Oocyte retrieval after heterotopic transplantation of ovarian tissue cryopreserved by closed vitrification protocol

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Abstract

Purpose A device for closed vitrification was designed to reduce the risk of contamination and investigated on its efficacy for ovarian function recovery after cryopreservation and heterotopic transplantation.

Methods Ovarian tissues from green fluorescence protein transgenic mice (10 GFP mice) were vitrified using the device, and warmed ovarian tissues were transplanted into the ovarian bursa region in wild-type female mice (6 mice). Fresh ovarian tissues were similarly transplanted as a control. After recovery of the estrous cycle, mice were mated with male mice. Ovarian tissues from six cynomolgus monkeys were vitrified and warmed with the device for autologous, heterotopic transplantation. Fresh tissue transplantation was not performed for the control. Ovarian function was examined by recovery of the hormonal cycle. Histological examination was conducted.

Results The number of live pups per recipient mouse was not significantly different after transplantation of fresh or vitrified-warmed ovarian tissue, although the pregnancy rate was reduced with vitrified tissues. The hormonal cycle was restored in 5/6 monkeys after heterotopic transplantation of vitrified-warmed ovarian tissue. Follicles were harvested at eight sites in the omentum and 13 sites in the mesosalpinx. In vitro maturation (IVM)/IVF produced embryo but did not develop.

Conclusions Resumption of the hormonal cycles, follicle development, and oocyte retrieval from vitrified-warmed ovarian tissue transplants may indicate that the use of vitrification for ovarian tissue in a closed system has a potential of clinical application without the risk of contaminations. More detailed analyses of the effects of vitrification on ovarian tissue, such as gene expression patterns in oocytes and granulosa cells, may be needed for establishing a standard procedure for cryopreservation of ovarian tissues in human.

Keywords Ovarian tissue cryopreservation · Vitrification · Closed device · Cynomolgus monkey · Fertility preservation · Autologous transplantation

Introduction

Medical and technical advances have given women better chances to preserve fertility and to have children at a later time, including the cryopreservation of embryos, oocytes, and ovarian tissue. Ovarian tissue cryopreservation benefits women more broadly, since ovarian tissue contains steroidogenesis and gametogenesis activities and a large number of primordial follicles and can be harvested irrespective of the stage of the menstrual cycle or be applied to pre-pubertal girls. Since the first successful transplantation of frozen–thawed ovarian tissues was reported by Oktay and Karlikaya in 2000 [1], about 130 live births have been reported after

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transplantation of cryopreserved ovarian tissue around the world [2], and the FertiPROTEKT network registry has listed more than 1000 ovarian tissue cryopreservation procedures [3]. Nonetheless, no validated standard protocol has been established for cryopreservation of ovarian tissue [4].

Some studies comparing slow freezing and vitrification by rapid freezing have shown that vitrification is superior for cryopreservation of ovarian tissue with respect to follicle density, cell proliferation, stromal cell preservation, and cell viability [5–8]. Recently, several protocols for vitrification have been reported [5, 6, 9–11], and at least one vitrification system is currently available for clinical use. Cryopreservation by vitrification is achieved by elevating the cooling rate and concentration of cryoprotectants and forming a glassy, vitrified state by extreme elevation of viscosity during cooling [12]. There are two main challenges to improving vitrification techniques: (i) high concentrations of cryoprotectant, which may be toxic to oocytes, embryos, and ovaries, are required and (ii) intracellular ice may develop if the cooling or warming rates are incorrect [13, 14]. Several methods have been developed to overcome these problems. The logical way to make cooling more rapid is to use the smallest possible volume of cryoprotectant around the sample and to directly expose it to liquid nitrogen without any insulation. It is a process known as open vitrification [13]. Successful open vitrification of ovarian tissues has been achieved in mice [5, 15, 16], cattle [9], monkeys [10, 11], and humans [6]. Two healthy babies have been born from vitrified ovarian tissues preserved by open vitrification [17, 18]. However, there is concern about the sterility of liquid nitrogen and the risk of cross-contamination during long-term storage [19, 20]. Because ovaries are larger than oocytes or embryos, the risk of cross-contamination is increased with ovarian cryopreservation by direct contact between solution containing ovarian tissue and liquid nitrogen. To avoid the risk of contamination, a closed vitrification method was developed and used *in vitro* for development of preantral follicles from the macaque ovarian cortex [21]. However, *in vivo* tissue transplantation studies are needed to confirm long-term function and fertility of vitrified-warmed ovarian tissues.

In the present study, we developed a new closed vitrification protocol. The efficacy of the protocol was assessed using ovarian tissues of mice and cynomolgus monkeys for long-term functions of vitrified-warmed ovarian tissues after transplantation.

Materials and methods

This investigation of closed vitrification process was based on previous research into open vitrification by Suzuki et al. [11]. The study protocol was approved by the ethics committee of St. Marianna University School of Medicine (Kanagawa, Japan) and IVF Namba Clinic (Osaka, Japan). This study was performed in accordance with the guidelines for use of animals at

the experimental facilities of St. Marianna University School of Medicine (Kanagawa, Japan), Kinki University (Wakayama, Japan), and Eve Bioscience Ltd. (Wakayama, Japan).

Studies in mice

Animal care and management

Female and male C57BL/6J mice and green fluorescence protein (GFP)-transgenic [C57BL/6J-Tg (CAG-EGFP)] mice were purchased from SLC Japan Inc. (Tokyo, Japan). Mice were housed under a 12-h light/12-h dark cycle at 22 °C with 55% humidity and received food and water *ad libitum*.

Collection, cryopreservation, and thawing of donor mouse ovaries

Donor ovaries were collected from 4-week-old C57BL/6J-Tg (CAG-EGFP) mice and placed into disposable Petri dishes containing Dulbecco's modified Eagle's medium/Ham's F-12 [1:1 (v/v)]. Then, each ovary was cut in half for cryopreservation using the new closed device. After 1–2 months, the cryopreserved ovarian tissues were warmed and stored in H199 supplemented with 20% (V/V) SSS medium until transplantation.

Transplantation of mouse ovaries

Orthotopic transplantation of vitrified-warmed half ovaries was performed in 6-week-old female C57BL/6J mice, while fresh ovaries harvested from 4-week-old CAG-EGFP female mice were cut in half and used as control grafts. Orthotopic transplantation was done as described previously [15] with some modifications. In brief, anesthesia was induced by intraperitoneal injection of pentobarbital sodium salt (7.5 g/kg body weight). A midline longitudinal skin incision was made in the lower back, followed by small bilateral incisions in the fascia and muscles immediately above each ovary through which the recipient's reproductive tract was lifted out. Then, a small slit was made in each ovarian bursa, and the recipient's ovary was removed under an operating microscope and was replaced with a fresh or thawed half ovary. Subsequently, the bursal membrane was sutured, the reproductive tract was replaced inside the body, and the skin incision was sutured.

Mating of mice

One recipient mouse was paired for 1 day with one male C57BL/6J mice 10 days after transplantation to allow recruitment of primordial follicles before mating. When mating was confirmed by the presence of a vaginal plug, pregnant females were moved to separate cages. The number of pups (both dead and alive) was recorded.

Monkey experiments

Animal care and management

Six cynomolgus monkeys (4 years old and weighing 3.0–3.5 kg) imported from Vietnam by Eve Bioscience (Wakayama, Japan) were used in this study. After importation, the monkeys were quarantined for 1 month and were examined for pathogenic bacteria and changes of appearance and/or behavior. Monkeys were housed individually in special cages (550 mm × 72 mm × 780 mm) in an animal room with a temperature of 25–38 °C, ventilation 5 times/h, and a 12-h light/12-h dark cycle (07:00–19:00). Food (AS; Oriental Yeast Co., Ltd., Tokyo, Japan) was supplied every morning, and drinking water (municipal tap water of Hashimoto City, Wakayama, Japan) was provided ad libitum. The estrous cycle was confirmed in each female by monitoring vaginal bleeding.

Anesthesia and surgery

Laparotomy was performed after induction of anesthesia by intramuscular injection of a 2:1 mixture of ketamine (20 mg/kg Ketalar 50; Sankyo Yell Pharmaceutical, Tokyo, Japan) and xylazine (1 mg/kg Celactal Bayer Yakuhin, Osaka, Japan). During surgery, additional doses of these anesthetics were administered as appropriate to maintain the depth of anesthesia. After resection of the bilateral ovaries, adherent tissues were removed, and the organs washed twice in modified Human Tubal Fluid (m-HTF, Kitazato BioPharma Co., Ltd., Shizuoka, Japan). Then, the cortical tissue was removed from each pair of ovaries and placed in m-HTF. Monkeys were recovered from anesthesia following suture. Relaparotomy for transplantation was performed generally after about 5 h, including 1 h for tissue preparation, 3 h of soaking in liquid nitrogen, and 1 h for warming.

Ovarian tissue cryopreservation and transplantation

The monkey ovarian tissue was cut into strips (10 mm × 10 mm × 1 mm) for vitrification, and cryopreservation was performed using the closed device by the same procedure as that for mice (Fig. 1d, e). Tissue strips were maintained in liquid nitrogen for at least 3 h. Laparotomy was performed for transplantation of vitrified-thawed ovarian tissue, with a minced graft (cut into 20–25 pieces measuring 1 mm × 1 mm × 1 mm) on the right side and an intact strip (10 mm × 10 mm × 1 mm) on the left side of the omentum or the mesosalpinx. Altogether, four tissue grafts, two preparations each for strips and minces, were transplanted in each monkey, one graft each on each side of two sites, the omentum, and the mesosalpinx. For omental transplantation, the graft was wrapped in the omentum that was sutured to form a bag. For transplantation into the mesosalpinx, an incision was made in the right side of the mesosalpinx to create a pocket, into which graft was placed and incision was sutured. To prevent adhesions,

Seprafilm® (Kaken Pharmaceutical Co., Ltd. Tokyo, Japan) was used during closure of the abdomen.

Stimulation of ovarian tissue

Ovarian stimulation was conducted after down-regulation with a gonadotropin-releasing hormone (GnRH) agonist (1.88 mg of Leuplin injection 3.75; Takeda Pharmaceutical, Osaka, Japan). Six female monkeys were administered various doses of recombinant human follicle-stimulating hormone (rhFSH; Follistim®; MSD, Osaka, Japan) after down-regulation in three trials, as described below. On the evening of the final day of rhFSH injection, 400 IU/kg of human chorionic gonadotrophin (hCG; Pubergen; Nippon Yakkyoku, Tokyo, Japan) was administered, and ova were collected 40 h later. In the first trial, 25 IU/kg of rhFSH was administered once daily in the morning on days 1 to 9 (initiation of FSH injection = day 0). The second and third trials were initiated 6 months and 1 year after transplantation. In the second trial, 50 IU/kg of rhFSH was administered once daily in the morning on days 1 to 9, after which 100 IU/kg of rhFSH was administered twice daily in the morning and evening on days 10 and 11. In third trial, 50 IU/kg of rhFSH was administered once daily in the morning on days 1 to 4, 75 IU/kg of rhFSH was administered twice daily in the morning and evening on days 5 to 11, and 100 IU/kg of rhFSH was administered twice daily in the morning and evening on day 12. Ovarian stimulation was initiated 2 weeks after estradiol elevation in the trial 1 and between estradiol elevations 6 months and 1 year after transplantation in the trials 2 and 3, respectively. All three trials were performed on all monkeys, except the monkey 6 which was subjected to only the trials 2 and 3.

Oocyte collection and transportation

Laparotomy was performed under general anesthesia as described above. After confirming mature follicle development, oocytes were aspirated, and a small piece of ovarian tissue containing the follicle growth area was excised and placed in the culture medium. Oocytes were collected by puncturing the follicle in a culture dish to avoid loss of oocytes. Aspiration of mature follicles was performed by using a 2.5-ml syringe with a 21-G needle that contained a small amount of HEPES-Tyrodé's albumin with lactate and pyruvate (HEPES-TALP, pH 7.4) and 0.3% bovine serum albumin, after pre-washing the needle and syringe with heparin sodium (Ajinomoto Pharmaceutical, Tokyo, Japan). The retrieved ova were transferred to HEPES-TALP in a dish on a warming plate maintained at 38 °C and maturation was examined by stereomicroscopy (×80). Then, the collected ova were placed into HEPES-TALP in a sealed round-bottom tube (2058, Becton Dickinson Labware, NJ, USA) kept in warm water (38 °C). The ovarian tissue was placed in 15-ml tubes (Polypropylene Conical Centrifuge Tube, BD Falcon, Bedford,

Fig. 1 Devices for vitrification. **a** Ova Cryo Device Type M (open device [11]). **b** Closed device. **c** Closed device in the pouch. **d** The sealed pouch. **e** Vitrified ovarian tissue

MA, USA) containing the same culture medium. The specimens were transferred from Eve Bioscience to Namba IVF Clinic (Namba, Osaka, Japan) within 2 h.

In vitro maturation of immature monkey oocytes

In the first and second trials, we examined whether oocytes obtained at the germinal vesicle (GV) stage or metaphase I (MI) stage could reach metaphase II (MII). The oocytes were incubated at 37 °C for 24–72 h under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂ in TALP medium containing L-glutamine, lactic acid, pyruvic acid, and 20% (v/v) fetal bovine serum. In the third trial, 20 ng/ml of epidermal growth factor (EGF; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was added to the abovementioned TALP medium, and the oocytes were cultured for 24–72 h.

Intracytoplasmic sperm injection: fertilization of monkey oocytes

Semen was obtained from a male monkey under anesthesia with ketamine HCl (10 mg/kg) by electrical induction of ejaculation. The methods used for freezing and thawing sperm were described previously [21]. Intracytoplasmic injection (ICSI) of cryopreserved sperm was performed as reported previously [11] with minor modifications. Briefly, cumulus cell-oocyte complexes were treated with 0.01% (w/v) hyaluronidase (H3757;

Sigma-Aldrich) dissolved in HEPES-TALP, and then oocytes were denuded by pipetting at room temperature. After maturation of the denuded oocytes was evaluated, culture was performed at 37 °C under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂ in TALP until the time of ICSI. The sperm suspension and oocytes were placed in HEPES-TALP and covered with mineral oil (M8410; Sigma-Aldrich). A single sperm with normal morphology was captured by an injection pipette (MIC-SI-30-NTP; Humagen Fertility Diagnostics, Charlottesville, VA, USA) and then was washed and immobilized in an injection pipette containing 10% polyvinylpyrrolidone (PVP360; Sigma-Aldrich) dissolved in HEPES-TALP. The immobilized sperm was injected into a mature oocyte in HEPES-TALP. After injection, culture was done for 24 h at 37 °C under an atmosphere of 5% O₂, 5% CO₂, and 90% N₂ in TALP. Fertilization was evaluated on basis of detecting two pronuclei and two polar bodies at 18 h after sperm injection, and cleavage was confirmed at 24 h after sperm injection. Then, the embryos were cultured for 144 h in CMRL-1066 medium (11530-037; Life Technologies, Carlsbad, CA, USA) containing L-glutamine, lactic acid, pyruvate, and 20% (v/v) fetal bovine serum under the same atmosphere.

Morphological investigation of monkey ovarian tissue

Monkey ovarian tissues were fixed overnight at 4 °C in Bouin's fixative (Polysciences, Warrington, PA, USA), embedded in paraffin, and cut into 1–5-μm-thick

Fig. 2 Cooling and warming rates of vitrification devices. **a** Cooling rate of the open (black) and closed (pink) devices. The cooling rates of the open device and the closed device were $-19,500$ and $-12,000$ °C/min,

respectively. **b** Warming rates of the open and closed devices were identical and shown in red. The warming rate was $42,000$ °C/min

sections. Sections were stained with toluidine blue (Certistain® Toluidine blue O (C.I.52040), Millipore, MA, USA) and hematoxylin-eosin (Sakura Finetek Japan, Tokyo, Japan), followed by observation under a microscope. Follicular development stages were defined as follows: primordial follicles were in a single layer of flattened pre-granulosa cells, primary follicles were follicles with only one granulosa cell layer, early secondary follicles had two to four granulosa cell layers without an antrum, late secondary follicles had more than four granulosa cell layers without an antrum, and antral follicles had multiple layers of cuboidal granulosa cells with an antrum [22].

Hormone assays

Estradiol (E_2) and progesterone (P_4) were measured by SRL Inc. (SRL, Tokyo, Japan) to confirm the hormone cycle of the monkeys before ovarian tissue cryopreservation and approximately every 2 weeks after transplantation of vitrified-thawed

ovarian tissue. This procedure was performed in the same manner as reported previously [11].

Open vs. closed vitrification

Loading with cryoprotectant

Cryopreservation of ovarian tissues using an open device was performed as described previously with some modifications [10, 11]. Strips of ovarian tissue were first equilibrated in HEPES-buffered 199 (H199, Invitrogen, Carlsbad, CA, USA) supplemented with 20% (V/V) SSS (Irvine Scientific, Santa Ana, CA, USA) and 1.61 mol/l ethylene glycol for 5 min, followed by equilibration in H199 supplemented with 20% (V/V) SSS and 3.22 mol/l ethylene glycol for 5 min. Subsequently, the ovarian strips were transferred into H199 supplemented with 20% (V/V) SSS, 5.64 mol/l ethylene glycol, 5% (w/v) polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, Mo, USA), and 0.5 mol/l sucrose EMPROVE®bio

Fig. 3 Orthotopic ovarian tissue transplantation in mice. Fresh and vitrified/warmed ovarian tissues were transplanted for functional evaluation. Graph shows the mean with the standard deviation for 6 mice (fresh tissue) and 10 mice (vitrified/warmed tissue). * $p < 0.05$. **a** Days required

(EMD Millipore, Billerica, MA, USA). All steps were performed at 26 °C.

Vitrification procedure

Open device Within 25 min, the strips were loaded into an ovarian storage device (Ova Cryo Device TypeM®; Kitazato BioPharma Co., Shizuoka, Japan; Fig. 1a) and the device was immersed vertically into liquid nitrogen. Then the frozen strips were individually placed into cryogenic vials and were stored in liquid nitrogen until further use.

Closed device The device was made from titanium (500 and 800 μm in thickness) for excellent thermal conductivity (CDT $\times 10$, titanium, Kitazato BioPharma Co., Ltd., Shizuoka, Japan). Two sizes of thickness (500 and 800 μm) were tested in consideration of conductance and structural stability. However, the similar stability and conductance were obtained with both thicknesses. The 500- μm thickness was used in mice. The 800- μm one was used only for one monkey as comparison after testing both thicknesses. For vitrification, a piece of ovarian tissue equilibrated with vitrification solution was placed on the titanium plate and covered with polypropylene film (Kitazato BioPharma Co., Ltd., Shizuoka, Japan). To prevent viral contamination, the device was placed in a bag made from the same polypropylene material as the film cover, which was heat-sealed and immersed in liquid nitrogen. This

before the initial increase of estradiol. **b** Rates of pregnancy. **c** Number of live pups per pregnancy. **d** Pups from transplants of fresh (**a**) or cryopreserved GFP mouse ovaries (**b**)

extra step for inserting vitrified tissue in a bag took only 10–30 s; thus, we did not feel there was any difference between open and closed device in the effect of cryoprotectants. The polypropylene material used for the film cover and bag was demonstrated to be safe by a cytotoxicity test before this study (Fig. 1b–e).

Warming procedure

For thawing, pouch is cut open and the device is directly placed into the thawing solution. The vitrified samples were thawed in 20 ml of H199 containing 20% (V/V) SSS and 0.8 mol/l sucrose, which had been warmed at 37 °C for 1 min in a water bath, followed by dilution in H199 containing 20% (V/V) SSS and 0.4 mol/l sucrose for 3 min at room temperature, and then dilution twice in H199 containing 20% (V/V) SSS for 5 min at room temperature. After removal of the cryoprotectant, the ovarian sections were placed in H199 containing 20% (V/V) SSS. No devitrification was observed in any tissue preparation.

Heat conduction rate

Cooling was assessed by measuring the heat conduction rate using a K-type thermocouple and a data logger GL900-4 (Graphtec Inc., Kanagawa, Japan), with the mean of 10 temperature measurements being plotted to generate a graph (Kitazato

Fig. 4 Hormonal cycles in monkeys. Estradiol (blue line) and progesterone (red line) in blood after ovarian transplantation. Number in green square: days required before hormone detection in blood. IVF cycles are indicated by arrows: GnRH (blue), FSH (red), and hCG (green)

BioPharma Co., Ltd., Shizuoka, Japan). The heat conduction rate was compared between the open device (Ova Cryo Device TypeM®) and the closed device (CDT × 10; Fig. 2a, b).

Statistical analysis

All statistical analyses were performed by using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

Results

Thermal conductivity

The cooling rates of the open and closed vitrification devices were $-19,500$ and $-12,000$ °C/min, respectively (Fig. 2a), while the warming rate of both devices was $42,000$ °C/min

(Fig. 2b). There were no differences of thermal conductivity, especially the cooling speed, between devices with a thickness of 500 or 800 μm.

Fertility of mice after transplantation of vitrified-warmed ovarian tissues

Six mice received transplantation of fresh ovarian tissue as controls. Vitrified-warmed ovarian tissue was transplanted into 10 mice. The estrous cycle was recovered in all recipients. Estradiol increased at 5–11 days after transplantation of fresh ovarian grafts and after 7–14 days in mice receiving vitrified-warmed grafts. There were no significant differences in the time required until recovery of the estrous cycle between mice receiving fresh grafts (8.2 ± 0.9 days) and vitrified-warmed grafts (8.1 ± 0.9 days) (Fig. 3a). Recipient mice were kept for 3 weeks after transplantation to allow recruitment of primordial follicles before mating. The delivery rate was significantly lower for mice with vitrified-warmed ovarian grafts

Fig. 5 Laparotomy for oocyte retrieval in monkey no. 4. Follicles are evident in the left mesosalpinx (a) and omentum (b) on the post-transplantation day 252. A pre-ovulatory follicle dissected from the omentum, shown in b, is shown in an enlarged scale (c)

(60%) than for mice with fresh grafts (83%) (Fig. 3b). However, the number of live pups delivered per recipient was not significantly different between the two groups (Fig. 3c, d).

Hormonal cycle in monkeys

Transplantation of vitrified-warmed ovarian tissues was performed in six cynomolgus monkeys. No monkey received fresh ovarian tissue as control due to the cost of each monkey. Five monkeys received ovarian tissue that had been vitrified using 500- μ m closed device, and one monkey (no. 3) received tissue vitrified using 800- μ m closed device. Follicle development was assessed by estradiol measurements every 2 weeks. Harvesting of ova was successful in five monkeys, while it failed in monkey no. 4. Resumption of the hormonal cycle was confirmed at 111 days (27–192 days) after transplantation (Fig. 4). The five monkeys used for egg collection exhibited periodic hormonal cycles by 367 days after transplantation. Oocyte collection was performed via laparotomy, with a small piece of tissue around the site of follicle formation being excised carefully while avoiding rupture of the follicle (Fig. 5). It was found that transplanted ovarian tissue had been incorporated into the omentum and the mesosalpinx, and minimal resection was required for oocyte harvesting. Three monkeys (no. 1, no. 3, and no. 6) were still alive at the time of writing, and their hormonal cycles had continued for >1240 days.

Follicle development

Follicle development was found at eight graft sites in the omentum and 13 sites in the mesosalpinx. In addition, it was detected at 10 sites after transplantation of minced grafts (1-mm cubes) and at 11 sites after transplantation of strip grafts (10 mm $\times$ 10 mm $\times$ 1 mm), while the number of oocytes recovered was eight and nine, respectively. There were no significant differences of follicle development between transplantation into the omentum or the mesosalpinx, between transplantation of strips or minced grafts, and between cryopreservation with the 500- or 800- μ m devices.

IVM and IVF

Seventeen oocytes were retrieved from five monkeys. To perform in vitro maturation (IVM), EGF was not used in the first and second trials in case immature oocytes were collected. A total of seven GV-stage oocytes, one MI oocyte, and two MII oocytes were obtained by the initial two egg collections. When the eight GV-stage oocytes were cultured for up to 72 h, only three reached MI stage but did not progress further. ICSI was performed with the two MII oocytes, and fertilization was confirmed by detection of two pronuclei, but development did not proceed beyond the one-cell stage. A total of six oocytes were obtained at the third egg collection, including four GV-stage oocytes, one MII oocyte, and one degenerated embryo. IVM was performed with four GV-stage oocytes by adding EGF at 20 ng/ml. One of these oocytes reached MII stage but failed to fertilize by ICSI (Table 1).

Table 1 IVM and IVF of oocytes retrieved from transplanted ovarian tissue in monkeys

Ovarian stimulation	No of Monkey	Thickness of device	Days between hormonal measurement and oocyte harvest	Serum estradiol (E2) level (pg/ml) / Serum progesterone (P4) level (ng/ml)	Days between hormone measurement and oocyte harvest	Site	Side	Follicle number	Gained oocyte number	Stage	IVM Reached to MII	ICSI Fertilized
Trial 1	1	500	110	E2: 76 / P4: 0.06	5	Mesosalpinx	Lt	1	1	GV	yes	No
	2	500		E2: 146 / P4: 0.1	5	Omentum	Rt	1	1	GV	yes	No
	2	500	258	E2: 223 / P4: 0.08	3	Mesosalpinx	Lt	4	4	GV	yes	No
Trial 2						Omentum	Lt	1	0			
	4	800		E2: 61 / P4: 0.04	3	Mesosalpinx	Lt	2	2	MI	No	Yes
						Omentum	Lt	1	1	MI	yes	No
						Mesosalpinx	Rt	1	1	GV	yes	No
						Omentum	Rt	1	1	GV	yes	No
Trial 3	1	500	377	E2: 34 / P4: 0.03	3	Omentum	Rt	1	1	MI	No	Yes
	3	800		E2: 116 / P4: 0.04	3	Omentum	Lt	1	1	Degeneration		No
						Omentum	Rt	1	0			
	6	500		E2: 49 / P4: 0.16	3	Mesosalpinx	Rt	4	4	GV	Yes	Yes
						Mesosalpinx	Lt	1	0			No
						Omentum	Rt	1	0			

Fig. 6 Post-transplantation histological analyses. **a** Ovarian tissue adjacent to pre-ovulatory follicles excised for oocyte harvest from the omentum on the post-transplantation day 258 that yielded a MI oocyte (monkey no. 4). **b** The omentum which yielded GV oocytes (monkey no. 2). **A** Toluidine blue staining. **b** H&E staining. Scale bar = 50 μ m

Histological findings

After collection of oocytes, the residual tissues were fixed for histological examination. At a site of ovarian tissue transplantation into the omentum, two primary follicles and one early secondary follicle were detected. Also, at another omental transplantation site, there were primordial follicles, primary follicles, and late secondary follicles, having the potential to develop for oocyte collection (Fig. 6a, b).

Discussion

A closed vitrification devise was developed using a titanium plate for housing ovarian tissue which is sealed inside a polypropylene pouch to avoid viral contamination during exposure to liquid nitrogen. Due to the excellent thermal conductivity of titanium, similar cooling and warming rates, which are important for maintaining the structural integrity of tissue, were observed by closed vitrification method to those by the standard open one.

The *in vivo* function was examined by transplanting ovarian tissues after cryopreservation by the closed vitrification devise on mice and cynomolgus monkeys. The number of live pups per litter was similar with both tissues in mice (Fig. 4a). However, the birth rate by cryopreserved tissue with the closed vitrification method was lower than that from fresh ovarian tissue in mice (Fig. 4b). Using similar vitrification conditions to the one used in the present study, follicle counts were similar to fresh tissue transplants, and pregnancy rates were higher than those of transplants from slow freezing or conventional vitrification and similar to the fresh tissue transplants [5, 16]. These studies may indicate that vitrification conditions influence the integrity and potentials of follicles. The lower birth rates observed in the current study could indicate interferences on follicle maturation by vitrification method utilized here, for which further studies may be needed for improvement of vitrification protocol.

In monkeys, vitrified-warmed ovarian tissues were transplanted into the mesosalpinx and the omentum which

were shown to be suitable sites for engraftment in our previous study [11]. Heterotopic transplantation was conducted with minced or strip grafts, but the type of graft did not affect oocyte collection. Grafts were wrapped with the omentum tissue, while they were put into a pouch by incision made in the mesosalpinx. These procedures made harvesting easier. Six follicles formed in grafts transplanted to the mesosalpinx and eight follicles were obtained after transplantation to the omentum (with no difference in follicle formation between the two sites). The ovarian cycle was restored after transplantation in most animals, while there were developments of pre-ovulatory follicles and mature oocytes which could fertilize after ICSI. Our study thus demonstrated the mesosalpinx and the omentum as viable options for transplantation of cryopreserved ovarian tissues which would be clinically available for those patients with bilateral ovariectomy or pelvic irradiation.

Multiple follicles were observed in both transplantation sites of the mesosalpinx and the omentum. Some are more mature or immature than others among these follicles. Therefore, the mechanism for selection of a dominant follicle could have been lost by the vitrification process. On the other hand, since the present study was performed by the IVF cycle, it is not apparent if the dominant follicle selection mechanism is preserved. Even in clinics, oocytes can be obtained from smaller follicles rather than the dominant follicle. As many follicles develop at one transplantation site due to ovarian stimulation by FSH, we tried to harvest as many oocytes as can be identified, thus resulting in the harvest of more immature oocytes. Thus, the number of follicles which could yield competent oocytes could have been very limited, even in the presence of cyclic steroid hormone production.

When oocytes were retrieved from transplanted vitrified-warmed ovarian tissues of monkeys after the return of the hormonal cycle, embryos did not develop after fertilization *in vitro*. Could the failure of embryogenesis be caused by injuries/damages during cryopreservation process? When murine ovarian tissues were processed for cryopreservation by different vitrification methods, transmission electron microscopy demonstrated varying degrees of damages in subcellular structures even in the presence of apparently normal delivery of pups [16]. Using

both murine and human tissues, changes in follicle morphology, particularly in granulosa and stromal cells, were seen after cryopreservation [5]. In ovarian tissue cryopreservation, stromal cells were demonstrated to be more vulnerable to cryoinjuries than primordial follicles [23, 24]. The ovary includes heterogeneous cellular and structural components with different properties, and their interactions are needed for follicle maturation and embryogenesis [25]. Apparent structural integrity of ovarian tissue after cryopreservation does not appear to guaranty the quality and function of follicles. It might be informative to analyze effects of cryopreservation on gene expression pattern of oocytes, stromal cells, etc. during maturation for establishing a standard procedure of ovarian tissue cryopreservation.

One of the six monkeys monitored for more than 1 year after transplantation showed a weak response to ovarian stimulation, but its estradiol level was still restored. It is possible that engraftment of ovarian tissue might have been poor in this animal. Among the monkeys monitored for less than 1 year after transplantation, one did not show stable recovery of the hormonal cycle by 8 months after transplantation, although it is possible that the hormonal cycle would have resumed if the follow-up period had been extended. Recovery of the hormonal cycle was confirmed in five of six monkeys, with menstruation returning between 50 and 90 days after transplantation. The time required for recovery of menstruation was shorter than that expected for growth of primordial follicles, indicating the presence of primary and secondary follicles in vitrified ovarian tissues as well as primordial follicles. In three of these monkeys, the hormonal cycle has persisted for over 1200 days since resumption after transplantation.

In conclusion, a closed vitrification procedure was developed to improve the efficacy for ovarian cryopreservation and to avoid the risk of cross-contamination during storage in liquid nitrogen. Live birth was achieved in mice, but our results were unsatisfactory in terms of fertilization and embryo development in primates. This outcome may have occurred because follicle development was only monitored by blood tests and might not have been fully matured. On the other hand, vitrification procedure may have caused damages to cellular structures and genetic activities interfering developmental processes in primates. As we confirmed persistence of the hormonal cycle for longer than 1 year, we are confident that heterotopic engraftment of ovarian tissue was successful. We hope that our closed device will help advance the vitrification technology for clinical application.

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Author's contribution Y.S., Y.M., A.T., and N.S. participated in the study conception and design; Y.S., A.U., M.N., S.H., and N.S. developed the device; N.O. and M.N. performed mouse studies; Y.S. and M.N. completed freeze–thawing of ovarian tissue in the monkey study; Y.S., N.S., C.N., and S.I. performed surgery on the monkeys; S.O. and M.T. performed ovarian stimulation and management in the monkeys; T.Y. performed collection of monkey oocytes and IVM/IVF; and Y.S. and T.S. conducted histological examination.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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