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Live births after autologous transplant of cryopreserved mouse ovaries*

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The fertility of mice after autologous transplantation of ovaries, before or after cryopreservation, was investigated in this study. Female mice were randomly assigned to either sham-operated (n = 14), ovariectomized (n = 11), fresh (n = 12) or cryopreserved (n = 11) ovarian transplant groups. Ovaries were cryopreserved in 1.4 M dimethyl sulphoxide (DMSO) by cooling to −55°C at 0.5°C/min (ice nucleation at −7°C), plunged in liquid nitrogen and then thawed at room temperature. Oestrous cyclicity was observed 7 days after sham operation or 15 days after fresh or cryopreserved ovarian transplant. Ovariectomized animals did not demonstrate oestrous cyclicity but were mated, and no pregnancies resulted. Live births were recorded from all sham-operated, all fresh transplant, and 8/11 (73%) cryopreserved transplant animals. Overall mean ± SEM litter sizes from fresh (4.32 ± 0.44) and cryopreserved (4.71 ± 0.57) transplant groups were smaller ($P < 0.05$) than those of sham-operated animals (12.54 ± 0.44), although the sizes were not significantly different ($P > 0.05$) from each other. Animals were mated at least four times, with four litters of live pups from 4/4 sham-operated, 1/10 fresh and 1/9 cryopreserved ovarian transplant animals. Litter sizes from pups of sham-operated and transplant animals were not significantly different from each other. Following autologous transplantation of mouse ovaries, before or after cryopreservation, offspring appeared normal, with high rates of fertility.

Key words: autologous transplant/cryopreservation/mouse/ovary

Introduction

Female oncology patients undergoing chemotherapy and/or radiation therapy for certain forms of cancer are at risk of suffering loss of ovarian function, resulting in menopausal symptoms and the associated adverse effects on general health, such as increased likelihood of heart disease, osteoporosis and loss of fertility (Jaffe, 1986; Byrne *et al.*, 1992; Nicholson and Byrne, 1993). Infertility and premature menopause can be both emotionally and physically devastating. Prior to oncology treatment, male patients have the option of cryopreserving semen samples for future use, i.e. insemination of a partner or for in-vitro fertilization (Rothmann, 1986; Rothmann *et al.*, 1986). A comparable option for women involves oocyte retrieval, IVF and embryo cryopreservation prior to chemotherapy, with or without gonadotrophin stimulation (Winkel and Fossum, 1993; Brown *et al.*, 1996). However, this is not always a feasible option, as some women do not have a designated partner, may be pre-pubertal and/or are concerned about cryopreservation of embryos. Cryopreservation of ovarian tissue prior to initiation of chemotherapy, followed by autologous transplantation after remission, could provide a means of protecting and replacing gonadal function and fertility. Steroidogenic function would be restored in these patients, thereby eliminating the need for exogenous hormone replacement therapy.

Pioneering work on the cryopreservation of ovarian tissue was carried out by Deansley (1954), Parkes and Smith (1954), Parkes (1957, 1958) and Parrott (1959, 1960). Improvements in the post-thaw viability of rat and mouse ovarian tissue were achieved with the addition of glycerol, a cryoprotectant, to the media in which the ovaries were cryopreserved (Parkes and Smith, 1954; Parkes, 1957). Following this pioneering work, little interest was generated in cryopreserving ovarian tissue until the 1990s, when live births derived from frozen–thawed mouse primordial follicles were reported (Carroll *et al.*, 1990; Carroll and Gosden, 1993). Few attempts at investigating in-vivo viability of cryopreserved ovarian tissue have been reported (Parrott, 1959, 1960; Miyamoto and Sugimoto, 1993; Gosden *et al.*, 1994; Harp *et al.*, 1994). Autologous transplantation of ovarian tissue in sheep resulted in delivery of a lamb derived from a cryopreserved ovarian graft (Gosden *et al.*, 1994). Harp *et al.* (1994) reported steroidogenic activity after autologous transplantation of cryopreserved mouse ovary, but did not comment on mating or pregnancies of these animals.

The present study was performed to evaluate the ability to cryopreserve whole mouse ovary using a slow cooling rate of 0.5°C/min. Cryopreserved ovarian tissue was transplanted autologously to the ovarian bursa, and subsequent oestrous cyclicity, pregnancies and live births monitored. Sham-operated, fresh and cryopreserved transplant animals were mated several times to determine the extent to which fertility was restored in terms of the ability to produce more than one

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litter. The fertility of the pups derived from ovarian tissue after fresh and cryopreserved transplants was also evaluated.

Materials and methods

Approval for this study was obtained from the Methodist Hospital Animal Research Committee. Albino ICR outbred female (aged 10–12 weeks) and male (breeders of proven fertility, aged 12–52 weeks) mice (Harlan Sprague-Dawley, Indianapolis, IN, USA) were housed with free access to food and water and with 14:10 h light:dark cycles. Vaginal smears were taken regularly to assess cytology and only di-oestrus mice, identified by the presence of leukocytes, were used for procedures. Female mice were randomly assigned to one of four groups; sham-operated (positive control, $n = 14$), ovariectomized (negative control, $n = 11$), fresh ovarian tissue autologous transplant ($n = 12$) or cryopreserved ovarian tissue autologous transplant ($n = 11$).

Surgical procedures

Anaesthesia was induced with an i.m. injection of Ace Promazine and Ketaset (Fort Dodge Laboratories, Fort Dodge, IA, USA) diluted with phosphate-buffered saline (Gibco, Grand Island, NY, USA). Each ovary and surrounding fat pad was exposed through a small dorsolateral incision and kept moist with sterile HEPES-buffered Tyrode's lactate medium (TL-HEPES; Bavister *et al.*, 1983). Sham-operated controls did not have any further surgery; each ovary and fat pad was replaced, incisions sutured and the animal allowed to recover. In all other experimental groups, a small slit was made in the bursa, and the ovary excised, taking care to remove all ovarian tissue. Excessive bleeding was reduced by applying pressure with a sterile gauze until a clot formed. Ovaries from the ovariectomy group were discarded, the incisions sutured, and the animal allowed to recover. Ovaries excised for fresh and cryopreserved transplant were placed in sterile TL-HEPES at 37°C. Fresh autologous transplants were performed within 5–7 min of removing the ovary. Ovaries to be cryopreserved were transferred to cryopreservation media, processed as described below, and transplants performed 24 h later. Fresh or cryopreserved–thawed ovaries were replaced within the bursa, which was secured with a suture, the fat pad replaced and the incision closed.

Cryopreservation and thawing protocol

Ovaries were cryopreserved using a protocol modified from Harp *et al.* (1994). Ovaries were placed in a 1.2 ml cryovial (Corning, Corning, NY, USA) with 1 ml of 1.4 M dimethyl sulphoxide (DMSO; Sigma, St. Louis, MO, USA) in TL-HEPES medium and held at room temperature for 5 min. The vials were sealed, placed in a programmable rate freezer (Planer, Sunbury-on-Thames, UK) and cooled from 25 to 10°C at 1°C/min, then at a rate of 0.5°C/min to –7°C and held at –7°C for 5 min. Ice nucleation was induced manually using pre-cooled forceps and the temperature was held at –7°C for a further 5 min, for release of latent heat fusion. The tissue was then cooled to –55°C at a rate of 0.5°C/min, plunged in liquid nitrogen at –196°C and stored for 24 h. Thawing was performed by removing the cryovial to room temperature until all ice had melted (15–20 min), and then transferring the tissue to 5 ml of fresh TL-HEPES at room temperature for 10 min, shaking gently to promote efflux of DMSO from the tissue. Tissues were then placed in TL-HEPES at 37°C until transplant, performed as described above. The thawing rate was found to be 48.8°C/min for the first 2 min, 9.1°C/min for the next 8 min and 0.91°C/min for the last 10 min, by which time all the ice had melted.

Mating and pregnancy evaluation

Animals were allowed to recover for 7–10 days after surgery, at which time vaginal cytology was examined for evidence of oestrous cycles (by the presence of epithelial cells). When vaginal cytology indicated early stages of pro-oestrus, or a majority of epithelial cells, females were paired with males. Matings were confirmed by the presence of a vaginal plug, at which time females were housed individually and weighed for 15 days to monitor weight gains indicating pregnancy. If no weight gain was seen after 15 days, the animals were re-mated at pro-oestrus, confirmed by visualization of epithelial cells in vaginal smears. If no vaginal plug was seen, females were left in continuous pairings with males for 21 days and then weighed for 15–21 days. Live births and numbers of pups were recorded. Animals from the sham-operated, fresh transplant and cryopreserved transplant groups were mated up to four times, after checking vaginal cytology at least 1 week after weaning of delivered pups. Pups from these groups were also mated (aged $\geqslant$6 weeks) with adult males or females, and deliveries and live births monitored.

Assessment of transplant tissue

Animals were killed 5–12 months after the initial transplantation or surgery, and the degree of adhesion, appearance of the bursa and the quantity and quality of ovarian tissue recovered were noted. Patency of the oviducts were determined by following the progress of 0.1% Trypan Blue (Sigma) through the tubes.

Data analysis

Data were analysed using the General Linear Models procedure of the Statistical Analysis System (*SAS User's Guide*, 1985) and expressed as means $\pm$ SEM. Comparisons were conducted through the least significant difference (LSD) approach. $P < 0.05$ was considered statistically significant.

Results

Vaginal cytology

Sham-operated animals all demonstrated clear oestrous cycles beginning 7 days after surgery. Vaginal cytology of ovariectomized animals did not show any evidence of epithelial cells. Smears from these animals contained mainly leukocytes, with some cornified epithelial cells. Epithelial cells were seen, 15 days post-operatively, in vaginal smears from fresh and cryopreserved autologous transplant animals. Vaginal cytology of the transplant animals did not always demonstrate clear oestrous cycles (as seen pre-operatively), but did show changes in the predominance of a particular cell type, i.e. majority of epithelial cells.

Body weight measurements

Animals with copulatory plugs did not always demonstrate increases in body weight, including one of the sham-operated control group (Figure 1). Conversely, body weight gains were recorded in some animals in which a vaginal plug was not seen, possibly missed by timing. In animals without visualization of a copulatory plug prior to delivery of live pups, days were calculated back, from the date of delivery as day 20. Animals which delivered at least one live pup demonstrated a 24% gain in body weight at delivery.

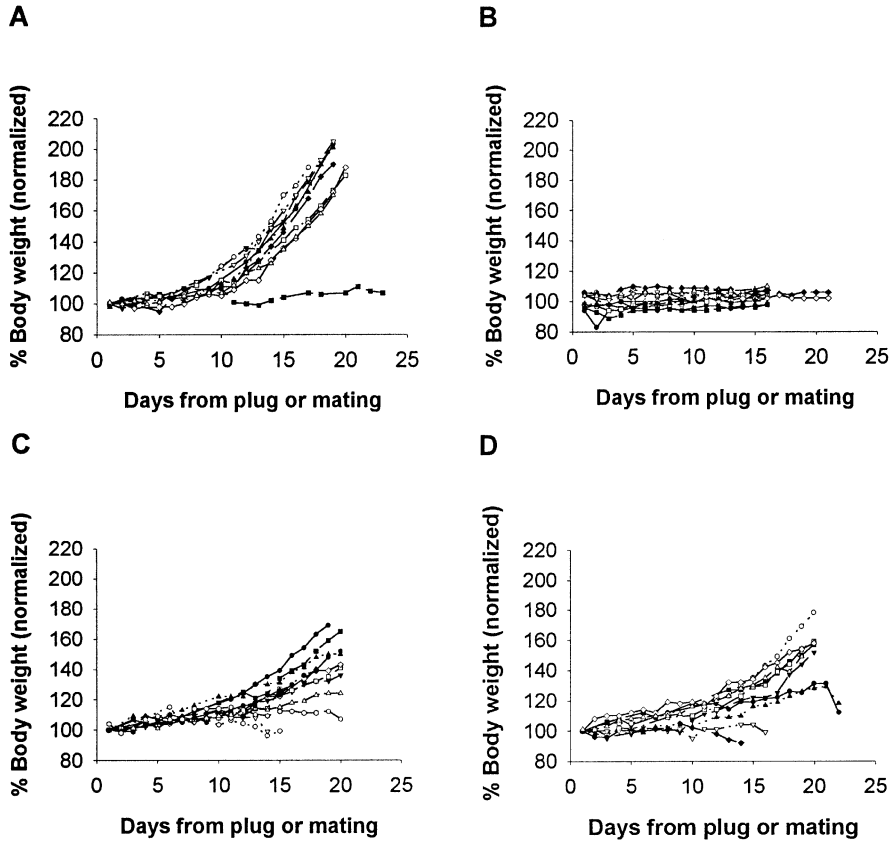

Figure 1. Body weight after visualization of a copulatory plug or end of a mating period. Body weights (normalized for each individual) for sham-operated (**A**), ovariectomized (**B**), fresh transplant (**C**) and cryopreserved transplant (**D**) animals.

Sham-operated (n = 14)

Thirteen (out of 14) sham-operated females became pregnant and had successful live births of large litters after the first mating. One sham-operated animal did not show increased body weight or signs of pregnancy after a confirmed mating (visualization of a vaginal plug) and was mated for a second time, with a resultant pregnancy and live birth.

Ovariectomized (n = 11)

Ovariectomized animals were placed in continuous pairings with male mice for 15 days. Ovariectomized animals were weighed for 15 days after removal from the male and monitored for a further 2 weeks. Two animals had vaginal plugs, indicating matings, but they did not demonstrate increased body weight after 21 days.

Fresh autologous transplant (n = 12)

After the first mating, nine out of 12 animals had live births. Three animals had confirmed matings but did not demonstrate an increase in body weight and were mated for a second time. After a second mating, all three animals delivered litters of live pups.

Cryopreserved autologous transplant (n = 11)

Six out of 11 animals with cryopreserved ovarian transplants had successful deliveries after the first mating. Two animals delivered live young after the second mating and two did not become pregnant after at least two further positive matings or continuous pairing with a male for at least 2 weeks. The remaining female demonstrated an increase in body weight followed by 19% and 24% loss overnight (19 days after visualization of the copulatory plug), together with evidence of cannibalized pups. The animals that did not become pregnant did demonstrate oestrous cyclicity, determined by the presence of epithelial cells in vaginal smears.

Litter sizes

Live births were recorded in 14/14 sham-operated, 12/12 fresh and 8/11 cryopreserved transplant animals. Overall mean $\pm$ SEM litter sizes were 12.54 $\pm$ 0.44 (range 8–17) for sham-operated, 4.32 $\pm$ 0.44 (range 1–9) for fresh transplant and 4.71 $\pm$ 0.57 (range 2–10) for cryopreserved transplant groups. Litters of the fresh and cryopreserved transplant groups were significantly smaller than those of sham-operated animals ($P < 0.05$), but were not statistically significantly different from each other.

Successive matings and litters

Animals from sham-operated, fresh and cryopreserved transplant groups were mated several times, with an overall live birth rate per mating of 26/28 (92.9%), 31/45 (68.9%) and 21/42 (50%) respectively. All animals achieved maximal pregnancy rates after the second mating (Figure 2): 100% for sham-operated and fresh transplant and 73% for cryopreserved transplant groups. The percentage of animals having more than one litter and the mean sizes of those

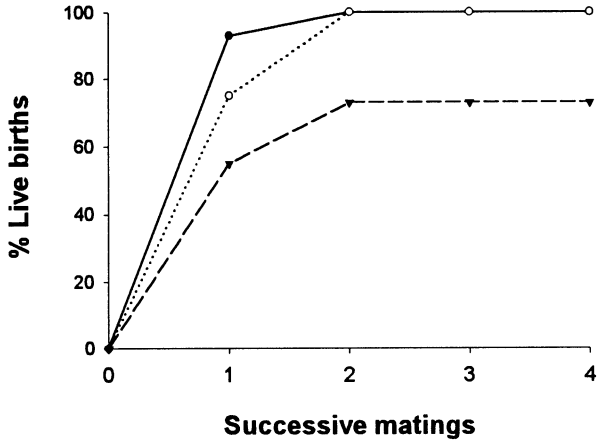

Figure 2. Cumulative pregnancy rate (% live births) after successive matings for sham-operated (●), fresh (○) and cryopreserved (▼) transplant groups.

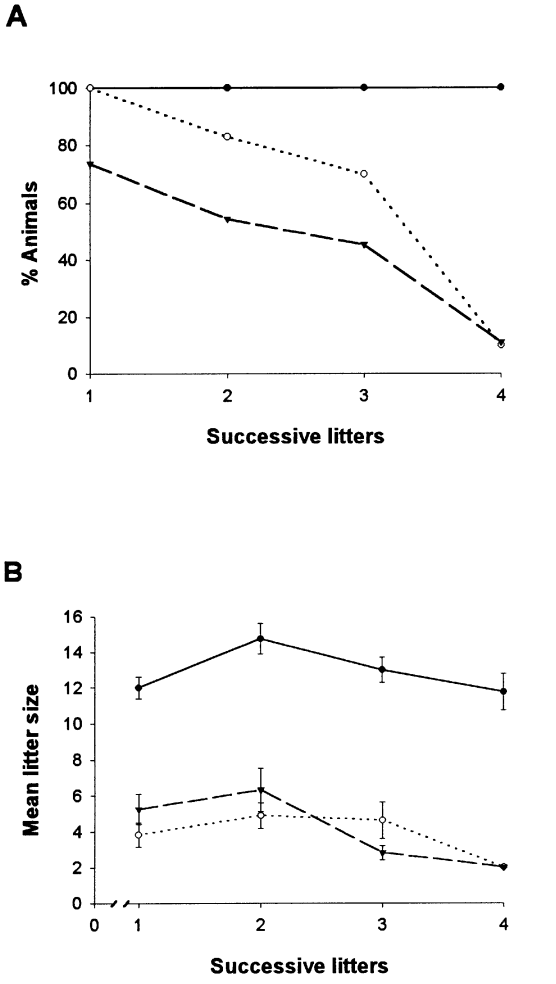

Figure 3. Percentage of animals delivering successive litters of live pups (**A**) and mean (±SE) litter sizes of successive litters (**B**) for sham-operated (●), fresh (○) and cryopreserved (▼) transplant groups.

successive litters are shown in Figure 3A and B. Sham-operated animals ($n = 4/4$) delivered four litters of live pups, whereas the numbers of successive litters from the ovarian tissue transplant groups decreased in a parallel manner (Figure 3A), to only 1/10 with fresh and 1/9 with cryopreserved ovarian tissue. Each litter of the transplant groups was smaller than those of the sham-operated animals ($P < 0.05$), but they were not significantly different from each other. The second sham litter (14.75 ± 0.85, $n = 4$, range 13–17) was significantly larger ($P < 0.05$) than the first (12.00 ± 0.62, $n = 14$, range 8–16) and fourth (11.75 ± 1.03, $n = 4$, range 9–14) litters, but was not significantly different from the third litter (13.00 ± 0.71, $n = 4$, range 12–15). There were no significant differences in sizes of successive litters from the fresh transplant animals. Second (6.33 ± 1.20) and third (2.83 ± 0.40) litters of cryopreserved transplant animals were significantly different ($P < 0.05$), perhaps reflecting a decline in ovarian function associated with age: third deliveries occurred at an age of 35–42 weeks, compared to 28–38 weeks for fresh transplant animals.

Second generation

Pups delivered from sham-operated, fresh and cryopreserved transplant groups were mated (aged ⩾6 weeks) with adult male or female mice. Mean litter sizes from first-generation male and female pups are shown in Table I. The litters (second generation) were large (from 8 to 16 pups) and not significantly different in size to litters from sham-operated pups; nor was there a difference in litter size between male and female pups. One pup was lost from at least one litter per group. Pups did not appear to have any obvious congenital defects.

Long-term ovarian function

Evidence of oestrogenic activity assessed by vaginal cytology (presence of epithelial cells) was seen in all but one animal (cryopreserved transplant) at the time of post-mortem investigation, 5–12 months after surgery. Moderate to severe adhesions were seen in three fresh transplant and five cryopreserved transplant animals, although they did not appear to preclude live births as one of these animals had four litters. Ovarian tissue was recovered from all but one animal in the cryopreserved transplant group, which had only trace amounts. Vaginal cytology of this animal demonstrated epithelial cells prior to its death. Tubal patency was established in at least one oviduct of all animals, with questionable patency of the other oviduct 4/12 fresh transplant and 3/11 cryopreserved transplant animals.

Discussion

In this study a slow cooling/rapid thaw protocol was used to cryopreserve ovarian tissue, with very high levels of subsequent in-vivo post-thaw function. Pregnancies, live births and subsequent fertility of the offspring derived from fresh (non-frozen) and cryopreserved ovarian tissue were evaluated. Although autologous transplantation of fresh (i.e. non-frozen) ovarian tissue resulted in a marked reduction in litter size and subsequent fertility compared to the sham-operated group, all animals demonstrated fecundity. Therefore, the surgical re-transplantation of ovaries has a detrimental effect on oocyte viability, compared to sham-operated controls. After transplant of cryopreserved ovarian tissue, 8/11 (73%) animals became

Table I. Litters resulting from pups derived from sham-operated, fresh or cryopreserved transplant groups. Values are mean ± SEM; n = number of animals delivering/number of animals mated. There were no significant differences between groups

Group	Litters from female pups (no. of pups)	Litters from male pups[a] (no. of pups)
Sham-operated	10.75 ± 0.25 (n = 4/5)	12.60 ± 0.51 (n = 4/5)
Fresh transplant	12.14 ± 0.51 (n = 7/7)	13.17 ± 0.79 (n = 5/6)
Cryopreserved transplant	12.00 ± 0.73 (n = 6/7)	11.80 ± 1.20 (n = 5/6)

[a]Male pups were mated with adult females, and resulting litters were evaluated.

pregnant and delivered live pups. However, three animals receiving cryopreserved ovarian tissue did not have successful litters, although evidence of oestrous cyclicity was seen (by vaginal cytology). Post-mortem investigation indicated an apparent loss of the majority of the tissue from the site of transplant, and one animal was found to have partially occluded oviducts. Litter sizes of the cryopreserved transplant group were not significantly different from those of the fresh transplant group, indicating that the negative impact of the transplant procedure (i.e. compared to sham) was not compounded by the freezing and thawing of the tissue. Subsequent fertility of fresh and cryopreserved transplant groups also appeared to decline in a parallel manner. Clearly, transplant trauma (delay in reattachment, cooling, exposure to culture media) to mouse ovary causes a loss of gamete number and/or function, resulting in smaller litters. Cryopreservation of mouse ovaries using the slow cool/rapid thaw protocol did not diminish the number of pregnancies and litter sizes (gross indices of ovarian function) to a greater extent than that caused by transplanting the non-frozen tissue.

Few reports of live births after transplant of cryopreserved ovarian tissue exist in the literature. Parrott (1960) first obtained live births after orthotopic allogeneic transplant of ovarian tissue cryopreserved using a protocol involving exposure to glycerol for 1.5–2.5 h and a cooling rate described as <2°C/min. Live birth rates following exposure to glycerol for 1 h prior to cryopreservation resulted in live births from 1/8 animals, which decreased to 0/10 when glycerol exposure was increased to 1.5–2.5 h. The choice of DMSO as the cryoprotectant, shorter duration of exposure prior to cooling (5 min) and a more controlled, slower cooling rate probably account for the marked improvement in pregnancies and live births obtained in the present study. Other studies evaluating in-vivo function of cryopreserved ovarian tissue reported some steroidogenic activity (Miyamoto and Sugimoto, 1993; Harp *et al.*, 1994; Candy *et al.*, 1995a), and the birth of a lamb derived from a cryopreserved ovine graft (Gosden *et al.*, 1994).

Successful cryopreservation of single cells or embryos requires the addition of permeating cryoprotectants. These compounds serve to protect the cells from high solute (primarily salt) concentrations which develop as water precipitates as ice. To achieve this end, the cell must attain an equilibrium state, in which the cryoprotectant and water concentrations, extracellularly and intracellularly, are equal (Mazur, 1984). Alternatively, vitrification may occur, in which high concentrations of cryoprotectants are used and rapid (usually one step) cooling to –196°C is performed. Successful post-thaw viability of the mouse ovary in this study may have been attained by (i) permeation of a majority of the cells with DMSO via a capillary network, and/or (ii) attainment of the equilibrium state in the cortical area containing primordial follicles. Evidence for the latter possibility is the time delay in restoration of oestrous cyclicity, 15 days post-transplant, which is close to the 3 week duration of growth and development of follicles from primordial to Graafian stage (Pedersen, 1969). Green *et al.* (1956) also reported a similar interval (13 days) between transplant of cryopreserved ovarian tissue and observation of the first period of oestrus. The mouse ovary has a large supply of primordial follicles and recruitment and ovulation of this population could be responsible for the pregnancy and deliveries obtained in this study. Post-thaw recovery of follicles in cryopreserved ovarian tissue has ranged from 5% (Green *et al.*, 1956) to 30% with vitrification (Sugimoto *et al.*, 1996). The highest recovery of follicles (50%) in cryopreserved mouse ovarian tissue was obtained when DMSO, propylene glycol, or ethylene glycol were the cryoprotectants used, compared to only 30% survival when glycerol was used (Candy *et al.*, 1995b). Although a significant proportion of the oocyte population may have been lost during cryopreservation, the recovery of sufficient viable oocytes to generate and maintain a viable pregnancy is possible. However, delivery of several litters is compromised, as seen in this study.

Cryopreservation of ovarian tissue is usually performed using empirically derived protocols, which may not be optimal for maximal post-thaw viability. Optimization of cryopreservation–thawing protocols can be achieved by determining the cryobiological properties of ovarian tissue. These include the rate at which water leaves (water permeability, L_p) and cryoprotectants enter (solute permeability, P_s) the cells. These investigations are currently underway, and preliminary data suggest that the permeability of the mouse ovaries to DMSO and ethylene glycol is greater than to propanediol.

Cryopreservation of ovarian tissue for autologous use by oncology patients could protect both steroidogenic and gametogenic functioning, and prevent premature menopause, which is often suffered by many of these patients. The ovary, or part of it, could be removed prior to treatment, cryopreserved and replaced when the patient had recovered from the primary condition. Replacement of the ovary may present a considerable challenge in locating a site for autologous transplant with adequate vascularization. Further problems would be the positioning of the tissue in relation to the Fallopian tube, if future fecundity is the goal. Alternatively, patients specifically seeking a pregnancy could have ovulation induction, recovery of oocytes and IVF. One potential and significant hazard is the possibility that cancerous cells from the original condition

may survive within the ovarian tissue and be reintroduced when the tissue is thawed and autologously replaced. Reintroduction of cancer is obviously a significant problem and requires further critical study.

Currently, efforts to prevent the extinction of endangered species rely on cryopreservation and banking of embryos (Dresser, 1988) or spermatozoa (Wildt, 1996). Ovarian tissue from an endangered species, e.g. snow leopard, could be cryopreserved and utilized to recover oocytes for IVF and transplant to surrogate animals, e.g. domestic cats. The drive to preserve biodiversity via generation of genome resource banks is intense, without which species extinction will continue relentlessly. Recent studies evaluating cryopreserved ovarian tissue from several species have demonstrated evidence of in-vivo oestrogenic function after xenotransplantation to immune incompetent mice (Gunasena *et al.*, 1996). Xenotransplantation to the immune incompetent mouse could serve as an in-vivo bioassay of cryopreserved ovarian tissue function, particularly from endangered species. Further work with cryopreserved ovarian tissue from endangered species is currently underway to evaluate the usefulness of this technique as a tool for genome resource banking.

The results of the present study suggest that high viability and function is retained in mouse ovarian tissue after cryopreservation. Litter sizes were not significantly different from those derived from non-frozen ovarian tissue, and deliveries of several litters were recorded. Pups derived from the fresh or cryopreserved transplants were also fertile, and the litter sizes from these animals were not significantly different from controls. The potential application of ovarian tissue cryopreservation for rescue of human gonadal function is immense, as is the possibility of generating genome resource banks to protect valuable genetic biodiversity.

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