

Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol–sucrose as cryoprotectants

Outi Hovatta¹, Rene Silye, Thomas Krausz, Ronit Abir, Raul Margara, Geoffrey Trew, Amir Lass and Robert M.L. Winston

Institute of Obstetrics and Gynaecology and Department of Pathology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

¹To whom correspondence should be addressed at: Infertility Clinic, The Family Federation of Finland, Kalevankatu 16, FIN-00100 Helsinki, Finland

Pieces of ovarian cortical tissue (0.3–2 mm in diameter) were obtained during gynaecological operations by biopsy or as a result of oophorectomy from 19 women aged 19–44 years. The tissue was frozen in a programmable freezer using one of two different cryoprotectants, either 1.5 M dimethylsulphoxide (DMSO), or a combination of 1,2-propanediol (1.5 M) and sucrose (0.1 M). After cryopreservation lasting from 24 h to 5 weeks, the ovarian pieces were thawed and studied histologically. Specimens taken before and after cryopreservation with either protectant showed no signs of tissue necrosis. Follicles at similar developmental stages were found before and after freezing. The proportions of follicles showing signs of atresia, 27% in the non-frozen tissue and 19% in the frozen-thawed tissue, were not significantly different. Oocytes, too, had the same appearance after freezing and thawing with both cryoprotectants as was seen in the specimens taken before freezing. These results suggest that cryopreservation of human ovarian tissue is feasible. However, the normality of the oocytes taken from tissue which has been frozen still needs to be established. Cryopreservation of ovarian tissue would be potentially an excellent method for storage of human oocytes once methods for their maturation *in vitro* have been developed.

Key words: cryopreservation/dimethylsulphoxide/ovarian follicles/ovarian tissue/propanediol

Introduction

Cryopreservation of oocytes could benefit many infertile patients. Cryopreservation of ovarian tissue before radiotherapy or chemotherapy might allow later replantation of ovarian cortex and restoration of fertility after cancer treatment in young women. If maturation of oocytes *in vitro* from thawed cryopreserved tissue were possible, many of the problems which limit the usefulness of egg donation would also be solved. Once techniques for in-vitro maturation of follicles and eggs have been developed and are reliable, frozen storage

of ovarian tissue could revolutionize treatment by in-vitro fertilization (IVF). Patients would no longer be required to undergo ovulation induction with exogenous gonadotrophins, and the clinical monitoring which makes IVF so demanding for patients would be greatly simplified. IVF would be cheaper and the side-effects from stimulatory drugs avoided.

After replantation of frozen–thawed ovarian grafts, successful pregnancies in mice were reported by Parrott (1960). In these early experiments grafts were stored at -79°C and glycerol was used as a cryoprotectant. Carroll and Gosden (1993) transplanted isolated mouse primordial follicles after cryopreservation using dimethylsulphoxide (DMSO). The recipient site used was the ovarian bursa, and normal offspring were born. Extra-ovarian maturation of cryopreserved mouse primary follicles had already been successful (Carroll *et al.*, 1990).

Sheep have been similarly treated. Ovarian tissue slices were preserved in liquid nitrogen after using DMSO as a cryoprotectant (Gosden *et al.*, 1994a). These animals became pregnant following transplantation of thawed ovarian tissue. Cryopreserved marmoset ovarian tissue has also been transplanted under the kidney capsule of immunodeficient mice (Candy *et al.*, 1995). Grafts recovered 21–32 days later contained follicles at all stages of folliculogenesis, including antral follicles 1–2 mm in diameter.

In the present study, human ovarian tissue was cryopreserved using one of two cryoprotectants, DMSO and a combination of 1,2-propanediol and sucrose. The latter is thought to be less toxic than DMSO and has been successfully used in the cryopreservation of human embryos (Lassalle *et al.*, 1985).

Materials and methods

Ovarian tissue was obtained during gynaecological operations by biopsy or from total oophorectomy. In all, 19 women, aged 19–44 years (mean 35 years), donated tissue after informed consent.

The ovarian tissue was placed in α -minimum essential medium (α -MEM; Gibco, Life Technologies Ltd, Paisley, Scotland) and 10% human serum in the operating theatre and transferred to the laboratory within a few minutes. The ovarian tissue was cut into pieces (0.3–2 mm in diameter) using a scalpel blade and needles and placed in an incubator in 5% CO_2 in air at 37°C . One or two pieces, depending on the size of the specimen, were immediately fixed in Bouin's solution for subsequent histology.

Freezing (DMSO)

Ovarian tissue from nine patients was frozen using DMSO (Sigma Chemical Company, St. Louis, MO, USA) as cryoprotectant (Gosden *et al.*, 1994a). After incubation for 30 min, ovarian tissue was transferred to freezing medium containing DMSO (1.5 M DMSO in

α -MEM with 10% human serum); this had been pre-cooled to 0°C in an ice bath. The vials (Nunc freezing vials; Nunclon, Roskilde, Denmark) containing the tissue were kept in this medium at 0°C for ~5 min until transfer to a programmable freezer (Kryo 10; Planer Biomed, Sunbury on Thames, UK). After ~10 min further at 0°C, the vials were cooled at 2°C/min to -7°C. The specimens were held at this temperature for 5 min, and ice crystal formation was induced manually by touching the cryotubes containing the freezing medium with forceps prechilled in liquid nitrogen. After a further 10 min they were cooled at 0.3°C/min to -40°C and then at -10°C/min to -150°C. They were then transferred to liquid nitrogen (-196°C) and stored.

Thawing (DMSO)

The vials were thawed in air at room temperature for 2 min and then immersed in a water bath at 37°C until the ice had thawed. The tissue pieces were then immediately transferred to culture medium. They were washed three times with new medium before further processing.

Freezing (propanediol)

Tissue specimens from 10 patients were frozen using the combination of 1,2-propanediol (Sigma) and sucrose (Sigma) as a cryoprotectant. After incubation, tissue pieces were transferred to medium containing 1.5 M propanediol and 0.1 M sucrose in α -MEM supplemented with 20% human serum at room temperature. After a further 10 min they were transferred to the programmable freezer, at a starting temperature of 18°C. They were cooled at 2°C/min to -8°C. After being held at that temperature for 5 min, ice crystal formation was induced. After a further 10 min they were cooled at -0.3°C/min to -30°C and then at -50°C/min to -150°C. They were then transferred to liquid nitrogen and stored.

Thawing (propanediol)

Vials were taken from the liquid nitrogen, kept in air for 30 s and immersed in a water bath at 30°C. When the ice had thawed, the tissue pieces were immediately transferred to medium containing 1.0 M propanediol and 0.2 M sucrose in α -MEM supplemented with 20% human serum. After 5 min they were transferred to medium containing 0.5 M propanediol and 0.2 M sucrose, and after another 5 min to medium with 0.2 M sucrose, where they were kept for 10 min. They were then transferred to medium containing 20% serum and kept in this for 10 min at room temperature. Further processing was in fresh medium in an incubator.

Once thawed, the tissue pieces were fixed in Bouin's solution. Both frozen-thawed tissue and control tissue were embedded in paraffin and cut into 2 μ m sections, which were then stained with haematoxylin and eosin. The primordial, primary, secondary and tertiary follicles were classified as described by Gougeon (1986) and counted per high power field (HPF) using a Zeiss Standard microscope and $\times 400$ magnification. The proportions of morphologically normal follicles before and after freezing were counted. Eosinophilia of the ooplasm, contraction and clumping of the chromatin material and wrinkling of the nuclear membrane of the oocytes were regarded as signs of atresia (Gougeon, 1986). The χ^2 test and Student's *t*-test were used for the statistical comparisons.

Results

There was no identifiable difference in histological examination of ovarian tissue before and after freezing. This was irrespective of the cryoprotectant used. There were no light microscopical changes in the tissue components. Central necrosis was not seen, even in pieces as large as 2 mm in diameter. Follicles at

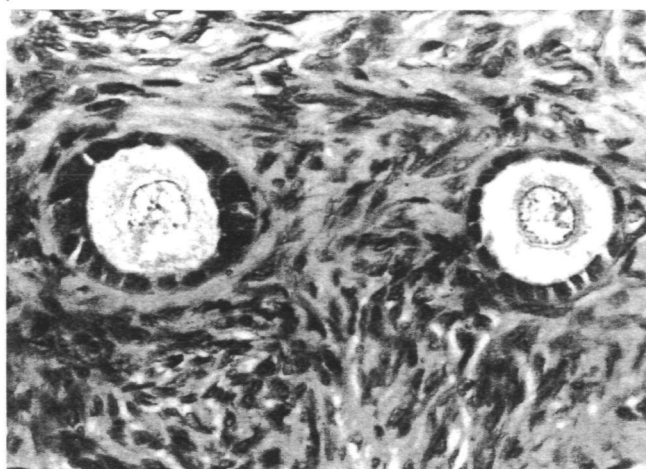


Figure 1. Primary follicles in ovarian tissue in a sample taken before freezing. Original magnification $\times 400$.

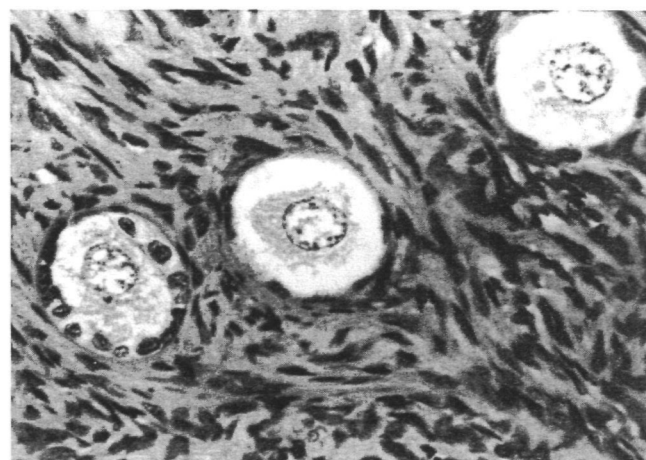


Figure 2. Two primordial and one primary follicle in an ovarian tissue sample cryopreserved using dimethylsulphoxide. The tissue is from the same patient as that in Figure 1. Original magnification $\times 400$.

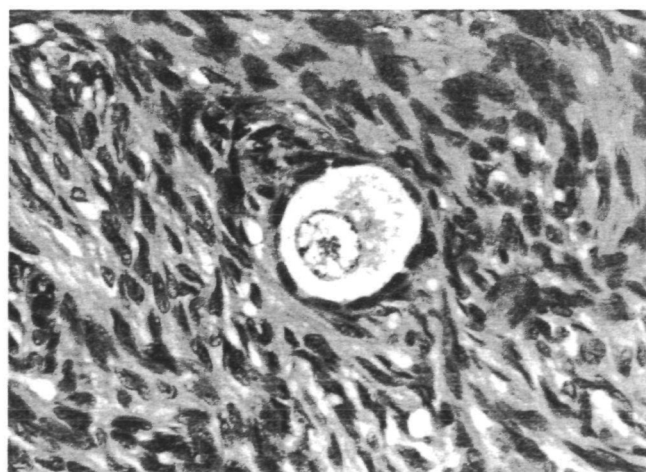


Figure 3. A primordial follicle in ovarian tissue in a sample taken before cryopreservation. Original magnification $\times 400$.

varying developmental stages were found similarly before and after freezing (Table I). In non-frozen pieces, 27% of the follicles showed signs of atresia, whereas 19% of the follicles

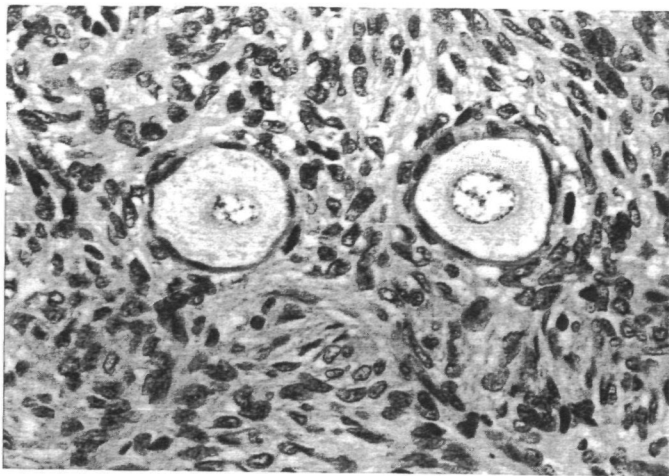


Figure 4. Primordial follicles in ovarian tissue cryopreserved using propanediol and sucrose. The tissue is from the same patient as that in Figure 3. Original magnification $\times 400$.

Table I. Numbers of follicles per high power field over 10 observations in ovarian tissue before and after cryopreservation. Tissue from 10 women was frozen using propanediol and from nine with dimethylsulphoxide (DMSO). Values are means \pm SD

	Propanediol		DMSO	
	Before	After	Before	After
Primordial follicles	5.52 \pm 6.15	4.22 \pm 2.36	7.15 \pm 13.8	4.76 \pm 4.22
Primary follicles	2.23 \pm 2.14	2.26 \pm 1.59	1.47 \pm 1.18	2.96 \pm 3.37
Primordial and primary follicles	7.75	6.48	8.62	7.72

in frozen-thawed specimens were atretic. This difference was not significant. Oocytes with similar morphology were seen in the follicles before freezing (Figures 1 and 3), after freezing with DMSO (Figure 2) and after freezing with propanediol and sucrose (Figure 4).

Table I shows the numbers of primordial and primary follicles per HPF in the sections studied. The ovarian tissue of 10 patients fixed before propanediol freezing contained on average 7.75 primordial and primary follicles/HPF. The corresponding number after cryopreservation was 6.48. In the samples taken before DMSO freezing, a mean of 8.62 primordial and primary follicles/HPF was seen, and after cryopreservation 7.72/HPF. In one biopsy taken from a 40 year old woman, no follicles were found. After freezing and thawing in DMSO, however, one follicle was discovered. The majority of the follicles seen in these biopsy specimens were primordial. Primary follicles were found less frequently, and secondary and tertiary follicles were seen only occasionally. The mean diameters of the follicles and oocytes are shown in Table II. There were no significant differences between the groups.

Discussion

The normal appearance of oocytes and follicles after an attempt at freezing and thawing suggests that human ovarian tissue can be cryopreserved. Consequently, oocytes should be capable

Table II. Diameters of the follicles and oocytes before and after cryopreservation. Values are means \pm SD

	Propanediol ($n = 11$)		DMSO ($n = 15$)	
	Before	After	Before	After
Diameter of the follicles (μm)	42.1 \pm 2.8	42.9 \pm 3.6	36.6 \pm 10.1	46.6 \pm 4.7
Diameter of the oocytes (μm)	16.9 \pm 1.1	17.1 \pm 1.5	14.6 \pm 4.0	16.4 \pm 1.0

of being matured later either naturally after replantation, or by maturation *in vitro* at the time this becomes technically feasible.

Our findings are reassuring. Previously, in cryopreserved marmoset ovaries (Candy *et al.*, 1995), vacuoles were described in the cytoplasm of the oocyte. There was no vacuolation of thawed material in the oocytes in our present study. This could be due to species difference or because of slight differences in the freezing protocols. No differences were seen in the size of oocytes or follicles before and after freezing; this finding is similar to that of Gougeon (1986).

To assess whether primordial and primary follicles retain their developmental ability, ovarian biopsies have been transplanted under the kidney capsule of severe combined immunodeficiency mice. Gosden *et al.* (1994b) showed the feasibility of this with biopsies taken from cats and sheep, and Candy *et al.* (1995) did similar work with the marmoset. This approach is now needed to assess the functional viability of cryopreserved human ovarian tissue.

Replantation of cryopreserved ovarian tissue slices after radiotherapy or chemotherapy should offer the possibility of success, as has been the case in sheep (Gosden *et al.*, 1994a). This would probably not give long-lasting fertility, but biopsies could be replanted at laparoscopy without great technical difficulty. Presumably, such a procedure could be repeated several times, giving an added chance of repeated pregnancy.

An alternative to freezing of ovarian tissue is oocyte cryopreservation. This has been done successfully in the mouse and live young were born (Whittingham, 1977). Live young have also resulted from rabbit (Al-Hasani *et al.*, 1989), cow (Fuku *et al.*, 1992) and human frozen oocytes (Chen, 1986). However, success in freezing mature oocytes has been limited. Fertilization rates of human oocytes after using either propanediol or DMSO have been reasonable (75 and 50% respectively), but polyploidy has been very common (Al Hasani *et al.*, 1987; Mandelbaum *et al.*, 1988). Both cooling and DMSO have adverse effects on the microtubular system of mice oocytes (Johnson and Pickering, 1987; Pickering and Johnson, 1987). In human oocytes the structure of the filaments is thought to make them more susceptible to damage of the meiotic spindle with a risk of aneuploidy (Pickering *et al.*, 1988). The meiotic spindle of human oocytes has also been shown to be sensitive to cooling (Sathanathan *et al.*, 1988). Increased polyploidy was reported in mouse embryos derived from frozen-thawed oocytes (Glenister *et al.*, 1987; Carroll *et al.*, 1989; Trounson and Kirby, 1989; Bouquet *et al.*, 1992), and this was thought to be due to an increased risk of digyny after retention of the second polar body.

For all these reasons, freezing of ovarian tissue containing immature oocytes may be a technology worthy of exploration. Cryopreservation of the oocytes at an immature stage has been suggested to overcome spindle damage, and there has been greater success (Mandelbaum *et al.*, 1988). Human prophase I oocytes from an IVF programme have been frozen using propanediol (Toth *et al.*, 1994). These oocytes showed an 83% rate of maturation and a 58% rate of fertilization after thawing.

In the mouse, cryopreservation of immature oocytes and in-vitro maturation after thawing have reportedly been successful. However, the developmental capacity was lower than that of fresh oocytes matured *in vitro* (Schroeder *et al.*, 1990). In a recent report (Candy *et al.*, 1995), the overall survival of mouse oocytes frozen at the germinal vesicle stage compared favourably with the estimated overall survival rate of oocytes. Candy *et al.* (1995) explained the increased post-implantation loss as due to suboptimal conditions of maturation *in vitro* rather than freezing injury. Cryopreservation of immature human oocytes could be possible as well, and, in particular, cryopreservation of very small oocytes in primordial and primary follicles might save the cells from spindle damage.

Although DMSO has been shown to have toxic effects at temperatures $>4^{\circ}\text{C}$ and is possibly genotoxic, other cryoprotectants, such as propanediol with sucrose, look promising (Lassalle *et al.*, 1985; Shaw *et al.*, 1995). However, propanediol has not been used for cryopreservation of ovarian tissue. Our results show that propanediol with sucrose is as good a cryoprotectant as DMSO.

Although it has been possible to mature viable oocytes from frozen primary follicles in the mouse (Carroll *et al.*, 1990), until recently it has not been possible to mature human oocytes from primordial, primary or pre-antral follicles (Roy and Treacy, 1993; Eppig, 1994; Nayudu, 1994; Spears, 1994). The dense nature of human ovarian tissue has made preparation of follicles extremely difficult. A recent advance has been made by Abir *et al.* (1995). They matured pre-antral follicles, of 100–400 μm diameter, *in vitro* after harvesting them mechanically from human ovarian tissue. Antral follicles of up to 1.6 mm in diameter developed *in vitro*. The method is time-consuming and ovarian biopsies contain rather few pre-antral follicles. However, this technique should be worth further research.

If follicles can be cultured to antral stages, it might be possible to remove the cumulus-enclosed oocytes and develop them in a secondary culture system. In-vitro maturation of human germinal vesicle-stage oocytes has already been successful and children have been born (Cha *et al.*, 1991; Trounson *et al.*, 1994); further improvements are likely.

Human fetal ovarian tissue has been cryopreserved by an ultra-rapid freezing method after DMSO treatment by Zhang *et al.* (1995). They described follicular development afterwards, but fertilization was not attempted. The results of freezing were not as good as in our study, central necrosis of the tissue pieces occurring. The results with fetal ovarian tissue are probably not comparable with those obtained with adult tissue, and clinical use of fetal ovarian tissue raises huge ethical problems.

We consider that if our work is to have clinical applications, several biopsies will be needed to be cryopreserved. This is because we have found fewer primordial, primary or larger follicles than we expected. Moreover, in the human their distribution seems erratic.

The present study shows that cryopreservation of ovarian tissue is not a major problem. However, much effort will have to be devoted to the maturation of small follicles *in vitro*, before the potential of cryopreservation of ovarian tissue can be fully realized.

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