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Cryopreservation of ovaries from neonatal marmoset monkeys

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Abstract: The ovary of neonatal nonhuman primates contains the highest number of immature oocytes, but its cryopreservation has not yet been sufficiently investigated in all life stages. In the current study, we investigated cryodamage after vitrification/warming of neonatal ovaries from a nonhuman primate, the common marmoset (*Callithrix jacchus*). A Cryotop was used for cryopreservation of whole ovaries. The morphology of the vitrified/warmed ovaries was found to be equivalent to that of fresh ovaries. No significant difference in the number of oocytes retaining normal morphology per unit area in histological sections was found between the two groups. In an analysis of dispersed cells from the ovaries, however, the cell viability of the vitrified/warmed group tended to be decreased. The results of a comet assay showed no significant differences in DNA damage. These results show that cryopreservation of neonatal marmoset ovaries using vitrification may be useful as a storage system for whole ovaries.
Key words: comet assay, common marmoset, neonate, ovarian tissue cryopreservation, vitrification

Introduction

Since the mammalian ovary contains a large number of immature oocytes (follicles), ovarian cryopreservation is expected to provide an effective means for germ cell storage and fertility preservation. There are two main methods used for cryopreservation in reproductive biology and medicine. One is slow freezing performed using a programmable freezer that undergoes a controlled temperature change based on a computer program, and the other is vitrification, a rapid cooling method in which the tissue is directly plunged into liquid nitrogen after equilibration in high concentrations of cryoprotectants. Vitrification is known to be a simple and convenient protocol for preserving embryos and oocytes. This technology has been put to practical use for both oocytes and preimplantation embryos of various mammalian species [5, 36].

In recent years, ovarian cryopreservation has attracted attention as a promising reproductive technology for fertility preservation in mammals, because the ovary includes a large number of immature oocytes (follicles). This approach has been attempted not only in laboratory animals, such as rodents, but also in domestic and wild animals [25] and humans [29]. The methods for both slow freezing [3, 14, 17, 26, 34, 37, 38, 40] and vitrification [1, 9, 10, 12, 32–35, 40] have been investigated in nonhuman primates. The vitrification method is at least as effective as slow freezing in viability after thawing [40]. Furthermore, it is easy and more conve-

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nient than slow freezing because devices such as a programmable freezer are not required. These investigations have mainly targeted the follicles of adults or have, in rare cases, utilized prepubertal ovarian tissues [37, 38], but they have not utilized neonatal stage tissues.

The neonatal ovaries include a large number of primordial oocytes (follicles), more than are contained in adult ovaries. For instance, human ovaries at birth store approximately 130,000 to 500,000 primordial oocytes, and the number of ovarian follicles decreases gradually with the increase in age after puberty [7, 8]. Not all primordial oocytes stored in the ovaries achieve growth, maturation, and ovulation during reproductive life. A certain percentage of oocytes are present in dormant primordial follicles, and others undergo follicle atresia via an apoptotic process [19]. In fact, more than 99% of all follicles fail to reach the preovulatory stage, with most of these follicles undergoing atresia. Ovarian cryopreservation at the neonatal stage would therefore make it possible to store the largest possible number of oocytes.

The common marmoset (*Callithrix jacchus*) is a New World monkey that has attracted attention as a nonhuman primate model for biological and medical research [4, 22]. Among nonhuman primates, the marmoset has a comparative advantage as a laboratory animal because of its small size (300–450 g) and high reproductive efficiency. However, many monkeys die before or soon after birth in breeding. According to a study of mortality in the common marmoset [24], the frequency of fetal death was 10.5% of total births, and the highest ratios of fetal death occurred in quadruplet (37.5%) and singleton pregnancies (25.0%). In addition, some cases of infanticide occur immediately after birth. Therefore, cryopreservation of the neonatal ovaries may be useful for rescuing female germ cells when the individuals have a particularly important genotype.

The aim of this study was to investigate the effects of cryopreservation by a vitrification method on cryodamage in neonatal marmoset ovaries.

Materials and Methods

Animals

This study was conducted in accordance with protocols approved by the ethics committee for primate research at the National Institute of Neuroscience (NIN), National Center of Neurology and Psychiatry (NCNP), Japan. Female neonatal common marmoset monkeys (*Callithrix jacchus*, 0–10 days old) produced in the breeding colony of the NIN/NCNP were donated to us after they had been euthanized by intraperitoneal injection of sodium pentobarbital at doses of 100 mg/kg and utilized for neuroscience research. A total of 12 ovaries from 7 neonates were used in this study. Six ovaries were used for histology (3 ovaries were used as fresh controls). The remaining 6 ovaries were used for both a comet assay and analysis of ovarian cell viability (3 ovaries were used as fresh controls).

Cryopreservation and warming

A commercially available vitrification kit (Kitazato BioPharma Co., Ltd., Shizuoka, Japan) was used in these experiments. Neonatal ovaries were transferred into Leibovitz's L-15 medium supplemented with 10% heat-inactivated fetal bovine serum (L-15 and FBS; Life Technologies Corporation, Carlsbad, CA, USA). After a washing step, they were initially equilibrated in 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in a basal medium composed of HEPES-buffered TCM199 supplemented with 20% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA, USA) for 15 min at room temperature and then in a second equilibration solution composed of 15% EG, 15% DMSO, and 0.5 M sucrose in the basal medium for 30 min at 4°C [9]. The ovaries and a minimum volume of solution were placed on the polypropylene strip of the Cryotop (Cryotop-N, Kitazato BioPharma Co., Ltd., Shizuoka, Japan) and then immediately submerged into liquid nitrogen. For warming, the straw cap from the Cryotop was removed in liquid nitrogen, and the polypropylene strip was immersed directly into the basal medium supplemented with 1 M sucrose for 3 min at 37°C. The ovaries were then transferred into the basal medium with 0.5 M sucrose for 5 min and washed twice in the basal medium without sucrose for 15 min at room temperature.

Histology and evaluation

The ovaries were fixed in 4% formaldehyde in PBS for 24 h, and then embedded in paraffin, sectioned at a thickness of into 1–2 μm at 50 μm intervals, stained with hematoxylin and eosin, and then observed with a light microscope (Axio Imager M1, Carl Zeiss, Germany).

For the analysis, 9 to 10 sections per ovary were randomly selected, and the morphologically normal oocytes in each section were all counted. Oocytes were counted as morphologically normal if they showed no pyknosis

of the oocyte nucleus, they showed no large or multiple vacuoles in the oocyte, and they did not have shrunken ooplasm [38].

Ovarian cell viability

To evaluate the pre- and post-vitrification viability of ovarian cells that contain both ovarian somatic cells and primordial oocytes, each ovary was incubated in 5 ml HBSS containing 1.5 mg/ml collagenase (Type IV) for 15 min at 37°C with gentle agitation and was treated with HBSS containing 0.05% trypsin for 10 min. The trypsin was neutralized by adding 10% FBS, and the ovary was dispersed by pipetting. Floating cells were collected by centrifugation at 1,000 rpm for 5 min. The cells (10^5–10^6 cells/ml) were washed by centrifugation in PBS and were incubated in PBS supplemented with 2 μM calcein-AM and 4 μM propidium iodide (Double Staining Kit, Dojindo Molecular Technologies Inc., Kumamoto, Japan) for 15 min at 37°C. Cell viability was examined under fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan) after washing the cells. Surviving cells and the nuclei of dead cells were emitted green and red fluorescence, respectively. Lastly, the survival rate was calculated.

Comet assay

The cryodamage of genomic DNA in ovarian tissue after warming was assessed using an alkaline version of the comet assay for single cell gel electrophoresis (#4250–050-K; Trevigen, Inc., Gaithersburg, MD, USA). The tissue preparation and comet assay protocol described by Trevigen was adopted (http://www.trevigen.com/docs/protocol_4250-050-K.pdf). The ovaries were transferred into Ca^{2+}/Mg^{2+}-free PBS with 20 mM EDTA and thereafter were dissected into very small pieces (less than 1 mm^3) and allowed to stand for 5 min. Each ovary was incubated in 5 ml Hank's Balanced Salt Solution (HBSS; Life Technologies Corporation, Carlsbad, CA, USA) containing 1.5 mg/ml collagenase (Type IV, Sigma-Aldrich Corporation, St. Louis, MO, USA) for 15 min at 37°C with gentle agitation, and was treated with HBSS containing 0.05% trypsin for 10 min. The trypsin was neutralized by adding 10% FBS. After gentle pipetting for cell dissociation, the suspension was passed through a 70 μm cell strainer and centrifuged at 1,000 rpm for 5 min at 4°C. After removal of the supernatant, the pellet was resuspended in PBS at a concentration of 1×10^5 cells/ml and maintained at 4°C until the comet assay. These cells contained both ovarian somatic cells and primordial oocytes. Electrophoresis was carried out for 5 min at 25 V. Comet images of single cells were captured with a fluorescence microscope (BZ-9000, Keyence, Japan), converted to gray scale, and scored using the Comet Score software (ver. 1.5, TriTek, Sumerduck, VA, USA). Forty to 80 comets were measured per sample. We compared two different comet parameters. The percent of DNA in the tail was defined as the total acreage of tail intensity / (total acreage of head intensity + total acreage of tail intensity) × 100. The tail moment was defined as the tail length × the percent of DNA in the tail / 100.

Statistical analysis

Each experiment was repeated three times. All data were expressed as means ± SEM. Significance testing was carried out using one-way ANOVA. Percentage data were subjected to arcsine transformation, followed by ANOVA. Values of $P<0.05$ were considered to be statistically significant.

Results

Morphology and histology

The internal genital organs of a representative female neonatal marmoset are shown in Fig. 1A. In shape, the ovary was a concave ellipsoid (Figs. 1A–1D). The major and minor axes were 2.8 ± 0.1 μm and 1.2 ± 0.1 μm (n=3, mean ± SEM) in length, respectively. Therefore, it was possible to put an ovary on the Cryotop, a vitrification device (Fig. 1B). The vitrified/warmed ovaries had the same appearances as the fresh ovaries (Figs. 1C and 1D). A histological examination demonstrated the presence of primordial follicles (non-growing oocytes) and primary follicles (slightly grown oocytes). Follicles were also found enclosed by round cells and somewhat flattened cells. No preantral follicles were detected (Figs. 1E and 1G). In sections of cryopreserved ovarian tissue, some areas were cryodamaged by the vitrification/warming (Fig. 1F, Arrows), but areas with good preservation were found that showed a similar morphology to the fresh ovaries (Fig. 1H).

The number of morphologically normal oocytes per square millimeter was evaluated from histological sections (Fig. 2). There was no significant difference between fresh and vitrified/warmed ovaries (504.8 ± 112.6 vs. 394.8 ± 62.1, P=0.4402).

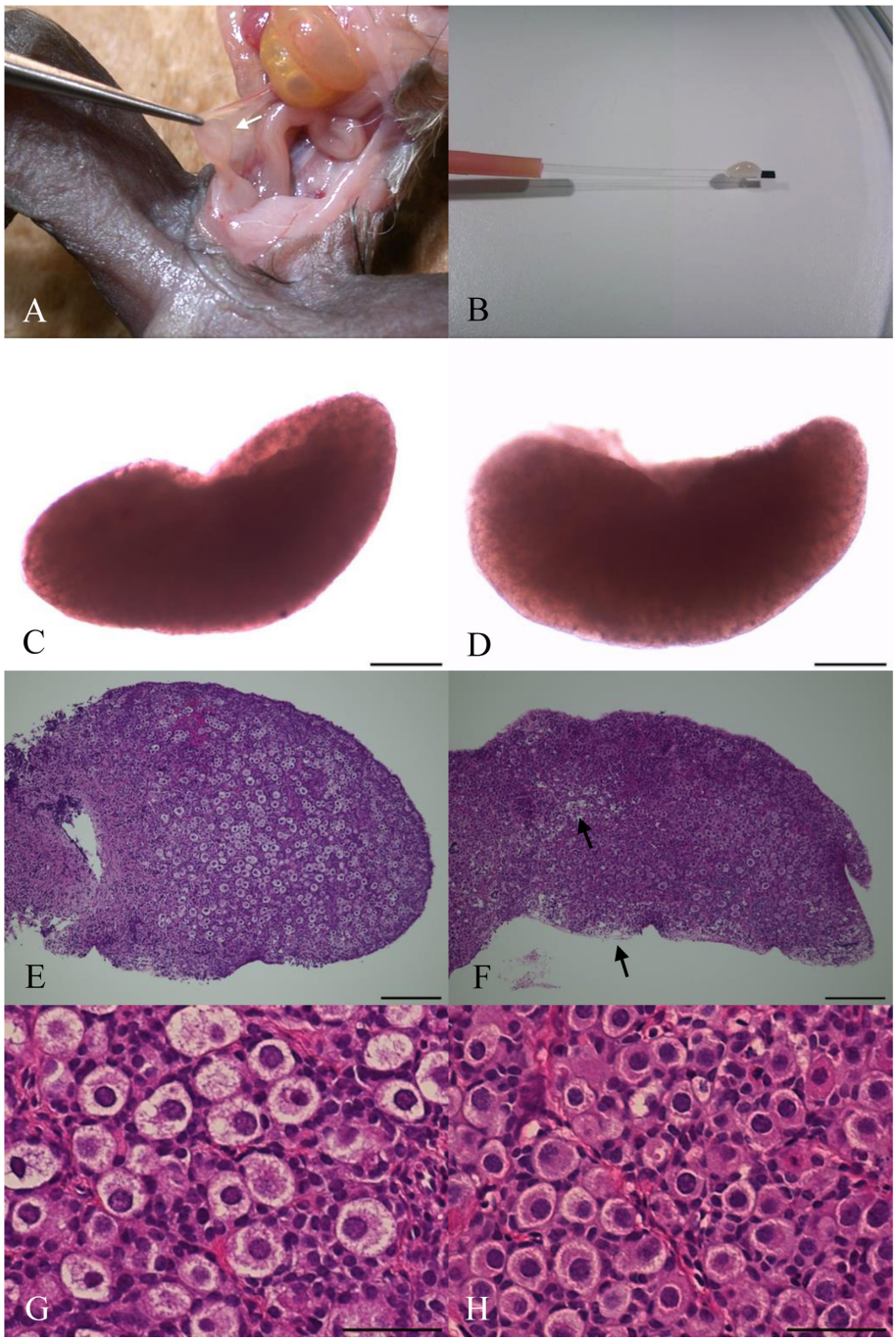

Fig. 1. The morphology and histology of fresh and vitrified/warmed ovaries from female neonatal marmosets. (A) The reproductive organs of a neonate. Arrow: an ovary. (B) An ovary placed on a cryotop. (C) The appearance of a fresh ovary. (D) The appearance of a vitrified/warmed ovary. (E) A histological section of a fresh ovary. (F) A histological section of a vitrified/warmed ovary. Arrows: cryodamaged areas. (G) An enlarged view of an area in E. (H) An enlarged view of the preserved area in F. The scale bars present 500 μm in (C, D), 200 μm in (E, F), or 50 μm (G, H).

Ovarian cell viability

The influence of vitrification/warming on the cell viability is shown in Fig. 3. The survival rate of cells from vitrified/warmed ovaries was significantly lower than that of the cells from fresh ovaries (86.0 ± 0.9 vs. 69.1 ± 6.3, P=0.0498).

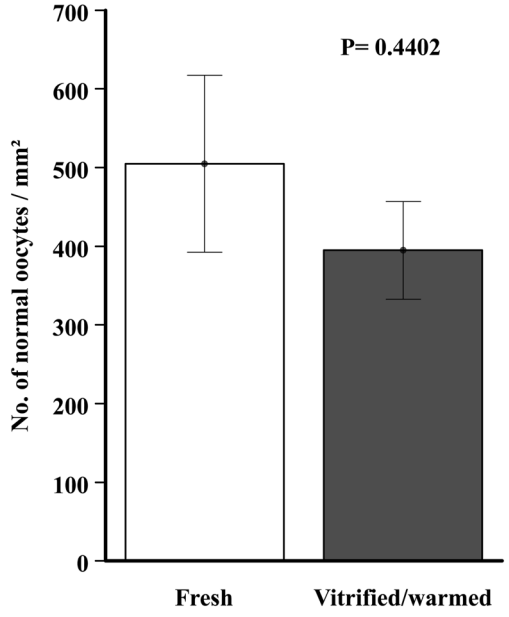

Fig. 2. The effects of vitrification/warming on the number of morphologically normal oocytes per unit area in the ovaries from female neonatal marmosets.

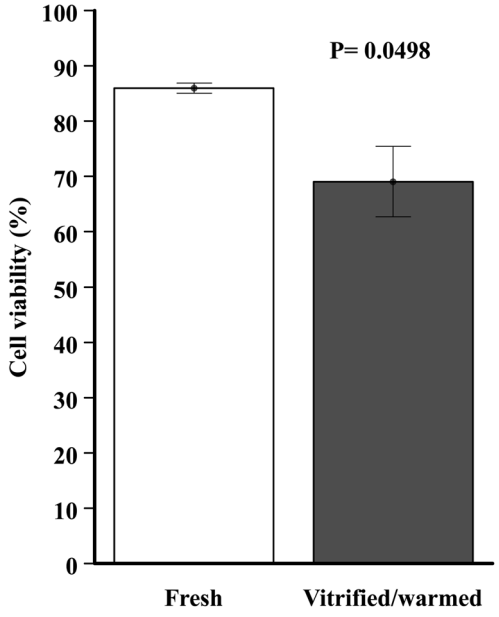

Fig. 3. The effects of vitrification/warming on the cell viability in the ovaries from female neonatal marmosets.

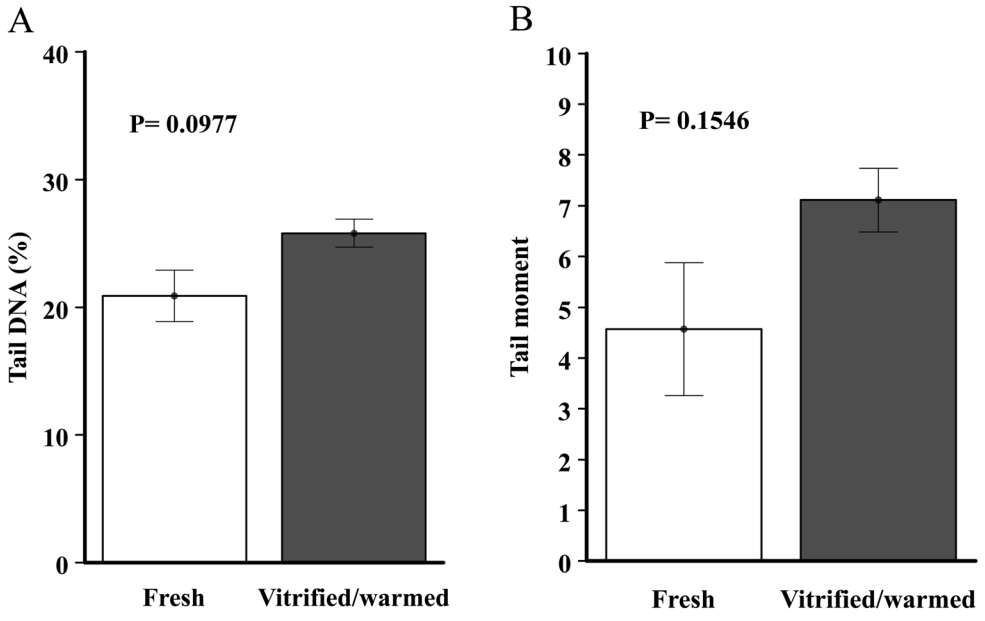

Fig. 4. The effects of vitrification/warming on the comet assay parameters of ovarian cells. (A) The percentages of tail DNA. (B) The tail moment.

Comet assay

The comet assay was performed to determine the extent of genomic DNA damage. The results of two different comet parameters are shown in Fig. 4. The comet parameter of the DNA, the percentage of DNA in the tail, was not significantly different between fresh and vitrified/warmed ovaries (20.9 ± 3.5 vs. 25.8 ± 1.9, P=0.0977, Fig. 4A). The tail moment parameters calculated from the mean values of each experiment were 4.6 ± 2.3 in the fresh group and 7.1 ± 1.1 in the vitrified/warmed group, which were also not significantly different (P=0.1546, Fig. 4B).

Discussion

To date, ovarian cryopreservation has been applied to mice [28, 39], humans [29] and other animals [25]. Initially, slow-freezing methods were utilized for the cryopreservation of ovarian tissues, and later, a rapid and simple cryopreservation method, vitrification, was tried. The Cryotop method is widely used as an ultra-rapid cooling "vitrification" system for preserving oocytes and embryos [15]. This method has also been applied to ovarian tissue cryopreservation, with modifications of the equilibration procedure. In mice, live pups were obtained through *in vivo* transplantation or *in vitro* oocyte growth after ovaries were vitrified/warmed using this method [9, 13]. The ovaries of neonatal marmosets resemble those of prepubertal mice in terms of tissue size and softness, which influence the permeability of the tissues to cryoprotectants. Our results indicate that this vitrification method using the Cryotop method and cryoprotectants (EG, DMSO, and sucrose) may be effective for cryopreservation of ovaries from neonatal marmosets, but carefull optimization of protocols is needed for maximum viability after thawing.

The comet assay, a single cell gel electrophoresis assay, is a simple and sensitive technique for detecting DNA damage at the level of the individual cell. The alkaline version of the comet assay enables detection of single- and double-strand breaks in single cells [30]. This technique has also been used to assess the cryodamage of the genomic DNA of cumulus cells [18], oocytes [2, 20, 27, 31], and sperm [16]. In addition, it is also possible to analyze the damage in cryopreserved somatic tissues [11, 23]. Therefore, the comet assay is useful for evaluating the cryodamage of cells following ovarian cryopreservation.

During the fetal stage in mammals, mitotically primordial germ cells develop into oocytes that enter the meiotic cell cycle and then are arrested at prophase I. Near the time of birth in rodents, and during mid-gestation in humans, the oocytes are enclosed by a single layer of flattened epithelial cells (progenitors of follicular granulosa cells). These oocytes and their surrounding follicles are called primordial oocytes and primordial follicles, respectively [21]. In the neonatal marmoset, numerous primordial oocytes (non-growing oocytes) are stored as primordial follicles or naked oocytes in the ovaries. Furthermore, the existence of primitive oogonia in the ovarian surface epithelium can be revealed by detecting markers of pluripotency and proliferation [6]. In breeding, many marmosets die before or soon after birth. Ovarian tissue cryopreservation is useful for rescuing female germ cells. One of the methods for recovery of animals is xenograft into immunodeficient mice. If oocytes are fully grown, it is possible to produce the offspring using *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo transfer (ET) [13].

In conclusion, cryopreservation of neonatal marmoset ovaries using vitrification will likely represent a useful storage system for whole ovaries. The results of this study are considered to provide useful information on the preservation of reproductive (genetic) resources for nonhuman primates.

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