propylene glycol	Glycerol
Follicle survival (%)	100	74	84	44	10

significantly different, though they were higher than in the glycerol group (P < 0.05). These differences were reflected in the rates of follicle survival in relation to the numbers in ungrafted control tissue, which were standardized to 100% (Table I). When expressed in this way, the best survival rate

(84%) was obtained in ethylene glycol and the worst in glycerol (10%). A total of 74% of the follicles survived in the grafted fresh tissue, although the data are not strictly comparable with the other grafts because they were based on a single donor.

The data were also expressed as the average number of follicles per section as an indication of follicle density and, hence, of the relative survival of follicular and stromal cells A statistically significant difference existed between groups (P < 0.001), which was mainly accounted for by a lower density in the glycerol group (Figure 3b). It was not possible to compare the sizes of the grafts because they were small and irregular in shape.

Discussion

The ovary is endowed with a store of primordial follicles before birth which cannot be replenished afterwards. The prospective number of menstrual cycles and timing of menopause are largely determined, therefore, by the size of the follicle store and its subsequent rate of attrition, setting a high priority for the conservation of germ cells in healthy women and cancer patients alike (Faddy and Gosden, 1995). Storage of ovarian tissue at low temperatures is an attractive strategy in theory because it offers the hope of restoring natural fertility by autografting the tissue once a patient has completed her course of sterilizing therapy. It is likely to be several years, however, before the techniques are fully validated when ovarian tissue is restored to the first patients.

Our results of xenografting frozen–thawed human tissue, which have produced follicle survival rates in the region of 50–80%, are encouraging because it is likely that human autografts will be at least, if not more, successful. In clinical practice, rather larger slices of tissue are likely to be used, as was the case in the aforementioned studies of sheep in which cryopreserved autografts restored fertility (Gosden et al.., 1994a). If the thickness of the slices is similar, cryopreservation and revascularization should be equally effective in large and miniature grafts, and good rates of follicular survival can be expected. This technology is emerging slowly, but it has been borne out by the results of animal experiments over many years

Pioneering work was carried out in London as long ago as the 1950s. Rat ovaries were frozen to -79°C in a glycerol–saline mixture and resumed follicle growth and oestrogen secretion after thawing and grafting into castrated hosts (Deanesly, 1957; Parkes, 1957). More dramatically still, mice that had been ovariectomized and received ovarian isografts that had been stored in the same way conceived and delivered normal pups (Parrott, 1960). However, the technology was ahead of the applications and there was only a brief flurry of interest in this work at the time.

Remarkable improvements in prognosis for many young cancer patients give new impetus to tissue banking, and advances in cryotechnology over the years have made prospects even brighter. More efficient cryoprotectants and the lower temperatures of liquid nitrogen have contributed to successful storage of murine ovarian tissue or isolated primordial follicles (Carroll and Gosden, 1993, Harp et al., 1994). In both cases,

follicles resumed growth and fertility returned after isografting to sterilized host animals. Despite these successes, doubts remained as to whether the human ovary could be cryopreserved because it is bulkier, more fibrous and the primordial follicles are more widely dispersed than in either rodents or marmoset monkeys, whose ovaries have also been cryopreserved successfully (Candy *et al.*, 1995). The restoration of fertility to oophorectomized sheep was reassuring because their ovaries resembled those of humans more closely, but experimental proof of the technique using tissue from the ovaries of humans or Old World monkeys has been needed.

Ovarian xenografts under the renal capsules of immunodeficient mice were used so that the viability of human ovarian tissue could be tested *in vivo* at a site in which good survival and development are possible (Gosden *et al.*, 1994b; Wade and Gosden, 1994; Candy *et al.*, 1995). The main drawback of this model is that fertility cannot be tested *in vivo*, and invitro fertilization of the oocytes recovered from grafts is ruled out because human primordial follicles may require many months of growth before they reach maturity (Gougeon, 1986). Nevertheless, the primordial follicles appeared to be cytologically normal and even the occasional growing follicle was present. It is probable that follicles that have weathered the rigours of cryopreservation and grafting to the stage at which the tissue has become revascularized will be long-term survivors.

The results show that under certain conditions the majority of follicles can survive and not just an odd individual at a favourable developmental stage or location. While strict comparisons are unjustified, such high rates of survival indicate that immature follicles may be more tolerant of cryopreservation than secondary oocytes. In theory, primordial follicles should be better suited to cryopreservation and grafting because they are small, lack a zona pellucida and are relatively metabolically quiescent and undifferentiated.

A cryoprotectant was essential for survival after freezing and thawing, and results varied with the substances tested. There were no significant differences between the results with ethylene glycol, DMSO and propylene glycol despite a large number of observations, but survival in glycerol was poor. Results may improve with a longer period of immersion to equilibrate tissue with the cryoprotectant; 30 min is not necessarily optimal but was chosen as a compromise between toxicity and sufficient penetration. Clearly, there are many permutations of the freeze–thaw protocol that could be tested in search of optimal conditions. However some 26% of the primordial follicles were lost as a result of grafting *per se*, which presumably reflects the acute effects of ischaemia, since virtually all of the follicles appeared healthy at the time of autopsy. It will therefore be important to investigate whether oxidative stress occurs as a result of ischaemia and reperfusion after grafting and whether antioxidants moderate any resultant cellular injury. Secondly, it will be important to minimize the period of ischaemia, since more follicles are lost at this stage than by freezing and thawing. Fortunately, the natural abundance of angiogenic factors (Koos, 1989) favours a rapid revascularization of ovarian grafts and the levels of pituitary gonadotrophins and ovarian steroids may also play a role (Sato

et al., 1982; Dissen *et al.*, 1994). There is therefore scope to improve results by controlling endocrine and/or antioxidant factors.

While there is room for improving both cryopreservation and grafting procedures, the results are sufficiently encouraging that tissue banking can now be considered as a way of conserving fecundity in appropriate cases. The advantages are many. Ovarian biopsies can be obtained without delay and the lengthy and expensive hormonal priming required for IVF is redundant. If autografting to an orthotopic site succeeds in restoring ovulatory menstrual cycles, hormonal replacement therapy and medical intervention in the process of conception will not be needed. Even child patients can benefit, and tissue storage is in some cases the only option for preserving fecundity. Indeed, the large numbers of primordial follicles and relatively quiescent state of prepubertal ovaries should increase the chances of success, although paediatric applications highlight the question of how long tissue should be stored.

Acknowledgements

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