Introduction

The cryopreservation of ovarian tissue is becoming a realistic means of storing female gametes. It is particularly applicable for women about to undergo cancer therapy which will severely reduce the number of viable oocytes in the ovaries and may cause chromosomal anomalies. To date, orthotopic or heterotopic grafting is the only practicable method of utilizing stored ovarian tissue. Recipients of orthotopic grafts of frozen ovarian tissue have normal ovarian cycles (mouse: Harp et al., 1994; sheep: Baird et al., 1999; human: Grischenko et al., 1987) and are capable of becoming pregnant (mouse: Cox et al., 1996). Live young have been born in mice (Parrott, 1960; Gunasena et al., 1997a,b; Sztein et al., 1998) and sheep (Gosden et al., 1994) after orthotopic grafting of cryopreserved ovarian tissue. However, the proportion of recipients subsequently shown to be fertile was variable, the number of live births was low (Sztein et al., 1998) and the long-term fertility of recipients was compromised (Gunasena et al., 1997a; Sztein et al., 1998).

The shortened fertile lifespan and reduced litter sizes in recipients of ovarian tissue are thought to be due to a loss of follicles caused by ischaemia during establishment of the graft (Jones and Krohn, 1960). Freezing and thawing also reduces the number of normal follicles in mouse (Green et al., 1956) and human ovarian tissue (Hovatta et al., 1996; Newton et al., 1996). Despite the survival of high proportions (>80%) of primordial follicles after freezing immature mouse ovaries in dimethylsulphoxide (DMSO), the number of follicles in grafts of both fresh and frozen tissue was markedly reduced compared with ungrafted ovaries (Candy et al., 1997a).

The aim of this study was to determine the effect of the loss of follicles on the long-term function of cryopreserved ovarian grafts and the subsequent reproductive lifespan of the recipient. Fresh ovaries and ovaries frozen in DMSO were grafted into genetically distinct recipients that were subsequently mated. Breeding performance was monitored. A preliminary report of these data appeared elsewhere (Candy et al., 1997b).

Materials and methods

Except where stated, all manipulations were carried out at 37°C in HEPES-buffered M2 medium (Wood et al., 1987) in which the bovine serum albumin was replaced with 10% fetal bovine serum (FBS; ICN Flow, High Wycombe, UK).

Animals

Ovaries from 10 day old C57BL/6J-Gpi-1a mice, homozygous for Gpi-1a, were used as the source of donor ovaries. The recipients of ovarian grafts were 42 day old female B6CB (C57BL/6JLac×CBA/CaLac) F1 hybrid mice, homozygous for Gpi-1a. These genetically distinct strains of mice were used in the study to enable identification of the source of ovary (i.e. native or grafted) from which pups were derived. All mice were bred at St George’s Hospital Medical School, London.

Ovarian tissue

Ovaries from C57BL/6J-Gpi-1a mice were dissected free of fat and mesentery. Intact ovaries (~2 mm³) were either frozen within 30 min of dissection or grafted into the ovarian bursae of recipient mice within 90 min of dissection.

Freezing and thawing

Ovaries were placed in 1.5 mol/l DMSO (BDH, Poole, UK) in M2 and held at room temperature (~20°C) for 20 min before being loaded into 1.8 ml cryovials (Nunc Intermed, Kamstrup, Denmark: two or three ovaries per vial) in 0.3 ml of the same solution. The cryovials

C.J.Candy, M.J.Wood1 and D.G.Whittingham

Department of Anatomy and Developmental Biology, St George’s Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK

1To whom correspondence should be addressed

Restoration of a normal reproductive lifespan after grafting of cryopreserved mouse ovaries

Human Reproduction vol.15 no.6 pp.1300–1304, 2000

© European Society of Human Reproduction and Embryology
were placed in a programmable freezer (R202; Planer Biomed, Sunbury-on-Thames, UK) at 0°C and cooled at a rate of 2°C/min to ~8°C. Ice crystal nucleation was initiated at the meniscus of the cryoprotectant solution 5 min later by touching the side of the cryovial with forceps previously cooled in liquid nitrogen. After a further 5 min, the cryovials were cooled at a rate of 0.3°C/min to ~40°C and then at 10°C/min to ~150°C. The cryovials were transferred into liquid nitrogen (~196°C) and stored for at least 24 h.

The cryovials were warmed in air at room temperature for 40 s and then immersed in water at 30–35°C until the ice melted (2–3 min). The ovaries were removed from the cryovials and placed in 1–2 ml M2 at room temperature for 5 min, and then washed twice in M2 at room temperature (1–2 ml per wash). After 5 min, the ovaries were placed in M2 at 37°C. The ovaries were grafted into the ovarian bursae of recipient mice within 45 min of thawing.

**Transfer to the ovarian bursa**

Fresh and frozen ovaries were transferred into the ovarian bursae of B6CBF1 mice using established techniques (Jones and Krohn, 1960). The recipients were anaesthetized by i.p. injection of 2.2,2,2-tribromo-ethanol (Aldrich Chemical Co., Poole, UK; 0.4 mg/g body weight) in tertiary amyl alcohol (BDH). Briefly, the ovarian bursa was exteriorized through a lateral abdominal incision in the lumbar fossa. A small incision was made just below the fat pad and the bursal membrane was reflected over the ovary. A pair of curved fine Watchmakers’ forceps was used to grip the hilum. A second pair of Watchmakers’ forceps was used to sever the native ovary from the hilum. Pressure was applied for ~10 s to control bleeding. The ovary for grafting was placed within the bursal cavity and the bursal membrane replaced over it before returning it to the peritoneal cavity. The contralateral ovary was removed in a similar manner. Some animals received an ovarian graft on this side too (bilateral grafts) but others did not (unilateral grafts).

**Breeding of recipients of ovarian grafts**

Seven days after the orthotrophic grafting of ovarian tissue, the recipients were paired continuously with sexually mature, fertile B6CBF1 males. The date of birth and size of each litter was recorded. The pups were culled at 10 days postnatally and their spleens were removed and stored in 1.8 ml Eppendorf tubes at ~20°C for subsequent analysis of the glucose phosphate isomerase (GPI) isozymes. When a female failed to produce a litter within 60 days of the initial mating or after her last recorded parturition, the male was replaced. If no litter or visible signs of pregnancy were observed within a further 30 days, the female was autopsied and examined for ovarian tissue. Any remaining ovarian tissue was placed in Bouin’s fixative for histology. If found, the number of fetuses was recorded and tissue from each was analysed for GPI isozymes.

**Glucose phosphate isomerase analysis of offspring**

GPI assay was used to determine the origin of offspring from recipients of ovary grafts. PIG is an enzyme involved in the glycolytic pathway which, in the mouse, commonly exists as two isozymes GPI-1A and GPI-1B, encoded by the allelic genes Gpi-1a (Gpi-1a) and Gpi-1b (Gpi-1b) respectively. The two isozymes differ electrophoretically and can be separated using cellulose acetate electrophoresis and then stained to reveal the GPI bands (Buehr and McLaren, 1981). Cells homozygous for Gpi-1a or Gpi-1b will show only the GPI-1A or GPI-1B isozyme bands. Offspring derived from grafted ovaries are heterozygous for Gpi-1a and Gpi-1b and contain a copy of both isozymes. After electrophoresis three bands can be seen; the GPI-1A band and the GPI-1B band and also a hybrid band. Offspring derived from any native ovary not completely removed by oophorectomy are homozygous for Gpi-1b and show only the GPI-1B band.

Frozen-thawed spleens were homogenized in 50 mmol/l Tris–HCl (Sigma Chemical Co., Poole, UK) containing 0.1% Triton X-100 (Aldrich) then centrifuged at 600 g for 2 min. Aliquots (10 μl) of the supernatant were run on cellulose acetate plates (Titan III; Helena Laboratories, Tyne and Wear, UK) in a Tris-glycine buffer (0.2 mol/l Tris base, 0.2 mol/l glycine, pH 8.5; Sigma) for ~1 h at 150 V. The plates were overlaid with ~5 ml 1.5% agarose (Type II; Sigma) in 50 mmol/l Tris–HCl buffer containing 1 mg NADP (monosodium salt; Sigma), 10 mg fructose-6-phosphate (disodium salt; Sigma), 5 mg MgCl2.6H2O (BDH), 1 mg MTT (3-[4,5-dimethyl-thiazol-2-yl]-2.5-diphenyl tetrazolium bromide; Sigma), 0.25 mg phenazine methosulphate (Sigma) and 6 units glucose-6-phosphate dehydrogenase (Sigma). The GPI bands were revealed after ~15 min incubation in the dark.

**Vaginal smears**

Vaginal smears were taken daily from some of the recipients of frozen ovarian tissue. The vaginal wall of each recipient was scraped gently using a plastic probe and the cells smeared onto a clean glass slide. The air-dried smears were stained with an aqueous solution of 0.1% methylene blue. The stage of the oestrous cycle was determined from the cell types observed in the vaginal smear (Rugh, 1990).

**Histology**

At autopsy, any remaining ovarian tissue was placed in Bouin’s fixative for at least 24 h, then embedded in paraffin wax, serially sectioned at 5 μm and stained with haematoxylin and eosin. The sections were examined for the presence of follicles and corpora lutea.

**Experimental design**

Ovaries from ten, 10 day old C57BL/6Lac-Gpi-1a mice were frozen. The frozen ovaries were transferred into the ovarian bursae of 42 day old B6CBF1 mice on two separate days: six recipients received 12 ovaries (bilateral grafts) and six recipients received six ovaries (unilateral grafts). Freshly collected ovaries from 10 day old C57BL/6Lac-Gpi-1a mice were transferred into ovarianectomized recipients on two separate days: seven recipients received 14 ovaries from seven donors (bilateral grafts) and six recipients received six ovaries from three donors (unilateral grafts). Recipients of ovarian tissue were paired with B6CBF1 males from 7 days after grafting to assess breeding performance. Four, 42 day old B6CBF1 and three, 42 day old C57BL/6Lac-Gpi-1a females were paired continuously with B6CBF1 males as control breeding pairs. One B6CBF1 control female was culled due to illness and one C57BL/6Lac-Gpi-1a control female died before breeding ceased.

In a separate experiment, frozen ovaries from 10 day old C57BL/6-Gpi-1a mice were transferred unilaterally into the ovarian bursae of six, 42 day old B6CBF1 recipients. These recipients were also paired with B6CBF1 males from 7 days after grafting but were culled after two litters had been born. Vaginal smears were taken from each recipient from 3 days after grafting until the observation of cornified epithelial cells, indicating resumption of oestrogenic activity, or observation of a copulation plug.

**Statistical analysis**

All results are expressed as means ± SE. χ²-test with Yates’ correction for small samples was used to compare the proportion of fertile females. A one-way analysis of variance for unequal sized groups (Armitage and Berry, 1987) was used to compare the number of litters per female, the time interval between litters and the number of pups per litter. Differences between groups were compared using...
Gabriel’s test (Kendall and Stuart, 1968). Differences were considered significant when $P < 0.05$.

**Results**

**Fertility of recipients of ovarian grafts**

There was no difference in the fertility and graft acceptance between recipients receiving one or two ovaries and the combined data are shown in Table I. Overall, a similar proportion of recipients of fresh and frozen ovaries were fertile (92% versus 83%; $\chi^2_{[1]} = 0.01$, not significant). More than half of these (fresh: 67%; frozen: 60%) had litters containing pups which were derived only from grafted ovaries (GPI-1A/GPI-1B). A similar number of fertile recipients of fresh and frozen ovaries had either litters derived from both grafted and native ovary (GPI-1A/GPI-1B and GPI-1B respectively) or only from remnants of native ovary (GPI-1B; $\chi^2_{[1]} = 0.12$, not significant). One recipient of fresh ovary and two recipients of frozen ovary were infertile.

**Time before observation of first litters**

Recipients of fresh and frozen ovaries and control females were paired 7 days after grafting. There was no difference in the latency between pairing and the birth of the first litters containing offspring derived from grafted tissue (fresh: 46.5 ± 8.6 days, range 23–106 days; frozen: 42.3 ± 6.4 days, range 24–70 days; $F_{[1,17]} = 0.14$, not significant). Litters in which the offspring were derived from native ovary only were observed after 33.5 ± 9 days (range 22–60 days). All the unoperated controls littered within 25 days of mating (B6CBF1: 20.5 ± 1.5 days; C57BL/6-Gpi-1a: 22 ± 1 days).

**Number of litters**

There was no difference in the total number of litters containing offspring derived from ovarian grafts born to each recipient of fresh (6.2 ± 1.2 litters, range 1–10) or frozen (8.4 ± 0.5 litters, range 5–10) ovary or to C57BL/6-Gpi-1a control females (6.3 ± 1.8 litters, range 3–9). B6CBF1 control females had significantly more litters per female (12.0 ± 2.0 litters, range 6–15; $F_{[3,24]} = 3.61, P = 0.03$). Recipients that had offspring derived from native ovary only, appeared to have fewer litters (mean 4.3 ± 1.3 litters, range 2–7) compared with recipients with litters containing pups derived from grafted ovaries, but the difference was not significant ($F_{[1,21]} = 3.14$). There was no significant difference in the number of litters born to recipients of one or two ovarian grafts (unilateral: 8 ± 1.1 litters, bilateral: 6.5 ± 1 litters, $F_{[1,17]} = 1.09$).

The number of females with litters containing offspring derived from grafted ovaries as a proportion of the number of females mated is shown in Figure 1. B6CBF1 recipients of fresh and frozen C57BL/6-Gpi-1a ovaries and the number of ungrafted B6CBF1 and C57BL/6-Gpi-1a control mice with successive litters, as a proportion of the number of females mated in each group.

**Time interval between litters**

Recipients of fresh and frozen ovarian grafts reproduced continuously for up to 11 months. Overall, the time interval between litters was similar for the females with litters containing offspring derived from grafted ovaries (fresh: 27.2 ± 1.4 days; frozen: 26.9 ± 1.1 days) and control females (B6CBF1: 26.8 ± 1.5 days; C57BL/6-Gpi-1a: 26.7 ± 1.6 days; $F_{[3,22]} = 0.11$, not significant). The time interval between litters tended to increase as the females neared the end of their reproductive life. As the time intervals between litters were similar for all the groups, this suggests that recipients of frozen ovaries did not have increased pregnancy loss.

<table>
<thead>
<tr>
<th>Ovary</th>
<th>No. of females transplanted</th>
<th>No. of fertile recipients (%)</th>
<th>No. of fertile recipients with litters of each GPI isozyme type (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>13</td>
<td>12 (92)</td>
<td>8 (67)</td>
</tr>
<tr>
<td>Frozen</td>
<td>12</td>
<td>10 (83)</td>
<td>6 (60)</td>
</tr>
</tbody>
</table>

**Table 1.** The fertility of ovariectomized B6CBF1 (Gpi-1a/Gpi-1b) females grafted with fresh or frozen ovaries from C57BL/6-Gpi-1a females and mated with B6CBF1 (Gpi-1a/Gpi-1b) males, and the glucose phosphate isomerase (GPI) isozyme type of the litters.

![Figure 1.](image-url)
**Litter size**

There was no significant difference in the number of pups per litter derived from grafted ovaries in recipients of fresh (5.4 ± 0.7 pups/litter) or frozen (6.3 ± 0.7 pups/litter) grafts, compared with the litter size of control C57BL/6-Gpi-1<sup>a</sup> females (6.3 ± 1.0 pups/litter). B6CBF1 control females had significantly larger litters (9.8 ± 0.6 pups/litter; \( F_{[3,24]} = 4.5, P = 0.014 \)). Fewer pups derived from remnants of native ovary were born (2.9 ± 0.5 pups/litter; \( F_{[1,21]} = 7.87, P = 0.011 \)). Overall, 26.6% of pups from recipients of fresh ovary and 11.6% of pups from recipients of frozen ovary were derived from remnants of native ovarian tissue. None of the pups in this study appeared abnormal. Towards the end of the reproductive life of the female, litters tended to decrease in size.

**Histology of ovarian grafts after cessation of breeding**

Ovarian tissue was recovered from all three infertile females. Histological examination of the tissue showed the presence of follicles, albeit in low numbers. In one female a partial blockage in the oviduct was the most likely cause of failure to breed, since an ectopic pregnancy was identified with a fetus developing within the oviduct. On cessation of breeding, ovarian grafts were recovered from all recipients of ovarian grafts and examined histologically. In three recipients of bilateral grafts (two received frozen and one received fresh tissue) only one piece of ovarian tissue was recovered from each mouse. In general, very few follicles remained in the recovered grafts, but actual numbers were not counted since it was impossible to distinguish the follicles from grafted on the fertility of mice after orthotopic grafting of frozen ovaries. Resorptions were observed in four recipients of fresh study). The freezing methods in the different studies are very different. This study, namely: (i) the number of viable follicles in the lutea. Resorptions were observed in four recipients of fresh and frozen ovarian grafts were fertile, and more than 80% of the recipients had litters that were derived from the grafted ovaries. Recipients of fresh and frozen ovaries and unoperated C57BL/6-Gpi-1<sup>a</sup> females had similar numbers 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). These observations confirm previous reports that fertility is restored in females receiving grafts of mouse ovaries frozen in DMSO (Cox et al., 1996; Gunasena et al., 1997a, b; Sztein et al., 1998) and glycerol (Parrott, 1960). Previously only a few successive litters (mean 1.5–2.5) have been obtained from recipients of frozen ovary (Gunasena et al., 1997a; Sztein et al., 1998). Although it was reported (Parrott, 1960; Gunasena et al., 1997a) that recipients of fresh and frozen ovarian grafts had litters of similar size, others suggest that cryopreservation reduces litter size (Sztein et al., 1998).

In contrast with previous reports of reduced litter sizes and a shortened reproductive life span after orthotopic grafting of fresh tissue (Mussett and Parrott, 1961; Gunasena et al., 1997a), in the present study reproductive performance, i.e. mean number of pups per litter and mean number of litters per female, was similar in recipients of grafts of fresh and frozen ovaries and in untreated control females of the same strain as the ovary donors. This is despite the fact that there is a loss of functional follicles due to ischaemia during the establishment of orthotopic grafts of ovarian tissue (Jones and Krohn, 1960). In excess of 50% of the follicles are lost within 15 days of heterotopic grafting from fresh ovaries and ovaries frozen in DMSO (Candy et al., 1997a).

Historically, glycerol was used to cryopreserve mouse ovaries (Parrott, 1960). Only about 20% of follicles survived in ovaries frozen using glycerol (Candy et al., 1997a) and this may have contributed to the poor fertility achieved after orthotopic grafting (Parrott, 1960). Follicle survival is increased in ovaries frozen in DMSO (mouse: Candy et al., 1997a; human: Newton et al., 1996). The more recent studies on the fertility of mice after orthotopic grafting of frozen ovaries have used DMSO as the cryoprotectant (Cox et al., 1996; Gunasena et al., 1997a, b; Sztein et al., 1998; present study). The freezing methods in the different studies are very similar and other factors may explain the increased success in this study, namely: (i) the number of viable follicles in the grafted ovarian tissue and (ii) the breeding capability of the recipient. The number of follicles in grafted ovarian tissue is affected by the age of the donor and the amount of tissue transferred. The strain can also affect the number of follicles present in the ovary. The choice of recipient is an important factor. Here the recipients were young adult mice from an F1 hybrid strain. F1 hybrids tend to have larger litters and longer reproductive lifespans.

The latent interval before the resumption of oestrogen secretion after grafting 10 day old mouse ovaries (16.3 days) was similar to that reported by others (19–23 days: Cox et al., 1996; 15 days: Gunasena et al., 1997a) irrespective of the age of the donor ovaries (day 16 fetal ovaries: Cox et al., 1996; 10–12 week old ovaries: Gunasena et al., 1997a). The interval between grafting and the birth of the first litter (45–55 days) was also similar to that after grafting ovaries from 21–30 day old mice (47–52 days: Sztein et al., 1998). It was found (Cox et al., 1996) that recipients of day 16 fetal ovaries became pregnant within 28–40 days of grafting. These data suggest that the age of the ovary does not significantly affect the time before the grafted ovaries become functional. This may be important when contemplating heterochronic grafts of ovarian tissue, for instance when grafting human ovarian tissue that
has been frozen at a prepubertal stage of development back into an adult.

Fertile matings resulting in pregnancy did not occur immediately after the resumption of oestrogenic activity. Similarly, it was found (Gunasena et al., 1997a) that a proportion (~35%) of graft recipients which mated immediately after the first indication of oestrogenic activity did not establish pregnancy. This suggests that the oocytes which developed during the initial stages of graft establishment may be abnormal. Alternatively, an imbalance of hormones may have affected implantation. In this study, the interval between litters indicated that post-partum mating was taking place in graft recipients and control females. The interval between litters increased and the litter sizes decreased towards the end of the reproductive life of recipient and control females similar to that observed in conventional breeding colonies. This may be due to depletion of the follicle reserve, reduced fertility of ovulated oocytes from aged ovaries, a reduction in uterine receptivity leading to failure of implantation or failure to hold pregnancy to term (Finn, 1970). Also, post-partum loss of small litters or litters with abnormal pups may have been due to cannibalization.

In this study, it was possible to identify the source of the oocytes (i.e. from grafted and native ovarian tissue) that were fertilized and developed to term. This was important since ~20% of the fertile recipients of grafted ovaries had mixed litters containing offspring derived from grafted and native ovary. Some groups (Gunasena et al., 1997b; Sztein et al., 1998) were also able to determine the origin of offspring after orthotopic grafting, but others were not (Cox et al., 1996; Gunasena et al., 1997a). In this study, 3–12% of offspring were derived from the remnants of native ovary. In contrast, it was reported (Sztein et al., 1998) that ~36% of offspring were derived from native ovary. The method of grafting used can result in incomplete removal of the host ovary (Jones and Krohn, 1960). In this study, ovaries were grafted using an established method (Jones and Krohn, 1960) in which the ovary was exposed completely before excision and bleeding was controlled at the site of excision. This increases the likelihood of removing the recipient’s ovary completely. However, the possibility of contamination remains. The only certain way of eliminating a recipient’s functional ovary is by X-irradiation to destroy the germ cell population (Parrott and Parkes, 1960), although inadequate levels of irradiation may not render the recipients infertile (Aubard et al., 1998). Where ovarian grafting is being used to rescue a particular strain of animal, a precaution must be to ensure that the recipient is phenotypically or genetically distinguishable from the donor of the ovarian tissue.

Recipients of grafts of sheep ovarian tissue were hormonally active for up to 22 months (Baird et al., 1999). However, oestrous cycles can persist long after the fertile life of a female has ended (Jones and Krohn, 1960). In this study, recipients of fresh and frozen orthotopic ovarian grafts reproduced continuously for up to 11 months. This is the first conclusive demonstration that frozen ovaries may continue functioning for the normal reproductive lifespan of a species. It is encouraging for the use of cryopreserved ovarian tissue in clinical settings and also for animal breeding and conservation.

Acknowledgements

The authors would like to thank I.Harragan for the preparation of the histology sections and N.Woods for the husbandry of the breeding pairs. This work was funded by the Medical Research Council.

References


Received on December 1, 1999; accepted on March 10, 2000

1304