Live offspring from vitrified blastocysts derived from fresh and cryopreserved ovarian tissue grafts of adult mice

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Abstract

Ovarian tissue cryopreservation and transplantation can be used to preserve fertility for cancer patients. In this study, we assessed the viability and function of ovarian tissue from adult mice that was cryopreserved by solid surface vitrification or traditional slow-cooling using various *in vitro* and *in vivo* techniques, including allotransplantation, *in vitro* oocyte maturation, embryo culture *in vitro*, blastocyst cryopreservation, embryo transfer, and development. The importance of cumulus cells for oocyte maturation, fertilization, and embryo development was investigated. Graft recovery, follicle survival, and oocyte retrieval was similar in control, vitrified, and slow-cooled groups. High rates of oocyte maturation, cleavage, and blastocyst formation were achieved, with no significant differences between the control, vitrified or slow-cooled ovarian tissue grafts. The presence of cumulus cells was important for oocyte maturation, fertilization, and subsequent development. Cumulus–oocyte complexes with no surrounding cumulus cells (N-COCs) or with an incomplete layer (P-COCs) had significantly lower rates of oocyte maturation and blastocyst formation than cumulus–oocyte complexes with at least one complete layer of cumulus cells (F-COCs; maturation rate: 63, 78 vs 94%; blastocyst rate: 29, 49 vs 80%). Live births were achieved using vitrified blastocysts derived from oocytes taken from vitrified and slow-cooled ovarian tissue heterotypic allografts. Successful production of healthy offspring from these vitrified blastocysts suggests that this technique should be considered as a useful stage to pause in the assisted reproduction pathway. This provides an alternative protocol for restoring fertility and offering cancer patients a better indication of their chances of pregnancy and live birth.

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Introduction

About 1 in 600 girls develop cancer within the first 15 years of life, but of these, more than 90% are cured using surgery and/or radiochemotherapy (Bath *et al.* 2002). However, these cancer patients are at risk of severely damaging or losing their ovarian function. Currently, the main options for them to preserve fertility are ovarian tissue, oocyte, or embryo cryopreservation. Many factors, such as female age, nature of disease, partner status, and moral or religious views, need to be considered when choosing the most suitable treatment (Donnez *et al.* 2006). Ovarian tissue cryopreservation has often been the only available option, especially for prepubertal girls and women who cannot delay their cancer treatment (Donnez *et al.* 2006). Improved ovarian tissue cryopreservation and transplantation protocols are needed to ensure the best outcomes for these patients (Donnez *et al.* 2006). Despite considerable interest, however, only five healthy babies have been born after cryopreservation and transplantation of human ovarian tissue (Donnez *et al.* 2004, Meirow *et al.* 2005, Demeestere *et al.* 2007, Andersen *et al.* 2008).

In animals, ovarian tissue cryopreservation and transplantation have been used for breeding, research, and conservation purposes in various species, including mouse, rats, rabbits, sheep, cats, wombats, and monkeys (Candy *et al.* 1995, 2000, Aubard *et al.* 1998, Wolvekamp *et al.* 2001, Salle *et al.* 2003, Almodin *et al.* 2004, Bosch *et al.* 2004). Slow-cooling, usually the method of choice for cryopreservation of ovarian tissue, is based on the principle of optimizing cooling rates to control injury from ice crystal formation, while minimizing chemical toxicity and osmotic stress from exposure to high concentrations of salts and solutes during freezing (Demirci *et al.* 2003).

Despite successes using slow-cooled cryopreserved ovarian tissue, vitrification has gained popularity. Vitrification is a process which aims to transform intra- and extracellular fluids into a glass-like matrix without forming ice crystals. It is faster than slow-cooling and can be an effective method for cryopreserving ovarian tissue (Chen *et al.* 2006, Kagawa *et al.* 2007, Li *et al.* 2007). High concentrations of cryoprotectant and fast cooling rates are the most important elements in achieving vitrification. Different vitrification methods involving

a variety of devices have been used for oocytes, embryos, or ovarian tissues, including electron microscope grids (Martino *et al.* 1996), open pulled straws (Vajta *et al.* 1998), nylon loops (Lane *et al.* 1999), solid surfaces (Al-aghbari & Menino 2002), or dropping samples directly into liquid nitrogen (LN₂; Papis *et al.* 2000). Of these methods, solid surface vitrification has achieved good results with ovarian tissue. Using this method, tissue samples are normally placed in a very small volume of vitrification solution and cooled on a sterile metal surface to maximize the cooling rate, and minimize the potential for contamination of samples by LN₂. Measures of survival of vitrified ovarian tissue after cryopreservation have included morphological examination (histology), viability assessment by viability staining or development *in vitro* or *in vivo* culture (Yeoman *et al.* 2005, Santos *et al.* 2007, Aerts *et al.* 2008). There are, however, limited data on the effects on the function of ovarian tissue vitrified using solid surface vitrification systems.

Cumulus cells are important for *in vitro* or *in vivo* oocyte maturation, fertilization, and subsequent embryo development. Removal of cumulus cells before *in vitro* maturation (IVM) procedures reduces the developmental potential of oocytes of many species, including mouse (Anderiesz & Trounson 1995, Chang *et al.* 2005), rabbit (Tao *et al.* 2008), pig (Wongsrikeao *et al.* 2005), cow (Chian *et al.* 1994), and human (Johnson *et al.* 2008). Cumulus cells are of critical importance for the final stages of oocyte nuclear and cytoplasmic maturation (Goud *et al.* 1998, Moor *et al.* 1998), because compounds such as amino acids and pyruvate enter the oocytes through the cumulus cell foot processes (Gilchrist *et al.* 2004). With the decrease in the layers of cumulus cells surrounding rabbit oocytes, the maturation rates declined (Tao *et al.* 2008). The presence of cumulus cells and corona cells also significantly improves the developmental potential of matured oocytes post-fertilization (Anderiesz & Trounson 1995).

In the mouse grafting model, healthy pups have been reported from F-COCs retrieved from fresh or cryopreserved ovarian grafts (Kagawa *et al.* 2007) while no attention has been drawn to those N-COCs or P-COCs. However, such selective criteria cannot always be used clinically, because the main aim is to maximize the number of matured oocytes for fertilization *in vitro*. In clinical settings, it is also normal to remove cumulus cells in order to assess their maturation status and for ICSI (Chin & Chye 2004). This study will investigate the developmental potential of oocytes (with or without cumulus cells enclosed) derived from fresh and cryopreserved ovarian grafts using a mouse model and the effect on live birth rate of vitrifying and storing blastocyst prior to embryo transfer.

In this study, a solid surface vitrification technique was compared with a traditional slow-cooling method. The cryopreserved adult mouse ovarian tissues were

transplanted into severe combined immune-deficient (SCID) mice. Fresh mouse ovarian tissues were used as controls. The aims were: 1) to evaluate two cryopreservation strategies for mouse ovarian tissues, by determining the subsequent graft development and survival, and the viability of the oocytes recovered from these grafts, 2) to test the need for cumulus cells to mature and fertilize oocytes derived from unfrozen and cryopreserved ovarian grafts, and 3) to assess the effect on live birth rate of vitrifying and storing pre-implantation embryos derived from cryopreserved ovarian tissues prior to embryo transfer.

Results

Graft survival and oocyte recovery

Subcapsular grafts of ovarian tissue were successfully retrieved from the kidney after transplantation in all treatment groups. Recovery and survival rates for the vitrified and slow-cooled ovarian grafts were similar to those of the fresh controls (Table 1).

A total of 308, 303, and 267 oocytes were collected from vitrified, slow-cooled, and fresh grafts respectively. In fresh controls, 46% of the oocytes recovered were cumulus-enclosed oocytes, and the remainders were either partially surrounded by cumulus cells or were cumulus-free. Similar proportions were found in recovered oocytes from the vitrification and slow-cooling treatment groups. The mean number of oocytes retrieved per survived quarter-ovarian graft in the vitrification group (3.56 ± 1.29) was not significantly different from the slow-cooling (4.42 ± 0.78) or fresh controls (3.66 ± 0.61 ; Table 1).

Morphological examination

Most of the fresh or cryopreserved quarter-ovarian grafts were successfully recovered and contained growing follicles after 12 days transplantation under the kidney capsule in SCID mice. Antral follicles were obvious and easily identified, and revascularization of the grafted tissue could also be seen. Histological examination

Table 1 Comparison of the number of grafts recovered and survived and oocytes retrieved from fresh, vitrified, and slow-cooled ovarian grafts after 12 days transplantation under the kidney capsule.

Treatment group ^a	Number of grafts recovered/number grafted	Number of grafts survived/number recovered	Total number of oocytes collected	Number of oocytes retrieved/survived graft
Fresh grafts	84/88 (95%)	74/84 (88%)	267	3.66 ± 0.61
Vitrified grafts	98/104 (94%)	86/98 (83%)	308	3.56 ± 1.29
Slow-cooled grafts	72/80 (90%)	69/72 (96%)	303	4.42 ± 0.78

^aNo significant difference was observed in any of these parameters between the two treatment groups and the fresh control grafts.

Figure 1 The morphology of grafts on the kidney of SCID mice after 12 days transplantation. (A) Eight pieces of slow-cooled grafts are visible with obvious antral follicles. Different number indicates the position of each graft. (B) Histological section of fresh grafts with multiple follicle growth. (C) Higher magnification of histological section showing antral follicle formation in a vitrified graft. A, antrum; AF, antral follicle; BV, blood vessel; CO, cumulus oophorus; O, oocyte; and SF, secondary follicle.

demonstrated that multiple follicles were developed after 12 days transplantation and antral follicles were easily distinguished by antrum cavity formation adjacent to full-sized oocytes (Fig. 1).

Oocyte maturation, fertilization, and embryo development

A total of 308 oocytes with or without cumulus cells were retrieved from 86 vitrified ovarian grafts and 77% matured *in vitro*. Of these, 73% cleaved to 2-cell embryos after IVF, and 62% of the 2-cell embryos developed to blastocysts. A total of 69 slow-cooled grafts produced 303 oocytes, of which 84% matured *in vitro*. After insemination, 80% of the matured oocytes cleaved to form 2-cell embryos, and 63% of these developed to blastocysts. From the 74 fresh control grafts, a total of 267 oocytes were recovered, 83% were matured *in vitro*, resulting in 76% 2-cell embryos, of which 58% developed to blastocysts. No significant difference was found in the incidence of oocyte maturation, embryo cleavage, and blastocyst development between control and different cryopreservation treatment groups (Fig. 2).

The effect of cumulus cells on oocyte maturation, early cleavage, and subsequent embryo development was also examined (Fig. 2). A total of 354 F-COCs, 269 P-COCs, and 255 N-COCs were retrieved from the different treatment groups. F-COCs had a significantly higher maturation rate (94%) when compared with P-COCs (78%, $P < 0.01$) and N-COCs (63%, $P < 0.001$). No statistical difference was found in cleavage rate between F-COCs (83%), P-COCs (67%), and N-COCs (76%). With embryos cultured until day 4 or 5, F-COCs (80%) yielded a significantly higher percentage of blastocysts than that of P-COCs (49%, $P < 0.01$) and N-COCs (29%, $P < 0.001$).

Blastocyst recovery and viability after cryopreservation and embryo transfer

All blastocysts (graded 1 or 2) developing at day 4 or day 5 from the control and treatment groups were vitrified and stored in LN₂ for 1–2 weeks before warming.

After 2 h post-thaw culture in blastocyst medium, a total of 80 vitrified blastocysts derived from F-COCs from the fresh or cryopreserved groups that showed signs of re-expansion were transferred into five surrogate recipients (15–16 embryos/recipient). All recipients became pregnant. Eight (25%; 4 male and 4 female) healthy pups were born from 32 vitrified-warmed blastocysts derived from vitrified grafts, and four (12.5%; 2 male and 2 female) pups were born from the same number of vitrified-warmed blastocysts from slow-cooled grafts. In the fresh grafting controls, five male pups (31%) were born after transferring 16 vitrified-warmed blastocysts (Table 2). All the pups developed normally to weaning age. Four pups (2 from vitrification and two from slow-cooling group) were mated with their siblings and all produced normal-sized healthy offspring.

Discussion

When attempting to preserve the fertility of cancer patients, ovarian tissue cryopreservation has many potential merits over embryo or oocyte cryopreservation. The ovary harbors numerous immature oocytes that can maximize the production of gametes; and most of those oocytes are quiescent and have relatively low metabolic rates, which make them more tolerant to cryopreservation injury, and most important is that no ovarian stimulation is required so that patients do not have to delay their cancer treatment (Gosden *et al.* 2002, Amorim *et al.* 2003). However, there is still contention about which cryopreservation methods are the most suitable for ovarian tissue. This research examined whether the solid surface vitrification procedure produced different results from the standard slow freezing protocols currently being used clinically and whether vitrification of blastocysts produced from oocytes retrieved from ovarian tissue allografts can be used successfully to provide a pause in the procedure prior to embryo transfer. Our data show that the viability and function of ovarian tissue cryopreserved using the solid surface vitrification method are similar to those in slow-cooled tissue after transplanting

Figure 2 Developmental competence of oocytes collected from fresh and cryopreserved ovarian grafts with different oocyte classification. Note: oocyte maturation rate, total number of oocyte matured/total number of immature oocytes collected; cleavage rate, total number of 2-cell embryos/total number of oocytes matured; blastocyst rate, total number of blastocysts/total number of 2-cell embryos. Mean values with different superscript letters (a, b, c) are significantly different from each other ($P < 0.05$). N-COCs, oocytes with no surrounding cumulus cells; P-COCs, oocytes with partial cumulus mass; F-COCs, oocytes with at least one complete layer of cumulus cells.

in vivo suggesting that it can be an efficient and cost effective alternative to slow-cooling.

In this study, SCID mice were used as 'living incubators' for the fresh and cryopreserved adult mouse ovarian tissue allografts to observe oocyte development and maturation. Some successes have been reported by maturing oocytes *in vitro* (Liu *et al.* 2001) or *in vivo* (Snow *et al.* 2004) from cryopreserved ovaries from immature mice. However, the success for mature adult mice is still limited (Lee *et al.* 2005, Kagawa *et al.* 2007). The ovaries of adult mice have more collagen fiber compared with those of immature mice, making them relatively harder and difficult to revascularize quickly after transplantation (Kagawa *et al.* 2007),

Table 2 Number of pups born after transferring vitrified-thawed blastocysts from fresh or cryopreserved mouse ovarian grafts.

Treatment group	Number of recipients	Number of blastocysts transferred	Number of pups born/recipient (%)
Vitrified grafts	2	32	2/16 (12.5%) 6/16 (37.5%)
Slow-cooled grafts	2	32	2/16 (12.5%) 2/16 (12.5%)
Fresh grafts	1	16	5/16 (31%)

No significant difference ($P < 0.05$) was found within or between each treatment group.

fibrous connective tissue might also affect the permeability of cryoprotectant, compromising the tissue recovery after cryopreservation procedures.

Therefore, it is important to choose the 'right' size of tissue for cryopreservation and transplantation. A previous study by Kim *et al.* (2002) found that tissue ischemia and fibrosis formation is obvious after transplanting large piece of ovarian grafts (5 mm in size). Kagawa *et al.* (2007) suggested minimizing the size of mouse ovarian graft (0.2–0.3 mm) to accelerate revascularization speed, based on the texture difference between mature and immature mice ovary. Here, we cut the adult mouse ovary into medium size pieces (1–1.5 mm/piece) and transplanted these under the kidney capsule of SCID mice. Histological examination showed numerous follicles with full size oocytes developing in revascularized grafts. Our results showed that mouse ovarian grafts that were 5–10 times larger than the minimum size recommended by Kagawa *et al.* (2007), were easier to handle than smaller pieces, and showed very good recovery, vascularization, and survival after heterotypic allografting. These grafts also produced large numbers of oocytes that were competent for use in IVM and IVF tests.

Ovarian tissue contains multicellular and heterogeneous components (Gosden *et al.* 2002). To protect different types of follicles and support cells from cryoinjury, a subtle balance is needed between the choice of cryoprotectants and carriers, concentrations and equilibration periods for cryoprotectants, and cooling and warming rates. The cryologic solid surface vitrification method was found to be a simple and convenient freezing method for cryopreservation of ovarian tissue in this study, and has also been used successfully to cryopreserve embryos and oocytes with a high post-thaw survival rate in other species (Fry *et al.* 2005, Beebe *et al.* 2006, Costigan *et al.* 2006). Aerts *et al.* (2008) used the cryologic solid surface vitrification method to cryopreserve mouse ovarian tissue and assessed the survival of thawed tissues using histological examination and viability staining 7 days after heterotypic allografting in the kidney. Their results showed the fraction of intermediary and primary follicles significantly increased in both vitrified and slow-cooled grafts, but slow-cooled tissues yielded a significantly higher proportion of further developed follicles, including secondary and antral follicles, suggesting that the solid surface method may have inhibited follicle growth.

By contrast, in this study, follicle survival and development in ovarian tissue vitrified using the cryologic solid surface vitrification method was comparable with the traditional slow-cooling method. Our results were superior, in terms of graft recovery and the proportion of oocytes maturation in F-COCs group, to those reported by Kagawa *et al.* (2007) who vitrified mouse ovarian cubes using a cryotop vitrification method. The cleavage rate in our study was also higher

than that of Kagawa *et al.* (2007) (94/110, 85% vs 57/76, 75%). It is difficult to compare the embryo developmental competence between these two studies as Kagawa *et al.* (2007) transferred the embryos at early cleavage stage, whereas we further cultured all the embryos to blastocyst stage. Out of 94, 76 (81%) cleaved embryos developed to blastocysts originating from F-COCs from vitrified grafts indicating high embryo developmental competency. This increased competency was also indicated in our embryo transfer study, even though our embryos were vitrified prior to transfer, the live-pup birth rate from fresh (5/16, 31%) and vitrified grafts (8/32, 25%) were similar to Kagawa *et al.* (2007) (18% from vitrified grafts, 26% from fresh grafts). This relatively low success rate is likely to reflect the combined effect of transplantation, IVM, IVF, and *in vitro* culture to the blastocyst stage since we achieve a live-pup birth rate of 71% (22/31) when we transfer vitrified-warmed blastocysts derived from *in vivo* fertilized oocytes cultured *in vitro* from the 2-cell stage (X Wang, unpublished observation).

Although there was no difference in oocyte maturation, fertilization, and subsequent embryo development between vitrification and slow cooling cryopreservation methods, significant differences were found between oocyte types (with or without cumulus cells). The F-COCs had significantly higher maturation, cleavage, and blastocyst rates than N-COCs or P-COCs confirming other studies that matured mouse oocytes *in vitro* with cumulus cells (Anderiesz & Trounson 1995) or without cumulus cells (Chin & Chye 2004, Chang *et al.* 2005). However, even oocytes recovered without cumulus cells showed some degree of developmental competence (29% blastocyst rate). In this study, only blastocysts from F-COCs originating from fresh or cryopreserved ovarian grafts were used for embryo transfer. It will be interesting to investigate if those blastocysts developed from P-COCs or N-COCs have similar implantation competence as F-COCs. The lack of developmental competence of N-COCs or P-COCs are most probably associated with ooplasm defects because transfer of pronuclei from those oocytes into the enucleated cytoplasm of *in vivo* matured oocytes significantly increased the number of embryos that developed to blastocysts (Chang *et al.* 2005). Co-culture of N-COCs or P-COCs with granulosa cells (Dandekar *et al.* 1991, Yamazaki *et al.* 2001) or addition of serum (Kito & Bavister 1997, Yamazaki *et al.* 2001) may enhance their meiotic maturation and subsequent developmental potential *in vitro*.

Embryo cryopreservation is a well-established protocol and many IVF clinics now reduce their multiple pregnancy rate by performing single embryo transfers and cryopreserving excess embryos (Youssry *et al.* 2008). In humans, combination of oocyte and early stage of embryo cryopreservation have been used to produce healthy babies (Levi Setti *et al.* 2005, Chen *et al.* 2008).

In this study, we have used two different cryopreservation steps – ovarian tissue and blastocyst cryopreservation – to re-establish fertility after ovariectomy. We chose to cryopreserve all the embryos derived from fresh and cryopreserved ovarian grafts at the blastocyst stage, because blastocysts are pre-implantation embryos that have passed through most of the genomic activation period and have a relatively high developmental potential (Ménézo 2004). The live births achieved in this study from vitrified blastocysts transferred to recipient mice are the first to be reported using the cryologic solid surface vitrification method. Our result emphasizes the importance of cryobiology in reproductive science and the potential advantages of combining ovarian tissue cryopreservation and embryo cryopreservation in preserving fertility of women undergoing treatment for cancer.

In conclusion, adult mouse ovarian tissues can be successfully cryopreserved by the cryologic solid surface vitrification method and successfully transplanted under the mouse kidney capsule. The existing IVM system has been successfully used to mature oocytes derived from cryopreserved grafts. Oocytes from these grafts had high oocyte maturation, cleavage, and blastocyst rates in both the vitrification and slow-cooling groups. The presence of cumulus cells correlated closely with the capacity for oocyte maturation and fertilization *in vitro*. The combination of ovarian tissue cryopreservation and a standard blastocyst freezing protocol can yield offspring at a high rate. Such findings are important to understand the usefulness of cryobiology in assisted reproduction and to consider their use in clinical treatments.

Materials and Methods

All the chemicals and reagents used for this study were purchased from Sigma Chemical (Sigma) unless otherwise stated.

Animals

The study was approved by the Monash Medical Centre Animal Ethics Committee (approval number A2008/15). Six-to-seven-week-old F1 female hybrid (C57BL×CBA) mice were used as ovarian tissue donors ($n=40$), and 8–10-week-old SCID female mice were used as graft recipients ($n=20$). Eight-week-old F1 hybrid (C57BL×CBA; $n=5$) female hybrids were mated with proven vasectomized F1 hybrid (C57BL×CBA) males and used as pseudo-pregnant embryo transfer recipients. Twelve-week-old F1 male hybrids (C57BL×CBA) were used as sperm donors for IVF. All mice were obtained from Monash University Animal Services and housed in specific pathogen-free conditions with a relative humidity of 30–60%, a temperature range from 21 to 24 °C and a 12 h light:12 h darkness light cycle. All mice had free access to sterile food and water.

Ovarian tissue collection

Female donor mice were killed by cervical dislocation, and ovaries were collected and placed into HEPES-buffered KSOM handling medium (KSOM HM; Summers *et al.* 1995) supplemented with 0.5% w/v BSA. Each ovary was then cut into quarters using a razor blade (ProSciTech, Brisbane, QLD, Australia) and transplanted under the kidney capsule of SCID mice either immediately (controls) or after cryopreservation.

Ovarian tissue cryopreservation

Vitrification

For vitrification, the tissue fragments were initially equilibrated in KSOM HM containing 10% v/v ethylene glycol (EG) and 10% v/v DMSO for 20 min at room temperature (RT) and then transferred to vitrification solution containing 17% v/v EG and 17% v/v DMSO and 0.75 M sucrose in KSOM HM for 3 min. Each ovarian tissue fragment was then transferred with a minimum volume (1–3 μ l) of the vitrification solution on to the hook-end of a plastic Fiberplug and vitrified by contacting the hook-end of the Fiberplug with the surface of metal block (CVM kit, Cryologic, Melbourne, VIC, Australia) pre-cooled by practical immersion into LN₂ (Fig. 3). Fiberplugs were then inserted into corresponding storage straws and plunged into pre-cooled goblets for storage in LN₂. To warm the ovarian tissue fragments, Fiberplugs were removed from the storage straw and immersed in 0.5 M sucrose in KSOM HM for 5 min at 37 °C, and then the tissue fragments were washed through stepwise solutions in KSOM HM (0.25, 0.125, 0 M sucrose, 5 min each). After washing and prior to grafting, the tissues were held in KSOM HM at 37 °C.

Slow-cooling

For slow-cooling, we used the method of Snow *et al.* (2004) with minor modifications. Briefly, up to eight ovarian fragments were placed into the slow-cooling solution containing 10% v/v DMSO and 0.1 M sucrose and loaded into 0.5 ml plastic straws (IMV, L'Aigle, France). Straws were sealed with polyvinyl alcohol powder and after equilibration for 10 min at RT, placed in a programmable freezer (Freeze Control CL8800i, Cryologic) and cooled at 2 °C/min from RT to –7 °C. Straws were held at –7 °C for 10 min at which stage the straws were seeded (Fig. 3), and then taken down to –40 °C at a rate of 0.3 °C/min. Straws were then removed from the cryochamber and plunged directly into LN₂ and stored. For thawing, each straw was held

in LN₂ vapor for 10 s, placed at RT for 30 s, and then immersed in a 37 °C water bath for 20 s. The end of each straw was cut using sterile scissors and the thawed ovarian fragments were expelled into 30 mm petri dish (Becton Dickinson, Franklin Lakes, NJ, USA) containing 0.5 M sucrose solution in KSOM HM for 5 min at RT and then subsequently moved to 0.25, 0.125, and 0 M sucrose in KSOM HM for 5 min each. All the thawed tissues were kept in KSOM HM before grafting.

Ovariectomy and transplantation

Ovarian grafting was performed using a standard procedure (Cox *et al.* 2000, Snow *et al.* 2002). In brief, tissue transplant recipients (8–10-week-old female SCID mice) were anesthetized (Avertin (20 mg/ml 2, 2, 2-tribromoethanol and 2% v/v tert-amyl alcohol), 0.4 mg/g body weight, i.p.). The dorsal skin over the flank was sterilized using 70% v/v ethanol and a 1.5 cm dorsal incision was made through the skin. The skin was separated from the underlying tissue to allow access on each side to the lateral body wall about 0.5 cm from the mid-line to the right or left. A 0.5 cm incision was made through the abdominal wall to expose the ovarian fat pad and bilateral ovariectomy was performed (Nagy *et al.* 2003). Eight ovarian fragments from each donor mouse selected from one of the three groups (fresh controls, vitrified, or slow-cooled) were inserted under the capsule of one kidney in the recipient. A similar number of ovarian fragments from a donor taken from one of the two remaining groups were inserted under the capsule of the contralateral kidney. Fresh ovarian fragments (controls) were transplanted within 15 min of removal from the abdominal cavity and cryopreserved ovarian fragments were transplanted within 15 min of thawing. Each kidney was returned to the body cavity, the body wall incisions closed with 5/0 suture (Ethicon, Somerville, NJ, USA) and the skin incision closed with Michel clips (Clay Adams, Sparks, MD, USA). Prior to the mice returning to consciousness, analgesic (Carprofen (Rimadyl, Dundee, UK), 5 mg/kg, i.p.) was used to provide post-surgery pain relief.

Oocyte collection and IVM

Ten days after transplantation, recipients were injected i.p. with 7.5 IU pregnant mares serum gonadotrophin (Folligon; Intervet, Boxmeer, The Netherlands) and killed by cervical dislocation 48 h later. Ovarian grafts were either removed and fixed *in situ* under the kidney capsule for histological assessment, or excised and placed in KSOM HM at 37 °C for oocyte collection and maturation. Antral follicles from the fresh and cryopreserved grafts were punctured with 26 gauge needles to release the oocytes. To assess the effects of cumulus cells on oocytes maturation, fertilization and subsequent embryo development, the retrieved oocytes were separated into one of the following groups: F-COCs; P-COCs; and N-COCs. For oocyte maturation, a modification of the method of Cortvrindt *et al.* (1996) was used. The maturation medium was Alpha-Minimum Essential Medium (Invitrogen), supplemented with 5% v/v heat-inactivated FCS (Invitrogen), 5 μ g/ml insulin, 5 μ g/ml transferin and 5 ng/ml selenium, 50 mIU/ml penicillin and 50 μ g/ml streptomycin, 2 μ g/ml FSH (Folltropin-V; BioNiche, Belleville,

Figure 3 Vitrification and slow cooling equipment. (A) Touching a Fiberplug to cold metal block during vitrification. (B) Manual seeding during slow-cooling.

ON, Canada), 1.5 IU/ml hCG (Chorulon, Intervet) and 5 ng/ml recombinant human epidermal growth factor (Invitrogen). Up to two oocytes were cultured in 20 μ l drops in 60 mm Falcon dishes under mineral oil at 37 °C in 100% humidity and 5% CO₂ in air for 18 h.

IVF and embryo culture

Male F1 (C57BL×CBA) mice were killed by cervical dislocation and the caudae epididymides were dissected, halved, and placed in 1 ml pre-equilibrated modified Tyrodes solution (MT6; Fraser 1984) in a 5 ml Falcon tube (BD Biosciences, San Jose, CA, USA) to recover sperm samples. After 30 min incubation at 37 °C in 100% humidity and 5% CO₂ in air, the supernatant containing high number of motile sperms was harvested and the sperm concentration and motility assessed before insemination. Insemination dishes were prepared the day before with 100 μ l MT6 medium droplets covered with a layer of mineral oil in 60 mm Falcon dishes and allowed to equilibrate at 37 °C in 100% humidity and 5% CO₂ in air for at least 12 h. After maturation, oocytes with or without cumulus from different treatment groups were inseminated in different dishes with 1×10^6 spermatozoa/ml for 2–4 h. At the end of this period when the cumulus had been dispersed, the maturation status of each oocyte was scored as follows: germinal vesicle visible (GV); GV absent but no polar body recognizable (GVBD/metaphase I (MI)); or a polar body presents (MII). All MII oocytes were washed and cultured in Sydney IVF Cleavage Medium (Cook Australia, Brisbane, QLD, Australia) for 2 days and transferred to Sydney IVF Blastocyst Medium (Cook Australia) from day 3 until day 5 at 37 °C, 6% CO₂, 5% O₂ atmosphere.

Blastocyst vitrification and warming

All day 4 or day 5 blastocysts were graded using the following criteria: Grade 1, blastocyst with tightly packed inner cell mass and trophectoderm with many cells; Grade 2, blastocyst with loosely packed inner cell mass and trophectoderm with few cells; and Grade 3, blastocyst with significant vacuoles, fragments, and/or signs of degeneration. Only Grade 1 or 2 blastocysts were vitrified and all the other embryos were discarded. For vitrification, blastocysts were initially equilibrated in KSOM HM containing 10% v/v EG and 10% v/v DMSO for 3 min at 37 °C and then transferred to vitrification solution in KSOM HM containing 17% v/v EG and 17% v/v DMSO and 0.75 M sucrose for 30 s at 37 °C. Each blastocyst was then placed in 2 μ l vitrification solution onto the hook end of the plastic Fiberplug, and vitrified by contacting the hook end of Fiberplug with the surface of metal alloy block pre-cooled by immersion into LN₂. Fiberplugs were subsequently placed into corresponding storage straws and then plunged into LN₂ in pre-cooled gobelets for storage. To thaw vitrified blastocysts, Fiberplugs were removed from the straw and submerged immediately into 0.3 M sucrose solution at 37 °C. Blastocysts were then quickly retrieved and transferred through a series of three solutions (0.25, 0.15, 0 M sucrose) for 5 min each at 37 °C. All the warmed blastocysts were cultured in Sydney IVF blastocyst medium at 37 °C in a 6% CO₂, 5% O₂ atmosphere. Two hours later all the blastocysts were scored and transferred.

Embryo transfer

Five F1 (C57BL×CBA) female mice (8-weeks-old) were used as embryo transfer recipients after mating with vasectomized F1 males from the same strain. The females were checked for the evidence of vaginal plugs after mating. Recovered blastocysts from different treatment groups were transferred into the uterus of day 2.5 pseudo-pregnant recipients using the method described by Nagy *et al.* (2003). Briefly, the recipients were anesthetized as described above. A 1 cm skin incision was made in the dorsal midline at the level of the last rib, the skin was separated from the underlying tissues using blunt dissection to the left and right until the ovarian fat pad was visible under the abdominal wall on each side. A 0.5 cm incision was made in the abdominal wall over the ovarian fat pad, which was gently pulled out to expose oviduct, ovary, and the upper part of uterus. Up to eight embryos were transferred into each uterine horn with a fine glass pipette. Each uterus was then returned to the body cavity, and the skin incision closed with Michel clips as previously described. Embryos in different treatment groups were transferred into different female recipient mice. All the recipients were allowed to deliver and raise pups. Live born pups were either raised to weaning age to check their normality or mated with their siblings from the same litter for fertility testing.

Histological examination

Fresh and cryopreserved-thawed ovarian grafts were fixed in Bouin's fixative overnight, dehydrated manually through a graded ethanol series, and embedded in paraffin. Five micron (5 μ m) serial sections were stained with hematoxylin and eosin and examined under an Olympus CX41 upright microscope.

Statistical analysis

Graft recovery and survival and number of pups born within and between treatment groups were compared using a χ^2 test. The result of oocyte collected per quarter-ovarian grafts was presented as mean $\pm$ S.E.M. and analyzed by one-way ANOVA. The data of oocyte maturation, cleavage and blastocyst rate in each oocyte classification of the different treatment groups were expressed as mean $\pm$ S.E.M. and analyzed by multivariate ANOVA. $P < 0.05$ was considered to be a significant difference. SPSS 12.0.1. (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Declaration of interest

We declare that there is no conflict of interest that can be perceived as prejudicing the impartiality of our research.

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