Blastocyst Development After Cryopreservation and Subcutaneous Transplantation of Mouse Ovarian Tissue

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Purpose: To investigate follicle survival and developmental potential with IVF of cryopreserved, subcutaneously transplanted mouse ovarian tissue.

Methods: Fresh and frozen mouse ovarian tissue was autologously transplanted into subcutaneous tissue. Two weeks after the transplantation, the morphology and histology of the fresh and frozen grafts were compared. Superovulation and IVF was performed to evaluate the fertility potential of the frozen ovarian graft.

Results: Both fresh and frozen grafts of ovarian tissue survived in 14 of 16 mice (88%). Morphologically, both types of grafts resembled fresh ovarian tissue and contained follicles at all stages of folliculogenesis. A total of 73% of follicles in fresh grafts and 62% in frozen grafts survived after transplantation compared with fresh ovarian tissue. Sixteen ICR mice underwent superovulation. A total of 56 oocytes from antral follicles were recovered from the subcutaneously transplanted cryopreserved ovarian tissue. Fourteen (25%) oocytes were in metaphase II stage, 6 were fertilized by IVF, and 2 progressed to the blastocyst stage.

Conclusions: Cryopreservation and subcutaneous transplantation of ovarian tissue provides a possible means of fertility preservation. The main loss of follicles occurred during grafting rather than during freezing and thawing.

KEY WORDS: Blastocyst; cryopreserved ovarian tissue; subcutaneous transplantation.

INTRODUCTION

Ovarian tissue banking is under investigation for many potential clinical applications. It may provide a solution for fertility preservation for patients with cancer whose treatment will impair their ovarian function. This is especially important for unmarried women or children who cannot use embryo cryopreservation. In addition to fertility preservation, restoration of endocrine function is also under investigation (1). Candy *et al.* reported successful re-

production after orthotopic transplantation of cryopreserved mouse ovaries (2). Liu *et al.* also demonstrated successful mouse IVF after transplantation of cryopreserved ovarian tissue into the renal capsule (3,4). However, orthotopic or renal capsular heterotopic transplantation of cryopreserved ovarian tissue is of little practical value since it makes ovum pick up (OPU) difficult. Subcutaneous transplantation would solve this difficulty (5,6). The aim of this study was to evaluate subcutaneous transplantation of cryopreserved mouse ovaries by investigating follicle survival and embryo development after IVF.

MATERIALS AND METHODS

Animal

Ethical approval for this study was obtained from the Animal Research Ethical Committee of Mackay

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Memorial Hospital. All studied animals are ordered from National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan. All mice used in this study were housed and bred in the Central Animal House at Department of Medical Research, Mackay Memorial Hospital, at 22°C with a 14 h light: 10 h dark regimen. Thirty-two 6- to 8-week-old female mice were used for autologous subcutaneous transplantation of cryopreserved ovarian tissue.

Harvesting of Mouse Ovarian Tissue

One gram of 2,2,2-tribromoethanol (Fluka, Aldrich Chemical Co., Poole, UK) was dissolved in 1 mL of tertiary-amyl alcohol (Baker Analyzed) to make avertin solution for anesthesia. The mice were anesthetized by intraperitoneal injection of 15 μ L/g of avertin solution. Both ovaries were removed surgically. After the fat was removed, each ovary was cut equally into 3 pieces in M2 medium under a dissection microscope, yielding 6 pieces of ovarian tissue per mouse. Two pieces were immediately implanted subcutaneously in the left inguinal area, two pieces were cryopreserved, and two pieces were preserved in 10% formaldehyde (Merck) to serve as histologic controls.

Freezing and Thawing

We adopted the method of Gosden *et al.* with some modifications (7). Two pieces of ovarian tissue from each mouse were equilibrated at room temperature for 20 min in M2 medium containing 10% fetal bovine serum (FBS; Sigma Chemical Co, St Louis, MO, USA) and 1.5-M dimethylsulfoxide (DMSO; Sigma Chemical Co., St Louis, MO, USA) before being loaded into a 0.25-mL plastic freezing straw (type-2A 175; NUNC). The straws were sealed with polyvinyl chloride powder and placed in a programmable biological freezer (CryoMed 1010, Forma Scientific, Inc., USA) at 0°C and cooled at a rate of 2°C/min to -7°C. They were held at -7°C for 5 min, seeded manually with previously cooled forceps into liquid nitrogen (LN2), and held at -7°C for a further 5 min. The straws were cooled at a rate of 0.3°C/min to -40°C and then at 10°C/min to -150°C. They were transferred into LN2 for storage at -196°C for at least 24 h.

One day later, the cryopreserved tissue was thawed as follows. The straws were removed from LN2, held in the air at room temperature for 20 s,

and then immersed in 37°C water bath until the ice melted. The tissue was then removed from the straws and placed in 2 mL of M2 medium at room temperature for 5 min, and then washed twice in 2 mL of M2 medium at room temperature. After washing, the tissue was placed in M2 medium at 37°C. Thawed tissue was autologously grafted into subcutaneous tissue in the right inguinal area within 45 min of thawing.

Transplantation of Ovarian Tissue

The same procedure was followed for autologous transplantation of fresh and frozen ovarian tissue. After the mouse was anesthetized with avertin solution, a small incision of about 0.5 cm was made in the inguinal region, followed by blunt dissection of a 1-cm subcutaneous pocket, using a pair of fine curved watchmaker's forceps. The ovarian tissue was inserted into the subcutaneous space, and the skin incision was closed with a stitch of nylon suture.

Experiment 1. Survival of Ovarian Tissue by Morphology and Histology

Two weeks after transplantation, 16 of the 32 mice were euthanized by cervical dislocation. The inguinal areas were opened bilaterally and the gross appearance of the grafted ovarian tissue was recorded, after which the grafts were removed. The ovarian tissue was fixed in 10% formaldehyde solution for at least 24 h, then embedded in paraffin wax, serially sectioned at 5 μ m, and stained with hematoxylin and eosin. One section from each of the treatment or control in the same mouse was examined for the presence of various follicles. The number of each kind of follicle was recorded and the follicular number loss was calculated according to the total number of various follicles recorded from all experimental mice.

Experiment 2. Superovulation of Cryopreserved Autologously Transplanted Ovarian Tissue

Two weeks after grafting, the remaining 16 mice underwent superovulation as follows. PMSG (pregnant mare's serum gonadotropin, China Chemical and Pharmaceutical Co., Hsin-Chu, Taiwan) 400 IU was injected intraperitoneally, and hCG (human chorionic gonadotropins, China Chemical and Pharmaceutical Co., Hsin-Chu, Taiwan) 200 IU

Fig. 1. Histologic section of (A) control fresh ovarian tissue, and, after 14 days of subcutaneous transplantation, (B) fresh graft, and (C) frozen graft. Scale bar = 200 μ m.

was given 48 h later. The mice were euthanized by cervical dislocation 10 h after hCG administration. The transplanted ovarian tissue was carefully removed from both inguinal areas. Oocyte-cumulus complexes (OCC) from the antral follicles of the grafts were isolated using 30-gauge needles (Becton Dickinson, USA). After washing with M2 medium, status of maturation of the oocytes was scored as a germinal vesicle (GV) being present, as the GV having broken down (GVBD), and as metaphase II (MII) when the first polar body had been extruded.

RESULTS

Experiment 1. Survival of Ovarian Tissue by Morphology and Histology

Two weeks after transplantation, 14 of 16 fresh and 14 of 16 cryopreserved ovarian tissues were easily identified on opening of the inguinal areas of the mice. Structurally, ovarian tissue was intact and was surrounded by a layer of fibrous tissue and blood vessels. The remaining 2 fresh and 2 frozen grafts showed were atrophic, with only fibrosis found. The proportion of both fresh and frozen grafts that survived was therefore 88% (data not shown).

The ovarian grafts were compared histologically with the control fresh ovarian tissue. Both fresh and frozen grafts contained follicles at all stages of follicu-

logenesis and corpora lutea (Fig. 1). Their appearance was similar to the control tissue. In the control tissue, the total number of primordial follicles, primary follicles, preantral follicles, and antral follicles in one high power field were 98, 55, 38, and 18; comparable numbers for the fresh grafts were 72, 44, 27, and 10 and for the frozen grafts, 65, 37, 21, and 6, respectively (Table I). Loss of follicles during cryopreservation and subcutaneous transplantation of ovarian tissue was noted, with approximately 27% fewer follicles in the fresh grafts and approximately 38% fewer in the frozen grafts, compared with the control tissue.

Experiment 2. Superovulation of Autologously Subcutaneous Transplantation of Cryopreserved Ovarian Tissue and IVF

The results of superovulation and OPU are shown in Table II and Fig. 2. A total of 96 oocytes from preantral follicles and 56 OCC from antral follicles were recovered. The OCC from grafts were loosely configured, with poor connections between the oocytes and their companion granulosa cells. After cumulus cells were denuded, 37 of 60 (60%) were found to be GV stage oocytes, 5 (9%) GVBD stage, and 14 (25%) M II stage. All 14 of the M II stage oocytes were subjected to IVF, resulting in 6 (43%) that were fertilized and underwent cleavage to the 2-pronuclear stage. Two of these zygotes progressed to the blastocyst stage.

Table I. Survival of Follicles After Grafting Fresh or Frozen-Thawed Ovarian Tissue

	Number of follicles per HPF				Total	% of control
	Primordial follicle	Primary follicle	Preantral follicle	Antral follicle		
Fresh	98	55	38	18	209	—
Fresh grafts	72	44	27	10	153	73
Frozen grafts	65	37	21	6	129	62

Table II. Outcomes of Subcutaneous Transplantation of Cryopreserved Ovarian Tissue in 16 Mice

Preantral follicle oocytes	96
Oocyte-cumulus complexes	56
GV stage oocytes	37 (66%)
GVBD stage oocytes	5 (9%)
M II stage oocytes	14 (25%)
Fertilized	6
Cultured to blastocyst stage	2

DISCUSSION

We have demonstrated that cryopreserved mouse ovarian tissue successfully survives subcutaneous transplantation. It is capable of responding to hormonal stimulation, yielding oocytes that can be fertilized, with development at least to the blastocyst stage.

Although there are well-established protocols for preserving human semen and embryos, there has

been little success in cryopreservation of mature oocytes (8). Undifferentiated primordial oocytes are relatively dormant developmentally, small in size, and do not have a zona pellucida or cortical granules. Owing to these characteristics, they tolerate freezing and thawing much better than mature oocytes.

Ovarian tissue is more resistant to damage during transplantation than other tissues. Primordial follicles are abundant and may be resistant to ischemia, as they normally develop within an avascular epithelium and a relatively hypoxic environment (9). A number of angiogenic factors were expressed in transplanted ovarian tissue (10). In addition, most primordial follicles are present in the periphery and are first to benefit from revascularization.

A series of successful animal experiments with ovarian tissue cryopreservation and transplantation have been published. In Candy’s mouse study (2), ovariectomized nude mice underwent orthotopic transplantation of frozen ovaries, mated with male

Fig. 2. Superovulation and IVF of ovarian frozen graft: (A) gross appearance of frozen graft after superovulation, (B) oocyte-cumulus complex, (C) preantral follicle, (D) GV stage oocyte, (E) GVBD stage oocyte, (F) M II oocyte, (G) four-cell stage embryo, (H) blastocyst after IVF. f: antral follicles, c: cumulus cells, g: granulose cells. The scale bar (=40 μ m) is shown for the oocyte and embryo (D–H).

mice, and delivered normal offspring. However, in orthotopic ovarian transplantation experiments of sheep, Baird *et al.* (11) found that reproduction was difficult from natural mating even though the ewes were hormonally active for up to 22 months. Salle *et al.* reported successful pregnancies and live births after transplantation of frozen-thawed ovaries in ewes, in spite of a dramatic reduction in the total number of follicles (12). In a human study, Oktay *et al.* developed techniques to orthotopically transplant frozen ovarian tissue by laparoscopy, maintaining ovarian function for 10 months, including ovulation and restoration of menses (13). He concluded that natural conception would be unlikely, since oocyte capture by the fallopian tubes following orthotopic transplantation would be problematic. However, IVF would still be feasible in such cases, which is why we specifically investigated heterotopic transplantation.

The mouse renal capsule is highly vascularized, providing an ideal site for rapid revascularization. A number of studies have demonstrated successful IVF in mice with such ovarian transplants, with delivery of offspring. Oktay *et al.* also developed human primordial follicles to the antral stage after transplantation under kidney capsule of nude mice (14,15). Both the ovary and renal capsules are rich in angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor, which further enhance survival and revascularization (1,3,10). But, as noted in the Introduction, grafts under the renal capsule made ovum retrieval difficult, leading us to investigate subcutaneous transplantation as an alternative.

Aubard *et al.* (16) performed subcutaneous transplantation of frozen ovarian tissue in sheep, but no embryos from IVF could be developed to the blastocyst stage. They concluded that the transplanted ovarian tissue yielded only poor oocytes. This was in contrast to our experiment in mice, in which the histologic appearance of frozen subcutaneous grafts was comparable to that of fresh grafts. Primordial follicles appear to be more resistant to the effect of ischemia than growing follicles, presumably by virtue of being dormant and having a low metabolic rate (1). In our study, fresh grafts had 73% and frozen grafts 62% as many follicles as the controls. The difference suggests that follicles are lost primarily in the grafting process rather than as a result of freezing and thawing. In the sheep's subcutaneous transplantation, Baird *et al.* found that the number of follicles was reduced by 65% after grafting the fresh tissue

and by only another 7% after freezing and thawing before grafting (11). Jeremias *et al.* and Laschke *et al.* also demonstrated that cryopreservation accounts for relatively little tissue injury in ovarian grafts (17,18). Fabbri *et al.* showed that human ovarian tissue antigenicity, cellular proliferation, and anti-apoptotic index were well preserved after cryopreservation (19). As mentioned above, therefore, it appears that the follicle loss in the cryopreserved grafts was primarily a result of slow revascularization, which may take more than one day to complete. So, while subcutaneous transplantation makes ovum retrieval easier, that advantage must be weighed against the risk of follicle loss to ischemia.

We preliminarily used a general dose of gonadotropin, 5 IU (20), to superovulation. However, follicles have no further development. In order to gain the ideal efficiency, we then modified the dosage and finally reached the higher doses of 400 IU. In orthotopic transplantation of human ovarian tissue, Oktay *et al.* also found larger dose of gonadotropin was needed in the stimulation cycle (13). This might be due to limited vascularization of the grafts in the subcutaneous space and poor ovarian reserve after freezing, thawing, and transplantation. However, even though there was a dramatic reduction in mature oocytes collected after superovulation, we still succeeded in fertilizing 6 of the oocytes and having 2 develop to the blastocyst stage. To our knowledge, this is the first report of blastocyst development after subcutaneous transplantation of cryopreserved ovarian tissue.

Oktay *et al.* also demonstrated that the human subcutaneous ovarian transplantation can preserve endocrine function (6,21) and the fertility was demonstrated by developing a four-cell embryo (21). They then transferred the embryo into the uterus but with no further illustration. In our study, we have transferred the blastocyst into the uterus of pseudopregnant mouse and also tried several times to transfer 2PN embryos into the oviduct but no offspring was found. Simultaneous transfer of multiple embryos might be needed to achieve a pregnancy.

Nugent *et al.* (22) tried to reduce damage by reactive oxygen species generated during ischemia and reperfusion by antioxidant vitamin E and achieved a significant improvement in follicle survival, from 45% to 72%. Schnorr *et al.* found that VEGF administered subcutaneously at the site of ovarian transplantation in a monkey model did not improve graft survival, although their sample size was small (23). Wang *et al.* demonstrated that ovaries

stimulated with gonadotropins before cryopreservation had improved follicle survival after thawing and subcutaneous transplantation, possibly by enhancing growth factors (5). In an unpublished study, we found that local transfection with a viral vector carrying platelet-derived growth factor C significantly improved mouse IVF embryo development.

Another possible cause of poor oocyte quality might be subtle functional impairment during freezing, thawing, and transplantation. Despite the survival of two-thirds of the primordial follicles after cryopreservation and transplantation, the number of follicles developing to mature M II oocytes was markedly reduced. In vitro maturation (IVM) of isolated preantral follicles to maturity might be another method for restoration of fertility. In the study of Liu *et al.* cryopreserved primordial follicles developed to preantral follicles by in vivo grafting but were then recovered and cultured to maturity by IVM (4). The same group also found apoptosis and impaired function in frozen and thawed granulosa cells after transplantation (24). It was suggested that granulosa cells are more active metabolically than primordial oocytes and thus were more subject to ischemic damage. Liu *et al.* found abnormal follicles in frozen grafts in terms of partial disconnection between oocytes and their companion granulosa cells (3), similar to our findings. The communication between oocytes and their granulosa cells is essential for proliferation and differentiation of granulosa cells as well as oocyte development (25,26).

CONCLUSION

Thus, while subcutaneous transplantation of cryopreserved ovarian tissue shows promise as a means of preserving fertility, other measures, for example, growth factors or anti-oxidant agents, are needed that will reduce ischemic damage during subcutaneous transplantation. In addition, successful transfer of embryos and maintenance of normal gestation must be demonstrated. If these obstacles can be overcome, cryopreservation of ovarian tissue may become a feasible alternative in women who lose their ovarian function because of cancer therapy.

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