Article

Production of the first offspring from oocytes derived from fresh and cryopreserved pre-antral follicles of adult mice

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Abstract

Although mammalian ovaries contain hundreds of thousands of pre-antral follicles, fewer than 1% of these reach maturity and ovulation. Obtaining immature eggs from the pre-antral follicles of ovarian tissue could increase the possibility of preserving fertility in women undergoing anti-cancer treatment, and in women who wish to delay pregnancy and child raising until they are older. This study reports the birth of 10 healthy mouse pups derived from oocytes obtained from pre-antral follicles after adult ovary tissue cryopreservation and allotransplantation. High in-vitro maturation (55.1%), fertilization (76.3%) and cleavage (98.3%) rates were achieved using these oocytes, and there was no significant difference between the vitrified and control samples except in maturation rate (55.1 versus 72.8%, $P < 0.05$). After an ultra-rapid vitrification procedure, the warmed tissue fragments were transplanted beneath the kidney capsule of severe combined immunodeficient mice for onward in-vivo culture. Within 10 days of culture, 138 full size oocytes developed from the 456 transplanted pre-antral follicles. In-vivo growth of follicles was followed by in-vitro oocyte maturation, in-vitro fertilization and subsequent embryo transfer, leading to the birth of 10 healthy pups. These results may lead to increasing the possibility of preserving fertility by cryopreservation of ovarian tissue.

Keywords: *adult*, *birth*, *Cryotop*, *follicles*, *pre-antral*, *vitrification*

Introduction

In contrast to the easy access to, large quantity of, and relatively efficient cryopreservation of male gametes in mammals, female gametes present serious challenges in reproductive biology, even though ovaries of adult mammals contain a large number of oocytes. The vast majority of these oocytes, however, are contained in pre-antral follicles in a very early phase of development. In past decades, many attempts have been made to produce offspring from these oocytes. Some success has been reported by using ovaries of fetal, newborn and immature mice (Rivera-Perez *et al.*, 1995; Sztein, 1998, 1999; Liu, 2001; Migishima, 2003; Hasegawa *et al.*, 2004), but in spite of repeated efforts, no developmentally competent oocytes have been obtained from pre-antral follicles of adult animals (Eppig *et al.*, 1996; Paris *et al.*, 2004). Accordingly, full in-vitro development of these oocytes has so far been considered impossible (Miyano, 2003).

Applied reproductive biology, i.e. preservation of fertility in humans, animal breeding, and preservation of endangered

species, does, however, require the use of adult ovaries. Utilization of immature oocytes from ovarian tissue would provide hope for young women suffering from malignant diseases to regain fertility by banking ovarian tissue before the initiation of radiotherapy and chemotherapy that may result in iatrogenic sterility (Hovatta, 2003). Current advances in the treatment of malignant diseases by chemotherapy and radiotherapy are saving an increasing number of lives, albeit at a cost; survivors now have to live with the sterility caused by their cancer treatment. Approximately 2% of all malignant diseases occur during childhood and adolescence, and cure rates can exceed 90% in this young group of patients (Linet *et al.*, 1999). One of the most significant long-term sequelae of these aggressive, but life saving, chemotherapy and radiotherapy regimens is ovarian failure and infertility (Apperley *et al.*, 1995). Cryopreservation of ovarian tissue can be carried out immediately, without the need to wait for numerous ovarian stimulation protocols, and may result in a vast number of oocytes. The same approach would also offer unique advantages for the conservation of endangered species. Ovaries could be collected from live individuals or immediately after death, cryopreserved and used as resource for re-creation of the species once the appropriate habitat and surrogate animals have been prepared. Animal husbandry would also need a large number of oocytes harvested from adult animals to enhance superior genetic traits such as high quality meat or increased milk production (Norman, 2004).

This paper reports the first birth of 10 healthy pups derived from the oocytes of pre-antral follicles obtained from cryopreserved ovarian tissue of adult mice. The study was conducted to help develop a method to utilize cryopreserved ovarian follicles at the pre-antral (secondary) stage obtained from ovarian tissue of adult mammals.

Materials and methods

Animals

Severe combined immunodeficient (SCID) mice (8–10 weeks of age; Clea Japan, Tokyo, Japan) were housed under controlled temperature (24 ± 2°C), lighting (14 h light: 10 h darkness), and humidity (80 ± 5%). During the experiment, each animal cage was covered with a high-efficiency particulate air filter (Clea Japan), and animals had free access to sterilized food and bottled water. Ovaries and spermatozoa were harvested from B6D2F1 (C57BL/6 × DBA/2) mice. ICR females mated with vasectomized males of the same strain were used as surrogate mothers of intracytoplasmic sperm injection (ICSI) embryos.

Ovarian tissue collection

Ovaries were aseptically removed from 56- to 70-day-old females killed by cervical dislocation. Follicular numbers were calculated by a method described previously (Senbon *et al.* 2004). Sections of ovaries prepared as cubes (approximately 0.2–0.3 mm edge length) containing only one secondary follicle 60–70 μm in diameter were dissected using surgical blades (Kai Industries Co. Ltd, Gifu, Japan) under a dissection microscope while immersed in M2 medium (Sigma, UK). The diameter of the follicles was determined by taking two perpendicular measurements to the nearest 10 μm with an ocular micrometre (Nikon, Tokyo, Japan) attached to a dissection microscope. Cubes were randomly distributed into two groups for allotransplantation, either with or without prior vitrification and warming respectively.

Cryopreservation of ovarian tissues by vitrification

The Cryotop vitrification technique established for cryopreservation of human oocytes and embryos was used with slight modifications according to the requirements of the tissue fragments (Kuwayama *et al.*, 2005a,b). Ovarian tissues were initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulphoxide (DMSO) in handling medium (HM; HEPES-buffered TCM-199 solution supplemented with 20% (v/v) synthetic serum substitute (Irvine Scientific, Santa Ana, CA, USA) for 20 min, and then a second equilibration in 15% EG and 15% DMSO for 3 min. Ovarian tissue cubes were then placed in minimum volume of solution onto a thin polypropylene strip of the Cryotop (Kitazato BioPharma Co. Ltd, Fujinomiya, Japan), and submerged directly into sterile liquid nitrogen. For warming, the protective cover was removed and the polypropylene strip of the Cryotop was immersed directly into 3 ml of 37°C HM solution supplemented with 1.0 1 mol/l/l sucrose for 3 min. Ovary cubes were subsequently transferred into 4.5 ml of 0.5 mol/l sucrose in 37°C HM solution for 5 min, and washed in 37°C HM twice for 10 min each before transplantation.

In-vivo culture (allotransplantation)

All vitrified secondary follicles survived warming. Thirty-six recipients (10-week-old female SCID mice) were anaesthetized with intraperitoneal injection of 0.17 ml/10 g body weight of 1.25% tribromo-ethanol and 1.25% tetramyl alcohol (Aldrich, Milwaukee, WI, USA) solution. The left kidney was exposed, and by using a glass pipette, 10–12 vitrified/warmed or fresh ovarian cubes were inserted under the renal capsule of 26 and 10 animals respectively.

Oocyte collection and in-vitro maturation

On day 9 after transplantation, recipients were injected intraperitoneally with 7.5 IU equine chorionic gonadotrophin (Serotropin, Teikoku-zoki Co. Ltd, Tokyo, Japan). At 48 h after treatment, these animals were killed by cervical dislocation, and the ovarian grafts (including tertiary follicles) were removed from the kidney capsule (Weissman *et al.*, 1999). Cumulus–oocyte complexes (COC) were recovered from antral follicles that had been developed in the grafts using a disposable 24-gauge needle. After three subsequent washings in modified HEPES-buffered Alpha-MEM medium (Invitrogen, Carlsbad, CA, USA), COC were cultured in Toyoda Yokoyama Hoshi (TYH; NaCl 119.4 mmol/l, KCl 4.8 mmol/l, $CaCl_2.2H_2O$ 1.7 mmol/l, KH_2PO_4 1.2 mmol/l, $MgSO_4.7H_2O$ 1.2 mmol/l, $NaHCO_3$ 25.1 mmol/l, Na pyruvate 1 mmol/l, glucose 5.6 mol/l, penicillin 100 IU/ml) medium supplemented with 10% (v/v) fetal bovine serum (Biowest, Nuaill, France) under mineral oil (Irvine Scientific) at 37°C in 5% CO_2 in air for 16 h (Toyoda *et al.*, 1971).

Collection and evaluation of spermatozoa

Spermatozoa were collected from the caudae epididymis of adult BDF1 mice, and prepared and evaluated as already described (Kimura *et al.*, 1995). The spermatozoa were then suspended in Chatot, Ziamok and Bavister (CZB) medium (Millipore, USA) added to 5 mg/ml BSA and examined for motility at room temperature (Chatot *et al.*, 1989). Those with distinct motility and vibrating or undulating tails were evaluated as being alive.

Intracytoplasmic sperm injection (ICSI)

ICSI was carried out by using a micropipette actuated by piezo-electric power according to Kimura and Yanagimachi, except that operations were performed at room temperature (Kimura *et al.*, 1995). Sperm heads were injected into oocytes after separation of the head from the tail by application of a few piezo-electric pulses to the head and tail junction. Injected oocytes were then transferred to 25-µl drops of CZB medium under mineral oil (10 oocytes per drop) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. At 6 h after ICSI, those with two distinct pronuclei and a conspicuous second polar body were recorded as normally fertilized. Fertilized oocytes were cultured to 24 h after ICSI until they reached the 2-cell stage.

Embryo transfer

At 0.5 days post-coitus, 2-cell stage embryos were transferred to the oviducts of ICR (albino) females that had been mated with vasectomized males of the same strain (for quantitative details, see **Table 1**).

Histological examination

For microscopic analysis, vitrified/warmed and fresh allotransplants recovered from one kidney were fixed in 10% phosphate-buffered formalin (pH 7.4; Wako Pure Chemicals, Osaka, Japan), dehydrated through a graded ethanol series and embedded in Histosec (Merck, Darmstadt, Germany). Serial sections 3 µm thick were stained with haematoxylin and eosin.

Statistical analysis

Data were analysed by the chi-squared test. *P*-values <0.05 were considered as significant.

Results

In-vivo growth (allotransplantation) of oocytes of pre-antral follicles

As shown in **Table 1**, within 10 days of in-vivo culture on the kidney of SCID mice in the vitrification group, 138 full size (70 µm or more diameter) oocytes developed from the 456 transplanted pre-antral follicles (n = 138/456, 30.3% development rate). A total of 103 oocytes developed from the 336 pre-antral follicles (n = 103/336, 30.7% development rate) in the non-vitrification group.

Histological examination

The transplantation of minimum-sized pieces of tissue (cubes with 0.2 to 0.3 mm edge length) into the kidney capsule resulted in the swift extension of a newly created vascular network to the transplanted ovarian tissues. Histological examination demonstrated loose connective tissue surrounding the ovary cubes with a large number of expanded capillaries (**Figure 1**). Similar expanded capillaries were also present in the vicinity of the theca layer. Histological examination demonstrated the presence of follicles with large antral cavities and with full size (approximately 70 µm diameter) oocytes.

In-vitro maturation, fertilization, culture and embryo transfer

After in-vitro maturation, fertilization by ICSI and in-vitro culture for 2 days, high maturation (n = 76/138, 55.1%), fertilization (n = 58/76, 76.3%) and cleavage rates (n = 57/58, 98.3%) were achieved by using oocytes originating from vitrified ovarian tissues. There was no significant difference between the vitrified and non-vitrified control groups in any parameter except for maturation rate (n = 76/138, 55.1% versus n = 75/103, 72.8%, $P < 0.05$).

Following transfer of a total of the 57, 2-cell stage embryos (**Figure 2**) originating from the vitrified pre-antral follicles into six surrogate recipients, all of the recipients became pregnant. The total number of implantation sites was 24 (implantation rate of 24/57, 42%), and eventually 10 normal pups (six males and four females, with average birth weight of 1.33 ± 0.14 g) were born (**Figure 3**). In a control experiment, 14 (26%) pups were born by implantation of 55, 2-cell stage embryos derived

Table 1. Development of oocytes derived from transplanted ovarian tissues.

Treatment	*Number (%) of oocytes or embryos*				
	Transplanted	*Recovered**	*Matured*	*Fertilized*	*2-cell stage*
Vitrified	456	138 (30.3)	76 (55.1)[a]	58 (76.3)	57 (98.3)
Non-vitrified	336	103 (30.7)	75 (72.8)[b]	60 (80.0)	55 (91.7)

[a,b]$P < 0.05$.
*Fully grown oocytes were recovered from the kidney of the severe combined immunodeficient mice.

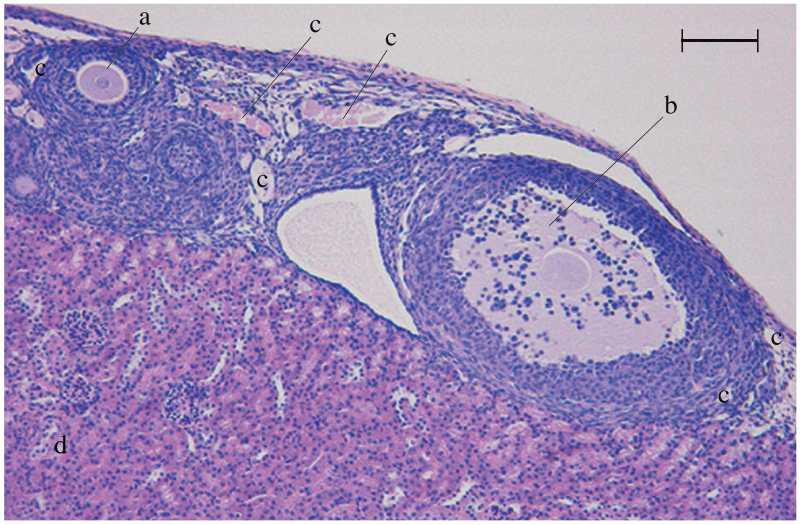

Figure 1. Cryopreserved ovarian tissues 10 days after allotransplantation. a, Pre-antral follicle; b, antral follicle; c, blood vessels filled with red blood cells in the theca layer area and in between neighbouring grafts; d, renal tissue. Haematoxylin–eosin staining, scale bar represents 100 μm.

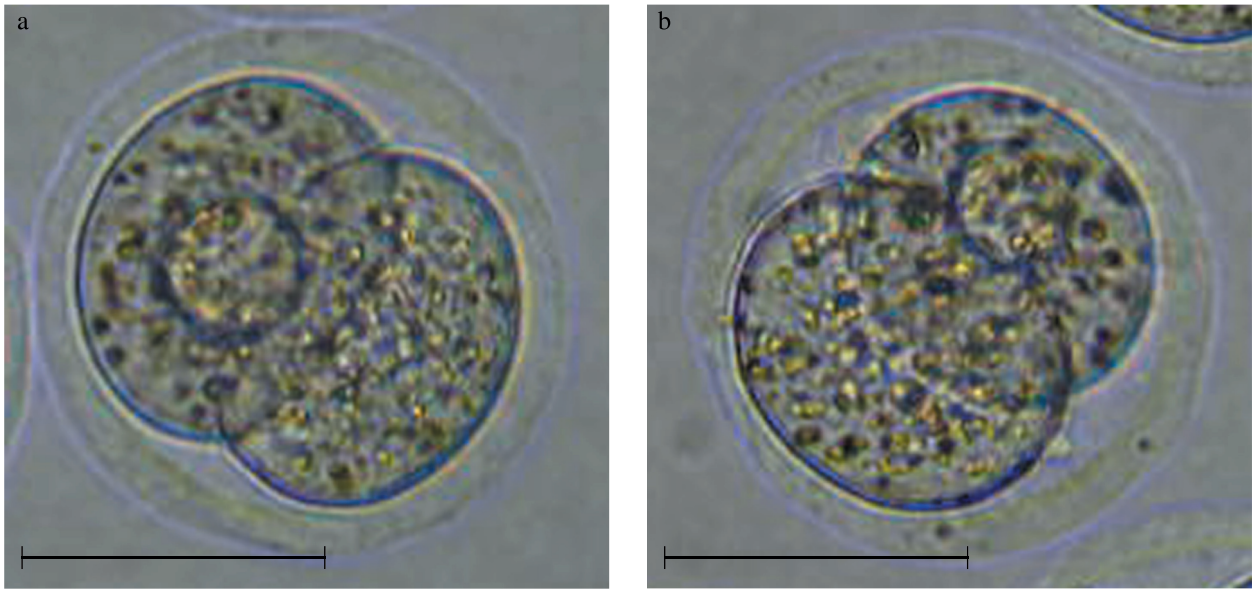

Figure 2. Light images of 2-cell stage embryos derived from the oocytes of (**a**) vitrified or (**b**) non-vitrified ovarian tissues after in-vivo growth, in-vitro maturation and fertilization by intracytoplasmic sperm injection (ICSI). Scale bars represent 50 μm.

Figure 3. Ten-day-old normal mice derived from the oocytes of pre-antral follicles of cryopreserved and allotransplanted adult ovarian tissues.

from non-vitrified ovarian tissues. The further development of all pups was normal, and all of them produced healthy offspring after mating with normal mice.

Discussion

Aggressive chemotherapy and radiotherapy or radical oncological surgery in young women with cancer has greatly enhanced these patients' life expectancy, but these treatments usually cause infertility or premature ovarian failure due to a massive destruction of the ovarian reserve (Donnez and Bassil, 1998; Maltaris *et al.*, 2006). It is therefore frequently recommended that ovarian tissue be cryopreserved in the hope that the women's fertility may be restored in the future.

An alternative approach is the collection and cryopreservation of oocytes from mature follicles after ovarian stimulation, as is routinely performed with IVF procedures in humans. However, this procedure results in only a limited number of oocytes, can only be performed once a month, and requires a period of waiting time that the cancer patient can ill-afford before starting chemotherapy or radiation. The results of this study indicate that healthy mouse pups can be obtained from vitrification of ovaries from adult mammals by the Cryotop method. By cryopreserving ovarian cortical tissue, thousands of immature oocytes can be stored without the need for ovarian stimulation (Oktay *et al.*, 1998, 2001, 2002; Aubard *et al.*, 1999; Kim *et al.*, 2001).

Morphological and functional analysis of the environment of the fetal versus adult pre-antral follicle may help to understand the reasons for their differing developmental competence. The pre-antral follicle consists of an oocyte wrapped in single or multiple granulosa cell layers and is surrounded by a vascular network outside the basement membrane of the granulosa cells. Follicular development and atresia are controlled through this vascular network, which transports signals to the granulosa cells, and the signal from the stimulated granulosa cells then reaches the oocytes through gap junctions. Accordingly, angiogenesis in the ovarian medulla is necessary for the selective growth of the cortical follicles (Shimizu *et al.*, 2003). The mesenchymal cortex surrounding the follicles of the embryo and newborn is soft, while in adult ovaries accumulated collagen fibres transform the connective tissue, making it hard and less permeable. A previous study demonstrated increased vascularization and progression of follicular growth to ovarian grafts in the kidney of SCID mice using immunohistochemical stain for CD34 antigen (Kagawa *et al.*, 2005). It is postulated that the initiation of follicle growth is determined by the environment surrounding the follicle, such as the density of vascular network and the softness of the interstitial tissue. Based on this hypothesis, an attempt was made to grow pre-antral follicles collected from adult animals by facilitating the development of an ample vascular network around the follicles, similar to that of newborn animals. This aim was achieved by in-vivo culture of ovarian tissue cubes beneath the renal capsule of SCID mice, where a dense capillary network is already present. The grafting condition was improved by minimizing the size of the ovarian section to be transplanted, so facilitating more rapid vascularization.

Follicle development after transplantation requires ample vascular network for the transplanted follicle. Otherwise, the follicle will regress within a few days due to ischaemia (Newton *et al.*, 1996; Gunasena *et al.*, 1997a,b). Kim *et al.* showed that transplanting large pieces of tissue (5 mm cubes) resulted in follicular atresia and generation of fibrosis of most grafts (Kim *et al.*, 2002). In contrast, this study has shown that the transplantation of minimum-sized pieces (cubes with 0.2–0.3 mm edge length) into the kidney capsule resulted in the swift extension of a newly-created vascular network to the transplanted ovarian tissues. Histological examination of these grafts showed numerous expanded capillaries surrounding the grafted tissue and the theca layer of the follicle (**Figure 1**). Follicles with large antral cavities and full size oocytes were also observed. These findings support the hypothesis that the rich vascular network surrounding the ovarian grafts induced growth and development of pre-antral follicles to the antral stage in the transplanted ovarian tissue. Scott *et al.* (2004) have

carried out some preliminary studies on culturing ovarian cortical tissue primordial follicles, but as of yet, no successful mature oocytes or live births have been achieved.

Another benefit of using very small samples of ovarian tissue is the ability to use the Cryotop method for cryopreservation. This method was originally developed for vitrification of embryos and oocytes, and was slightly modified for these solid tissue samples. After prolonged equilibration in cryoprotectant solution, the tiny cubes of ovarian tissue were contained within only a virtual volume of fluid on the surface of a thin plastic film and then submerged directly into sterile filtered liquid nitrogen. This allowed an extremely high cooling rate.

Ovarian tissue banking is a promising option for the preservation of ovarian function in women and in female children who are at risk of ovarian failure as a result of anti-cancer therapy. It does not require ovarian stimulation and can be accomplished without delaying medical treatment in most cases. Although embryo development after heterotopic ovarian transplantation and a live birth after orthotopic transplantation were reported recently (Meirow *et al.*, 2005; Donnez *et al.*, 2006), the overall success rates are very poor. However, there has been consistent success in humans using fresh cortical ovarian grafts between identical twins (Silber *et al.*, 2005; Silber and Gosden, 2007). The ideal technique for ovarian transplantation has not yet been determined. Future areas of development will probably involve research into whole ovarian cryopreservation, in addition to improving follicle survival after transplantation. Vascular transplantation may be the best approach to avoid follicular loss and extend the lifespan of ovarian grafts (Silber *et al.*, 2007). The cortical graft transplantation may result in extensive follicular loss due to ischaemic damage occurring before establishment of neovascularization (Wang *et al.*, 2002; Yin *et al.*, 2003). There have recently been two successful human cases of microvascular, intact whole ovary transplantation for which long-term follow-up may provide the answer (Silber *et al.*, 2007b).

This study has shown that oocytes of pre-antral follicles derived from cryopreserved ovaries of adult mice can yield offspring at a high rate. The in-vitro maturation (IVM) system, where individuals are produced from immature oocytes of antral follicles, has been established in humans, laboratory animals, in several domestic and wild mammalian species. This existing IVM system has been successfully combined with adult ovarian tissue cryopreservation and in-vivo follicle growth. The results have demonstrated the possibility of exploiting the abundant resource of adult female gametes. This model may open the way to increase the efficiency of animal breeding, contribute to the preservation of the ecological system through the conservation of endangered mammalian species, and help young female cancer patients to have offspring and restored ovarian endocrine function after chemotherapy and radiotherapy.

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