Fertility of mice receiving vitrified adult mouse ovaries

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Abstract

Cryopreservation of the ovaries is a useful technology for preservation of germ cells from experimental animals, because if the female founder is infertile or has mutated mitochondrial DNA, preservation of female germ cells is necessary. Although it is possible to cryopreserve immature mouse ovaries with a high degree of viability by vitrification with a mixture of several cryoprotectants, the viability of cryopreserved adult mouse ovaries is still unknown. Here, we investigated the viability of mouse ovaries at various ages after cryopreservation by vitrification techniques. Donor ovaries were collected from 10-day-, 4-week-, 10-week- and 7-month-old, female, nulliparous, green fluorescence protein (GFP)-transgenic mice and cryopreserved by vitrification. The vitrified-warmed ovaries were orthotopically transplanted to 4- or 10-week-old mice. GFP-positive pups were obtained in all experimental groups. In the 4-week-old recipients, the percentages of GFP-positive pups among the total pups from recipients transplanted with ovaries of 10-day-, 4-week-, 10-week- and 7-month-old donors were 44%, 9%, 12% and 4% respectively. In the 10-week-old recipients, the percentages of GFP-positive pups among the total pups from recipients transplanted with ovaries of 10-day-, 4-week-, 10-week- and 7-month-old donors were 36%, 16%, 2% and 9% respectively. Furthermore, GFP-positive pups also were obtained from recipients transplanted with ovaries of donors without normal estrous cyclicity. Our results indicate that cryopreservation of mouse ovaries by vitrification is a useful method for the preservation of female germ cells from mice of various ages.

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Introduction

Mutant or transgenic animals occasionally suffer from infertility. Therefore, the preservation of germ cells is a useful technology for maintenance or propagation of the experimental animal lines. Cryopreservation of spermatozoa, of which a large number from a few males are easily preserved, has been widely used to archive mouse strains (Okamoto *et al.* 1988, Nakagata *et al.* 1996, Nakagata *et al.* 1997). For reproduction, it is necessary to preserve female germ cells as well if the female founder is infertile or has mutated mitochondrial DNA (Inoue *et al.* 2002, Silva & Larsson 2002). Although the preservation of female germ cells and mitochondrial DNA can be accomplished through egg preservation, preservation of immature oocytes is the most effective method for those animals that do not respond to hormonal stimulation or that die unexpectedly. However, the oocyte–granulosa cell complex is required for the growth and maturation of oocytes (Richards *et al.* 1987, Matzuk *et al.* 2002). Therefore, it is necessary to preserve the ovaries and/or follicles and maintain their structure.

Ovaries from young mice that have been dissected and halved are cryopreserved by either the equilibrium freezing method (Cox *et al.* 1996, Gunasena *et al.* 1997, Sztejn *et al.* 1998, 1999, Candy *et al.* 2000, Shaw & Trounson 2002) or the vitrification method (Kagabu & Umezū 2000, Takahashi *et al.* 2001, Migishima *et al.* 2003). Recently, a new cryopreservation method for 10-day-old mouse ovaries by vitrification was reported (Migishima *et al.* 2003) using DAP213 (Nakagata 1989), a combination of the cryoprotectants dimethyl sulfoxide (DMSO), acetamide and propanediol in concentrations of 2, 1 and 3 mol/l respectively. It is the first report on the vitrification of whole ovaries. Additionally, this method has had a high success rate in mouse production via ovary transplantation. However, the viability of vitrified-warmed adult mouse ovaries is still unknown for this method.

In the present study, we investigated the viability of mouse ovaries of various ages after vitrification and the potential of this technology as a method for the preservation of female germ cells.

Materials and Methods

Donor ovaries

Donor ovaries were collected from 10-day-, 4-week-, 10-week- and 7-month-old, female, nulliparous, green fluorescence protein (GFP)-transgenic mice (genetic background: C57BL/6J Jcl). Donor ovaries were aseptically placed in disposable Petri dishes containing Whitten's medium (Whitten 1971). Vaginal smear samples were taken from all 7-month-old donor mice daily for 7 days before donation to determine their estrous cyclicity. Vaginal cell specimens were spotted onto glass slides by gently pipetting with a small amount of water and left to dry for a few minutes. After Giemsa staining, the specimens were classified into one of the following four categories: 1. proestrous, 2. estrous, 3. metoestrous and 4. diestrous. Seven-month-old donor mice that were classified as diestrous during their estrous cyclicity were regarded as donor mice without normal estrous cyclicity.

Ovary cryopreservation and warming

Ovaries isolated from the 4-week-, 10-week- and 7-month-old mice were cut into fourths (about 1 mm^3) to approximate the size of the ovaries of a 10-day-old mouse (Fig. 1). The ovaries were cryopreserved by vitrification with DAP213 (2 mol/l DMSO, 1 mol/l acetamide and 3 mol/l propanediol in PBI medium (Whittingham 1974)), as described by Migishima *et al.* (2003). Isolated ovaries were pretreated with $40\text{ }\mu\text{l}$ of 1 mol/l DMSO in PBI medium at room temperature for 5 min. This step was repeated twice. The ovaries were then transferred into 1 ml cryotubes (Nalge Nunc International KK, Tokyo, Japan) containing $5\text{ }\mu\text{l}$ of 1 mol/l DMSO in PBI medium. The cryotubes were placed in a 0°C Labtop cooler

(Nalge Nunc International KK, Tokyo, Japan) for 5 min. Then $95\text{ }\mu\text{l}$ DAP213 kept at 0°C were added to each cryotube, and the cryotubes were placed in the 0°C Labtop cooler for 5 min. After exposure to DAP213, the cryotubes were plunged directly into liquid nitrogen and stored. For warming, cryotubes containing the samples kept in liquid nitrogen were diluted with PBI medium containing 0.25 mol/l sucrose kept at 37°C . The recovered ovaries were transferred to the PBI medium for washing twice and then transferred to Whitten's medium before transplantation.

Ovary transplantation

Fresh or vitrified-warmed isolated ovaries were transplanted into 4- or 10-week-old female non-GFP C57BL/6J Jcl mice (CLEA Japan, Tokyo, Japan) by the transplantation procedure described by Migishima *et al.* (2003). Briefly, recipients were anesthetized with 10% sodium pentobarbital (Dainippon Pharmaceutical Co., Osaka, Japan) by i.p. injection. A single transverse incision of the skin at the dorsal, across the lumbar area, gave access to the ovaries on both sides. A small slit was made in the fat surrounding the ovarian bursa to expose the ovary. Half of the recipient's ovarian tissues were removed and a fresh or a vitrified-warmed ovary from a donor mouse was orthotopically transplanted into the respective ovarian bursa of a non-GFP recipient. Then, the small slit in the fat surrounding the ovarian bursa was closed by applying pressure with tweezers. One of the ovaries from a 10-day-old donor mouse was orthotopically transplanted into the respective ovarian bursa of a non-GFP recipient. Each quarter piece of ovary, from the 4-week-, 10-week- and 7-month-old donor mice was orthotopically transplanted into the respective ovarian bursa of non-GFP recipients.

Figure 1 Ovaries of GFP-transgenic mice at various ages. Ovaries isolated from 4-week-, 10-week- and 7-month-old mice were cut into fourths (about 1 mm^3), similar to the size of the ovaries of a 10-day-old mouse, to approximate the same permeability by DAP213. Bars: 1 mm.

The ovarian complex was replaced in the body cavity, and the incision was closed with wound clips (auto clip 9 mm; Nihon Becton Dickinson, Tokyo, Japan). Three weeks after ovary transplantation, the recipient mice were mated with C57BL/6J Jcl mice. Recipients in whom pregnancy was not detected during 8 weeks of pairing were regarded as infertile. The numbers of total pups, live pups and pups from donor ovaries that showed green fluorescence under ultraviolet light were counted.

Statistical analyses

Data presented in this study were analyzed statistically by the chi-square test and Tukey test for nonparametric multiple comparisons. In all statistical tests, a difference was considered significant when the two-tailed *P* value was <0.05.

All mice were housed in a controlled environment of light/dark (light 0500–1900 h), temperature ($24 \pm 1^\circ\text{C}$), and humidity ($50\% \pm 10\%$) with free access to standard laboratory chow (CE-2; CLEA Japan). The Animal Care and Use Committee of Chugai Pharmaceutical (Shizuoka, Japan) reviewed the protocols and confirmed that the animals used in the present study were cared for and used humanely.

Results

Fertility of recipients transplanted with fresh donor ovaries

All recipients transplanted with fresh ovaries gave birth to live pups with an average litter size normal for nontreated C57BL/6J Jcl mice (Table 1). Additionally, all of the experimental groups showed GFP-positive pups. The percentage of GFP-positive pups among the total pups delivered from 4-week-old recipients transplanted with ovaries from 4-week-old donors (61%) was significantly higher than that of 10-week-old recipients transplanted with ovaries

from 4-week-old donors (22%). There was no significant difference between the percentages of GFP-positive pups among the total pups delivered from 4- and 10-week-old recipients transplanted with ovaries from 10-week- or 7-month-old donors. For two of the ten 7-month-old donors, estrous cyclicity was not observed for at least 7 consecutive days from the vaginal smear test. GFP-positive pups were born to one of the two 4-week-old recipients and to the two 10-week-old recipients transplanted with ovaries from 7-month-old donors without normal estrous cyclicity.

The reproductive efficiency for the various combinations of recipient age and donor age was calculated by the following formula: reproductive efficiency = no. of GFP-positive pups / no. of donor mice used for transplantation. The reproductive efficiency of 4-week-old recipients transplanted with ovaries from 4-week-old donors (18.2) was nearly threefold higher than that of the 10-week-old recipients transplanted with ovaries from 4-week-old donors (6.4) (Fig. 2). The reproductive efficiency of 4-week-old recipients transplanted with ovaries from 10-week-old donors (15.1) was similar to the reproductive efficiency of 10-week-old recipients transplanted with ovaries from 10-week-old donors (13.1).

All recipients delivered GFP-positive pups for three litters. The cumulative GFP-positive pup birthrate including the third litter from recipients transplanted with the ovaries from 10-day-, 10-week- and 7-month-old donors showed similar rates for 4- and 10-week-old recipients (Fig. 3). GFP-positive pup birthrate per litter of 4-week-old recipients transplanted with ovaries from 4-week-old donors maintained a rate higher than that of 10-week-old recipients transplanted with ovaries from 4-week-old donors.

Fertility of recipients transplanted with vitrified-warmed donor ovaries

The percentage of impregnated recipients and the average litter size were not significantly different between

Table 1 Viability of transplanted fresh ovaries.

Experimental group		Recipients no.	Pregnancies no. (%)	Recipients bearing GFP-positive pups no. (%) [*]	Pups born total no.	Average litter size	GFP-positive pups among pups born no. (%) ^{**}
Donor	Recipient						
10-day-old		6	6 (100)	6 (100)	42	7.0	35 (83) ^{all}
4-week-old	4-week-old	14	14 (100)	13 (93)	96	6.9	59 (61) ^{abff}
10-week-old		15	15 (100)	15 (100)	101	6.7	55 (54) ^b
7-month-old		10	10 (100)	7 (70)	55	5.5	10 (18) ^c
10-day-old		5	5 (100)	5 (100)	26	5.2	14 (54) ^{abdll}
4-week-old		20	20 (100)	15 (75)	143	7.2	31 (22) ^{cff}
10-week-old	10-week-old	14	14 (100)	13 (93)	109	7.8	46 (42) ^{bd}
7-month-old		10	10 (100)	8 (80)	73	7.3	22 (30) ^{cd}

Fresh ovaries were transplanted into a recipient, grafted into the respective ovarian bursa.

^{*}Calculated from the number of pregnant recipients.

^{**}Calculated from the number of pups born.

^{a,b,c,d}Significant difference ($P < 0.05$) by Tukey's test.

Values with different superscripts are significantly different in the same parameter and recipient age.

^{ll}Significantly different ($P < 0.05$) by chi-square test.

Figure 2 Reproductive efficiency in mice with fresh ovary transplantation. Reproductive efficiency was based on the number of GFP-positive pups from a recipient's first delivery and calculated by the formula previously given. In 4-week-, 10-week- and 7-month-old donors, four recipients were used per donor mouse. Infertile recipients were included in the total number of recipients.

experimental groups of vitrified-warmed donor ovaries (Table 2). GFP-positive pups were obtained in all experimental groups. Additionally, the percentage of GFP-positive pups among the total pups delivered did not significantly differ for either 4- or 10-week-old recipients among all ages of donors tested. No estrous cyclicity was observed for at least 7 consecutive days in the vaginal smear test in two of the ten 7-month-old donors. GFP-positive pups were born to one of the two 10-week-old recipients transplanted with the ovaries from 7-month-old donors without normal estrous cyclicity.

The reproductive efficiency of the vitrified-warmed donors was calculated by the same equation as for fresh donors. The reproductive efficiency was not greatly different among recipients transplanted with ovaries from all ages tested (Fig. 4).

Figure 3 GFP-positive pup birthrate per three litters of recipients transplanted with fresh ovaries. GFP-positive pup birthrate for recipients transplanted with fresh ovaries from 10-day- (○), 4-week- (□), 10-week- (●) and 7-month-old (■) donors. Percent birthrate of GFP-positive pups = (no. of GFP-positive pups / total no. of pups born) × 100.

With the exception of recipients transplanted with ovaries from 10-day-old donors, the GFP-positive pup birthrate decreased greatly after the second litter (Fig. 5).

Discussion

The birth of live offspring after orthotopic transplantation of frozen-thawed mouse ovarian tissues has been widely reported (Cox *et al.* 1996, Gunasena *et al.* 1997, Sztejn *et al.* 1998, 1999, Candy *et al.* 2000, Takahashi *et al.* 2001, Shaw & Trounson 2002, Migishima *et al.* 2003), and the technology of vitrification is expected to be useful for the simple and rapid preservation of female germ cells in experimental animals. However, only a limited number of reports on the birth of live offspring after transplantation of ovaries cryopreserved by vitrification have been published (Takahashi *et al.* 2001, Migishima *et al.* 2003) compared with reports on the equilibrium freezing method (Cox *et al.* 1996, Gunasena *et al.* 1997, Sztejn *et al.* 1998, 1999, Candy *et al.* 2000, Shaw & Trounson 2002). Moreover, there has been only one report on successful cryopreservation by vitrification of adult mouse ovaries (Takahashi *et al.* 2001). For the experiments of Takahashi and his colleagues, the vitrification method of Rall and Fahy (1985) with VS1, a combination of the cryoprotectants 20.5% (w/v) DMSO, 15.5% (w/v) acetamide, 10% (w/v) propylene glycol and 6% (w/v) polyethylene glycol was used. However, this method requires many steps of vitrification and warming. DAP213 was developed from VS1 in order to simplify the procedure of vitrification for mouse oocytes and embryos. Although the toxicity of DAP213 in ovarian cells is unknown, a high degree of viability after orthotopic transplantation of cryopreserved immature mouse ovaries by vitrification with DAP213 has been reported (Migishima *et al.* 2003). In using this vitrification method to preserve germ cells, it is important to estimate the viability of cryopreserved adult mouse ovaries. If cryopreservation of adult mouse ovaries by

Table 2 Viability of transplanted vitrified-warmed ovaries.

Experimental group		Recipients no.	Pregnancies no. (%)	Recipients bearing GFP-positive pups no. (%)*	Pups born total no.	Average litter size	GFP-positive pups among pups born no. (%)**
Donor	Recipient						
10-day-old	4-week-old	5	5 (100)	4 (80)	32	6.4	14 (44) ^a
4-week-old		13	13 (100)	6 (46)	79	6.1	7 (9) ^b
10-week-old		12	10 (83)	4 (40)	67	6.7	8 (12) ^{bc}
7-month-old		10	8 (80)	2 (25)	55	6.9	2 (4) ^b
10-day-old	10-week-old	5	5 (100)	3 (60)	25	5.0	9 (36) ^{ac}
4-week-old		13	11 (85)	5 (45)	64	5.8	10 (16) ^{ab}
10-week-old		10	9 (90)	1 (11)	63	7.0	1 (2) ^b
7-month-old		9	5 (56)	2 (40)	33	6.6	3 (9) ^{bc}

Vitrified-warmed ovaries were transplanted into a recipient and grafted into the respective ovarian bursa.

*Calculated from the number of pregnant recipients.

**Calculated from the number of pups born.

^{a,b,c}Significant difference ($P < 0.05$) by Tukey's test.

Values with different superscripts are significantly different in the same parameter and recipient age.

vitrification is successful, it would be a valuable technology not only for reproduction, but also for archiving female germ cells and maintaining endangered mouse strains. In the present study, we compared the viability of cryopreserved adult mouse ovaries with that of immature ovaries. Our results indicate that adult as well as immature mouse ovaries cryopreserved by vitrification with DAP213 are viable for producing young. Further studies are necessary to investigate the effects from the number of donor ovaries transplanted into a recipient and the amount of cryoprotectant remaining in the ovarian tissue on the fertility of mice receiving donor ovaries.

Although GFP-positive pups were obtained from all vitrified-warmed donor ovaries examined in this study, the percentages of GFP-positive pups among the total pups delivered from recipients were lower than from fresh ovaries in all combinations of recipient age and donor age (Tables 1 and 2). These data indicate that vitrification and warming the ovaries reduced their viability, consistent with the results Migishima *et al.* (2003) reported, using

ovaries from 10-day-old, immature mice as grafts. This tendency showed especially when 4-week-, 10-week- and 7-month-old mice were used as donors (Tables 1 and 2). It is possible that the wide range of developmental stages of follicles in adult mouse ovaries is one of the causes of this tendency. Previous reports describing ovarian histology after freeze-thawing concluded that large mature follicles are more affected by freezing injuries and small immature follicles have a higher survival rate (Parrot 1960, Smith 1961, Gosden 1992, Harp *et al.* 1994, Cox *et al.* 1996, Candy *et al.* 1997). The number of large follicles that survive after freeze-thawing has been estimated to be about 5% of the total surviving follicles (Green *et al.* 1956). Additionally, analysis of the effect of cryoprotectants on follicle survival after freezing demonstrated that 81–94% of primordial follicles survived when DMSO was used as the cryoprotectant (Candy *et al.* 1997). In spite of a reduction in the number of donor-derived pups, the present results indicate that follicles in adult mouse ovaries are viable even after vitrification and develop normally

Figure 4 Reproductive efficiency in mice with frozen-thawed ovary transplantation. Reproductive efficiency was based on the number of GFP-positive pups from a recipient's first delivery and calculated by the formula previously given. In 4-week-, 10-week- and 7-month-old donors, four recipients were used per donor mouse. Infertile recipients were included in the total number of recipients.

Figure 5 GFP-positive pup birthrate per three litters of recipients transplanted with frozen-thawed ovaries. GFP-positive pup birthrate for recipients transplanted with frozen-thawed ovaries from 10-day- (○), 4-week- (□), 10-week- (●) and 7-month-old (■) donors. Percent birthrate of GFP-positive pups = (no. of GFP-positive pups / total no. of pups born) × 100.

after orthotopic transplantation into recipients. Additionally, GFP-positive pups were born to recipients transplanted with ovaries from 7-month-old donors without normal estrous cyclicity for at least 7 consecutive days, whether the ovaries of donors were fresh or cryopreserved. Therefore, it seems that follicles in ovaries from mice without normal estrous cyclicity also have the capability to develop normally even after vitrification and warming. However, further studies are required to elucidate the involvement of estrous cyclicity.

The most efficient reproduction was observed in 4-week-old recipients transplanted with fresh ovaries from 4-week-old donors with an efficiency rate nearly threefold higher than that of 10-week-old recipients transplanted with fresh ovaries from 4-week-old donors (Fig. 2). However, there was no significant difference in reproductive efficiency between 4- and 10-week-old recipients transplanted with fresh ovaries from 10-week-old donors. In recipients transplanted with fresh ovaries from 10-day- and 4-week-old donors, the percentage of GFP-positive pups among the total pups delivered from 4-week-old recipients was significantly higher than from 10-week-old recipients (Table 1). Additionally, GFP-positive pup birthrate per three litters of 4-week-old recipients transplanted with fresh ovaries from 4-week-old donors maintained a rate higher than that of 10-week-old recipients transplanted with fresh ovaries from 4-week-old donors. And recipients transplanted with ovaries from 10-day-, 10-week- and 7-month-old donors had almost the same birthrate whether the recipients were 4- or 10-week-old mice (Fig. 3). This suggests that 10-week-old recipients may have some negative effects on fresh ovarian grafts from 10-day- and 4-week-old donors. Thus, 4-week-old mice would be more suitable recipients for producing young via fresh ovary transplantation.

The above-mentioned negative effects of fresh ovarian grafts of young mice were not seen in 10-week-old recipients transplanted with vitrified-warmed ovaries (Fig. 4). Migishima *et al.* (2003) reported no difference between the percentages of donor ovarian oocytes among total oocytes collected from recipients transplanted with fresh ovaries and recipients transplanted with vitrified-warmed ovaries; however, the number of oocytes collected per recipient transplanted with vitrified-warmed ovaries was about one-third that per recipient transplanted with fresh ovaries. If the ovaries of donors are affected by cryopreserving injuries, the percentage of donor ovarian oocytes of total oocytes collected from recipients transplanted with vitrified-warmed ovaries would be lower than from recipients transplanted with fresh ovaries. Thus, cryoprotectants remaining in the ovarian tissue after warming and washing might have some effect on the recipients locally or systemically as well as on the viability of the graft itself. The reduced pregnancy rate for recipients with vitrified-warmed ovaries may also have been a result of such effects (Table 2).

Both adult and immature mouse ovaries cryopreserved by vitrification with DAP213 are viable for producing young, and follicles in ovaries of adult mice without normal estrous cyclicity also have the capability to develop normally even after vitrification and warming. Our results indicate that cryopreservation of mouse ovaries by vitrification with DAP213 is a useful method for preservation of female germ cells from mice at various ages. However, after a second litter, few pups were delivered from ovaries of donors from recipients transplanted with vitrified-warmed ovaries from adult mice. Further studies are necessary to improve ovary cryopreservation by vitrification in order.

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