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Successful Cryopreservation of Mouse Ovaries by Vitrification[1]

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ABSTRACT

We developed a new method of cryopreservation of whole ovaries by vitrification using DAP213 (2 M dimethyl sulfoxide, 1 M acetamide, and M propylene glycol) as a cryoprotectant. Four-week-old C57BL/6 mice that underwent partial ovariectomy were orthotopically transplanted with cryopreserved or fresh ovaries (experimental or control group) isolated from 10-day-old green fluorescent protein (GFP)-transgenic mice (+/+). GFP-positive pups were similarly obtained from both groups by natural mating or in vitro fertilization (IVF) followed by embryo transfer, indicating that the cryopreserved ovaries by vitrification retain their fecundity. However, a statistically significant difference ($P < 0.05$) was found between both groups with respect to the following parameters: the number of GFP-positive pups born by natural mating/grafted ovary (0.8 ± 0.3 for the experimental group versus 2.0 ± 0.7 for the control group, mean ± SEM), the number of collected oocytes by superovulation per mouse (7.0 ± 1.7 for the experimental group versus 22.7 ± 3.2 for the control group), the percentage of two-cell embryos obtained from GFP-positive oocytes by IVF (38.5% for the experimental group versus 90.0% for the control group). Histologically, normal development of follicles and formation of corpora lutea were observed in frozen-thawed grafts. However, estimated number of follicles decreased in frozen-thawed ovaries compared with fresh ovaries. Taken together, cryopreservation of the ovary by vitrification seems a promising method to preserve ovarian function, but further studies are required to overcome the possible inhibitory effects of this method on the growth of the ovarian graft.

in vitro fertilization, ovary, ovulation, ovum pick-up/transport, pregnancy

INTRODUCTION

The cryopreservation of ovarian tissue is potentially a useful technology for preservation of genetic resources of experimental, domestic, and wild animals. Moreover, it is employed in clinical medicine to restore the fecundity of young women suffering from infertility and premature menopause due to iatrogenic loss of ovarian function resulting from chemotherapy and/or radiation therapy of malignant neoplasms. Parrot reported the birth of live offspring after orthotopic transplantation of a slice of cryopreserved mouse ovary [1]. Since then, various cryoprotectants have been developed [2–7], and slow freezing methods to control freezing rate have been employed for the cryopreservation of ovaries.

The vitrification method was applied to the field of biology as a method of cryopreservation, and its recent development has greatly simplified the cryopreservation procedures. Vitrification can essentially be defined as the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling [8]. Extensive studies have proven the usefulness of this method for cryopreservation of oocytes and embryos. The survival rates of oocytes and embryos after the freezing-thawing procedure by vitrification are comparable with those by slow freezing methods [9].

Although the technical simplicity of vitrification has revolutionized cryopreservation of oocytes and embryos, its application to whole ovaries has been considered to be difficult. In the present study, we examined whether ovaries retain their fecundity after cryopreservation by vitrification utilizing DAP213 solution [10] (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol) as a cryoprotectant. For this purpose, we investigated the fertility of mice that had undergone orthotopic transplantation of the cryopreserved ovaries by natural mating and in vitro fertilization (IVF) followed by embryo transfer (ET). In order to distinguish graft-derived pups and oocytes and embryos from those derived from residual host ovarian tissue, we employed jellyfish green fluorescent protein (GFP)-transgenic mice [11] that express the GFP transgene in the entire body as sources of ovarian grafts.

MATERIALS AND METHODS

Animals

Female and male C57BL/6 and female ICR mice were purchased from CLEA Japan Inc. (Tokyo, Japan). GFP-transgenic C57BL/6TgN(act-EGFP) OsbY01 mice (+/+) [11], referred to as GFP-transgenic mice in this article, were kindly provided by Dr. Okabe of the Genome Information Research Center at Osaka University and were bred in our animal facilities under specific pathogen-free conditions. Mice were housed under a 12L: 12D regimen at 22°C and 55% humidity. Food and water were freely available at all times. All the experiments using animals were in accordance with the International Guiding Principle for Biomedical Research Involving Animals and the experimental protocol was approved by the Ethics Committee for Experimental Animals of our institute.

Collection of GFP+ Ovaries

In vitro fertilization and embryo transfer were performed to obtain the offspring of GFP-transgenic mice (+/+). Briefly, female homozygous GFP-transgenic mice were superovulated by injections of 5 IU pregnant mare serum gonadotropin (serotropin) and 5 IU hCG (gonatropin), obtained from Teikoku Hormone Mfg. Co., Ltd. (Tokyo, Japan), at 48-h

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intervals. The oocytes were obtained 15–16 h after hCG injection. Spermatozoa of male homozygous GFP-transgenic mice were collected from the cauda epididymis and suspended in TYH medium [12]. After 1 h, an aliquot of sperm suspension was added to TYH medium containing oocytes to make the final sperm concentration 150 cells/μl. Twenty-four hours after insemination, two-cell embryos were obtained, washed with Whitten medium [13], and then transferred into the oviducts of ICR mice prepared as pseudopregnant recipients.

Ten-day-old female homozygous GFP-transgenic mice born from the recipients of ICR mice were killed by cervical dislocation. Whole ovaries were aseptically removed and placed in 35 × 10-mm disposable Petri dishes (Becton Dickinson, Franklin Lakes, NJ) containing 3 ml of Whitten medium kept at 0°C.

Freezing and Thawing of Ovaries

We modified the simple vitrification procedure of Nakao et al. [14]. Briefly, two to four isolated whole ovaries were first pretreated with PBI medium [15] containing 1 M dimethyl sulfoxide (DMSO) at room temperature. Five microliters of the medium containing the ovaries was then transferred into a 1-ml cryotube (Nalge Nunc International KK, Tokyo, Japan), which was then placed in ice water for 5 min to allow DMSO to thoroughly permeate the ovaries. Then 95 μl of DAP 213 solution kept at 0°C was added to each cryotube. After placing the cryotubes in ice water (0°C) for 5 min, they were plunged directly into liquid nitrogen and stored for 2–7 days until use. The samples were taken from the liquid nitrogen and allowed to stand at room temperature for 30 sec, then diluted with 900 μl of PBI medium (37°C) containing 0.25 M sucrose. The recovered ovaries were transferred by pipetting to the PBI medium for washing, then to Whitten medium; both media were kept at 0°C.

Transplantation of Ovaries

We orthotopically transplanted frozen-thawed ovaries of 10-day-old female homozygous GFP-transgenic mice to 4-wk-old female C57BL/6 mice. Freshly isolated ovaries were used as control grafts. We employed the transplantation procedure of Behringer [16], with some modifications. Recipients were anesthetized with an i.p. injection of sodium pentobarbital (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) diluted at 1:10 (final concentration: 5 mg/ml) with isotonic sodium chloride solution (FUSO Pharmaceutical Industries Ltd., Osaka, Japan). The following surgical procedures were performed aseptically.

A single median longitudinal skin incision was made on the lumber portion to expose the subcutaneous tissue over the ovary. A small incision was made on the fascia and muscles immediately above the ovary, thereby exteriorizing the recipient's reproductive tract. One or two drops of 1:20 diluted epinephrine (final concentration: 50 μg/ml; Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan) with isotonic sodium chloride solution were applied to the ovarian bursa to prevent bleeding. A small slit was made on the ovarian bursa to expose the ovary and both of the recipient's ovaries were removed. A small portion (about 10%) of the recipient's ovarian tissue was left in situ in order to facilitate regeneration of blood supply into the grafts. A fresh or a frozen-thawed ovary isolated from a 10-day-old mouse was then inserted into each ovarian bursa of both sides. The bursal membrane was closed with tweezers without suturing, then the reproductive tract was returned to the abdominal cavity. The skin incision was closed with clips (9-mm auto clips; Becton Dickinson).

The ovarian transplant experiments were carried out by two persons. One performed the operation, while the other assisted by assigning each mouse to the experimental or control group and passing the ovarian graft to the operator. Only the assistant knew if each graft was a frozen-thawed or a fresh ovary. The assistant was not provided any information at all by the operator about the condition of surgical procedures. Thus, the surgeries were performed randomly and in a technically blind manner.

Mating and Recording

The recipient female mice were allowed to recover for 3 wk after the surgery and were then paired with male C57BL/6 mice. When mating was confirmed by the presence of a vaginal plug, the females were housed individually. If no vaginal plug was detected, pairing was continued for up to 8 wk. The numbers of recipients that gave birth to pups and of pups born alive were recorded. The pups were observed under 360-nm ultraviolet light using a hand-held illuminator (Ultra-Violet Products Ltd., Cambridge, UK). Although GFP requires a blue light (488 nm) for excitation, its fluorescence is visible to the naked eye under ultraviolet light [11].

In Vitro Fertilization and Embryo Transfer

Three weeks after weaning, the recipients that had given birth to GFP+ pups were superovulated and then IVF using C57BL/6 sperm was performed using the same procedure as described above. Two-cell embryos obtained were transferred into the oviducts of ICR mice prepared as pseudopregnant recipients. The numbers of two-cell embryos and of pups born alive were recorded.

Histological Examination

Recipients were killed by cervical dislocation 4 mo after transplantation of the ovaries. The ovary and the surrounding tissues in situ were observed under a stereomicroscope (Leica, Wetzlar, Germany) with 480-nm blue light. The ovaries were isolated and fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.2) at 4°C for 24 h. After washing with 0.1 M PB, the ovaries were immersed in 20% (w/v) sucrose in 0.1 M PB for 24 h for cryoprotection. The ovaries were embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) so that the maximum cross-sectional area could be cut. Then they were frozen using acetone bath cooled to −70°C with dry ice and stored at −80°C until use. Frozen sections (8 μm thick) of the prepared ovaries were cut using a cryostat (Leica) and GFP fluorescence was observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at excitation and emission wavelengths of 480 nm and 510 nm, respectively. Adjacent sections were stained with hematoxylin and eosin. In order to estimate the number of follicles of frozen-thawed or fresh ovarian grafts, we counted oocytes in primary, secondary, preantral, antral, and preovulatory follicles in the maximum cross-sectional area.

Statistical Analyses

We compared the recipients transplanted with frozen-thawed or fresh ovarian grafts with respect to the following parameters in each experiment. For natural mating after ovarian transplantation, we compared the number of recipients that had given birth to GFP+ pups by Fisher exact probability test and the percentage of GFP+ pups among pups born by chi-square analysis. We also compared the number of pups born per recipient and the number of GFP+ pups per ovarian graft by Mann-Whitney *U*-test. For the IVF-ET experiment, we compared the number of oocytes per each recipient after superovulation by Mann-Whitney *U*-test. We also compared the percentage of GFP+ oocytes among collected oocytes, the percentage of fertilized GFP+ two-cell embryos after IVF, and the percentage of finally obtained GFP+ pups after ET by chi-square analysis. For histological examination, we compared the number of GFP+ oocytes in maximum cross-sectional area of each ovarian graft by Mann-Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Fecundity of Frozen-Thawed Ovarian Graft

The ovaries isolated from 10-day-old GFP-transgenic mice were frozen by vitrification, recovered by thawing with 0.25 M sucrose in PBI medium, and transplanted into C57BL/6 recipients using the procedure described in *Materials and Methods.* A small portion of the recipients' ovarian tissue was left in situ. This procedure resulted in better establishment of the grafts compared with total ovariectomy (unpublished observation), probably by facilitating the regeneration of blood supply into the graft tissue.

Table 1 summarizes the fecundity of the recipients transplanted with frozen-thawed or fresh ovarian grafts (experimental or control group) examined by natural mating 3 wk after the transplantation. Seven of the 10 recipients (70%) of the experimental group gave birth to a total of 33 pups. Fetuses derived from the ovarian graft of GFP-transgenic mice can be easily identified by their strong GFP fluorescence (Fig. 1). Of the 33 newborns, 16 (48.5%) were GFP fluorescence positive (GFP+). GFP fluorescence-negative pups were judged to be derived from oocytes of the residual recipient's ovary and were therefore excluded from further analyses. Three of four recipients (75%) of the control group gave birth to 21 pups, 16 (76.2%) of which were

GFP+. The fertility (pups born/recipient) of the experimental group was significantly lower than that of the control group. The percentage of GFP+ pups among newborns and the mean number of GFP+ pups per grafted ovary were significantly lower in the experimental group than those in the control group, suggesting possible impairments of fecundity of frozen-thawed ovarian tissues. However, nearly one half of the pups derived by natural mating were GFP+ in the experimental group, clearly indicating that cryopreserved ovaries by vitrification maintained fecundity.

In Vitro Fertilization and Embryo Transfer

Next, we compared fecundity of recipients in the experimental group with that in the control group using in vitro fertilization and embryo transfer. Three weeks after weaning, the recipients that had given birth to GFP+ pups were superovulated to collect GFP+ oocytes, and in vitro fertilization using C57BL/6 sperms was performed. Obtained two-cell embryos were transferred to the oviducts of ICR mice prepared as pseudopregnant recipients. Results are summarized in Table 2. Forty-nine oocytes were obtained from seven recipients in the experimental group and 68 oocytes were obtained from three recipients in the control group. The number of collected oocytes per mouse was significantly lower in the experimental group (7.0) than that in the control group (22.7). The percentage of GFP+ oocytes among collected oocytes was not statistically different between the two groups (53.1% in the experimental group versus 57.4% in the control group). Fertilization rates were defined as the number of GFP+ two-cell embryos obtained by in vitro fertilization/total number of GFP+ oocytes. The rate was significantly lower in the experimental group (38.5%) than in the control group (90%). Finally, six GFP+ pups were obtained after transfer of 10 two-cell embryos derived from cryopreserved ovarian graft and 16 GFP+ pups were obtained after transfer of 35 two-cell embryos derived from fresh ovarian graft. The percentage of GFP+ pups to total number of transferred two-cell embryos was slightly higher in the experimental group (60%) than in the control group (45.7%), but the difference was not statistically significant.

TABLE 1. Fecundity of the recipients transplanted with fresh or frozen-thawed ovarian grafts examined by natural mating.

	Fresh ovaries*	Frozen-thawed ovaries†
Number of recipients‡	4	10
Number of recipients that gave birth to GFP+ pups	3 (75.0%)	7 (70.0%)§
Total number of pups born	21	33
Pups born/recipient (mean ± SEM)	7.0 ± 1.5	4.7 ± 0.9‖
Number of GFP+ pups among pups born	16 (76.2%)	16 (48.5%)#
GFP+ pups/grafted ovary (mean ± SEM)	2.0 ± 0.7	0.8 ± 0.3‖

* Freshly isolated ovaries were directly transplanted.
† Ovaries were frozen by vitrification and thawed immediately before transplantation as described in *Materials and Methods.*
‡ Two ovaries were transplanted into a recipient, one graft into each ovarian bursa on both sides.
§ No significant difference ($P > 0.05$) by Fisher exact probability test.
‖ Significant difference ($P < 0.05$) by Mann-Whitney *U*-test.
Significant difference ($P < 0.05$) by chi-square analysis.

Histology of Grafted Ovaries

The ovarian grafts were examined 4 mo after transplantation. Under a stereomicroscope, cryopreserved grafts were generally smaller than fresh grafts, suggesting that the growth of cryopreserved ovaries might be limited due to degeneration and necrosis of the tissue caused by the freezing-thawing procedure (Fig. 2). Histologically, GFP+ graft tissues could be readily identified by their strong fluorescence (Fig. 3, A–C). In spite of the smaller size, normal development of follicles and formation of corpora lutea was similarly observed in the cryopreserved grafts (Fig. 3, A and E) as in fresh ovarian grafts (Fig. 3, B and F). This

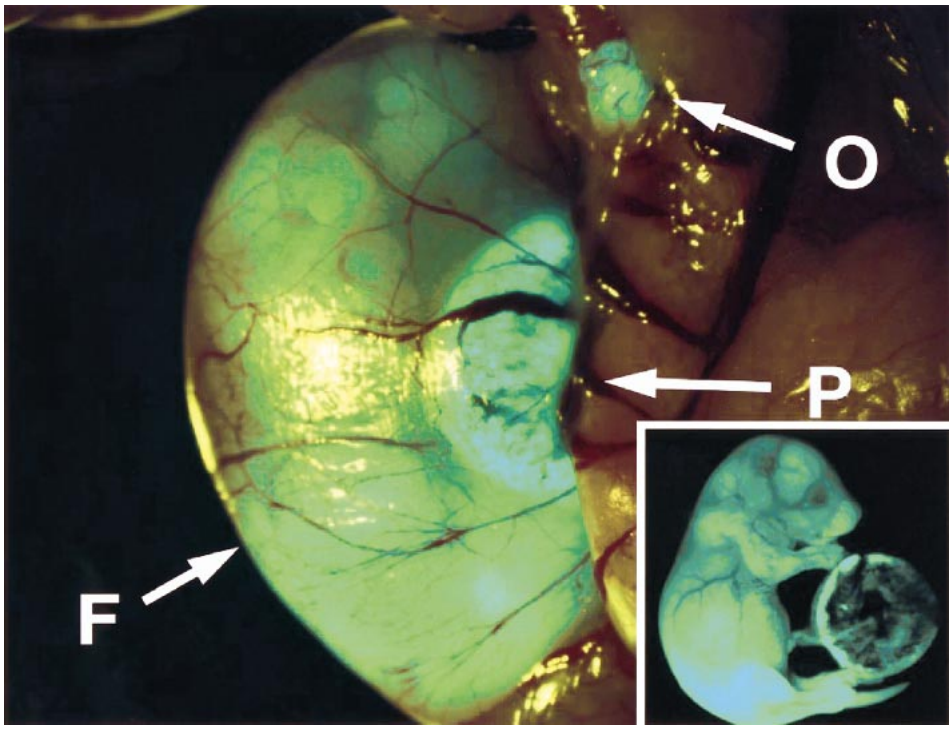

FIG. 1. A GFP+ embryo in the uterus of the recipient of cryopreserved ovarian grafts derived from a GFP-transgenic mouse. A fetus (F), a placenta (P), and an ovary (O) were all exhibiting strong GFP fluorescence 18 days after mating. An isolated embryo together with a placenta (inset).

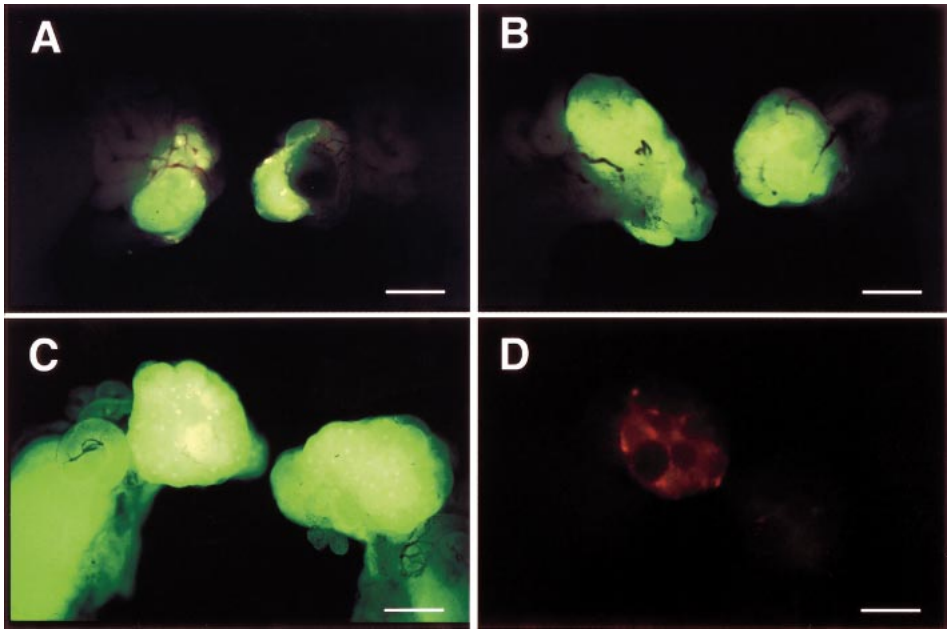

FIG. 2. Stereomicroscopic findings of GFP fluorescence of the ovarian tissue of the recipients and donor. **A**) Frozen-thawed ovarian grafts of GFP-transgenic mouse 4 mo after transplantation. Green fluorescence of GFP was clearly observed to be localized in the transplanted ovaries. Oviducts and uterus of recipients were GFP negative. **B**) Fresh ovarian grafts of GFP-transgenic mouse 4 mo after transplantation. Note that the size of the frozen-thawed ovaries is generally smaller than that of the fresh ovaries. **C**) Ovaries, oviducts, and uteri of GFP-transgenic mice. **D**) Ovaries, oviducts, and uteri of C57BL/6. Only a faint yellowish autofluorescence was detected in the ovaries. Bars in **A**–**D** = 1 mm.

suggests that surviving follicles after the frozen-thawed procedure could grow normally at least with respect to the morphological criteria. It should be noted that GFP fluorescence of granulosa cells in the follicles and some of the corpora lutea were very weakly or hardly detected (Fig. 3A). This was also the case with the fresh ovarian grafts of GFP-transgenic mice (Fig. 3, C and G), raising the possibility that the transgene promoter is not fully active in these cells.

In order to compare the numbers of follicles in the fresh and frozen-thawed grafts, we counted GFP+ oocytes in primary, secondary, preantral, antral, and preovulatory (graafian) follicles observed in the maximum cross-sectional area of each ovarian graft (Table 3). The average number of oocytes in frozen-thawed ovaries (4.0 ± 0.4) was significantly smaller than that in fresh ovaries (12.3 ± 1.3). This suggests that the loss of follicles is a highly possible reason for the smaller size of the cryopreserved grafts than that of fresh grafts.

DISCUSSION

Various reports on orthotopic transplantation of cryopreserved ovaries have been published since the first success in live births in mice using glycerol as a cryoprotectant [1]. It is critical to maintain the oocyte-granulosa cell complex and the survival of oocytes for good cryopreservation of ovaries. Successful slow freezing procedures of ovaries rely on the removal of intracellular and intercellular water to avoid the lethal effects of large amounts of ice formation during cooling. Therefore, cryoprotectants are used to remove and/or substitute the intracellular and intercellular water prior to the freezing procedure. In 1997, Gunasena et al. [17] reported live births after autologous orthotopic transplantation of cryopreserved whole ovaries using a modified procedure of Harp. In 1998, Sztein et al. [18] reported the successful cryopreservation and orthotopic transplantation of mouse ovaries with the coat color gene. There was no difference in histological findings between grafts of fresh and cryopreserved ovaries by slow freezing [19]. Thus, the cryopreservation of ovaries by slow freezing has been employed in clinical medicine [20] as well as in laboratories.

Cryopreservation of oocytes and embryos by vitrification has been widely conducted because of its simplicity and high survival rate of oocytes and embryos. However, there has been only one report about the successful orthotopic transplantation of cryopreserved ovary by vitrification [21]. Vitrification depends on the ability of a highly concentrated aqueous solution of cryoprotectants to supercool organs to an extremely low temperature. The very low temperature makes these solutions extremely viscous so they solidify without ice formation [22].

In the experiments by Rall and Fhay [23], a high survival rate of mouse embryos was observed for both the rapid and slow cooling method using highly concentrated VS1. In contrast, the warming rate of embryos also has very important effects on their survival rate. The survival rate was approximately 90% when embryos were subjected to a temperature change of 300°C/min, while it was 0% when subjected to a temperature change of 10°C/min. Such a poor survival rate is due to devitrification that occurred during the slow warming process [23]. In order to simplify the procedure and shorten the time of cryopreservation using VS1, Nakagata [10] developed DAP213 from VS1 for the cryopreservation of mouse oocytes and embryos. However, the toxic action of DAP213 on cells is not completely eliminated and the precise mechanism of its toxicity remains unknown. The mechanism of cellular toxicity of cryoprotectants can vary from a nonspecific impairment of cellular components (e.g., disruption of membranes, denaturation of proteins) to a specific action on distinct enzymes. The degree of chemical toxicity is directly related

FIG. 3. GFP fluorescence and hematoxylin-eosin staining of fresh and frozen-thawed ovarian grafts and an ovary derived from GFP-transgenic mice. **A**) A section of a frozen-thawed ovarian graft 4 mo after transplantation. Normal development of follicles was observed in the cryopreserved grafts. **B**) A section of a fresh ovarian graft. Primary, secondary, and antral follicle and corpora lutea were observed. **C**) A section of a GFP-transgenic mouse ovary. **D**) A GFP negative ovary of C57BL/6 mouse. **E**–**H** were stained by hematoxylin-eosin using sections adjacent to those shown in **A**–**D**. Pri.F, Primary follicle; Sec.F, secondary follicle; Pre.F, preantral follicle; Ant.F, antral follicle; CL, corpora lutea. Bars in **A**–**C** and **E**–**H** = 200 μm.

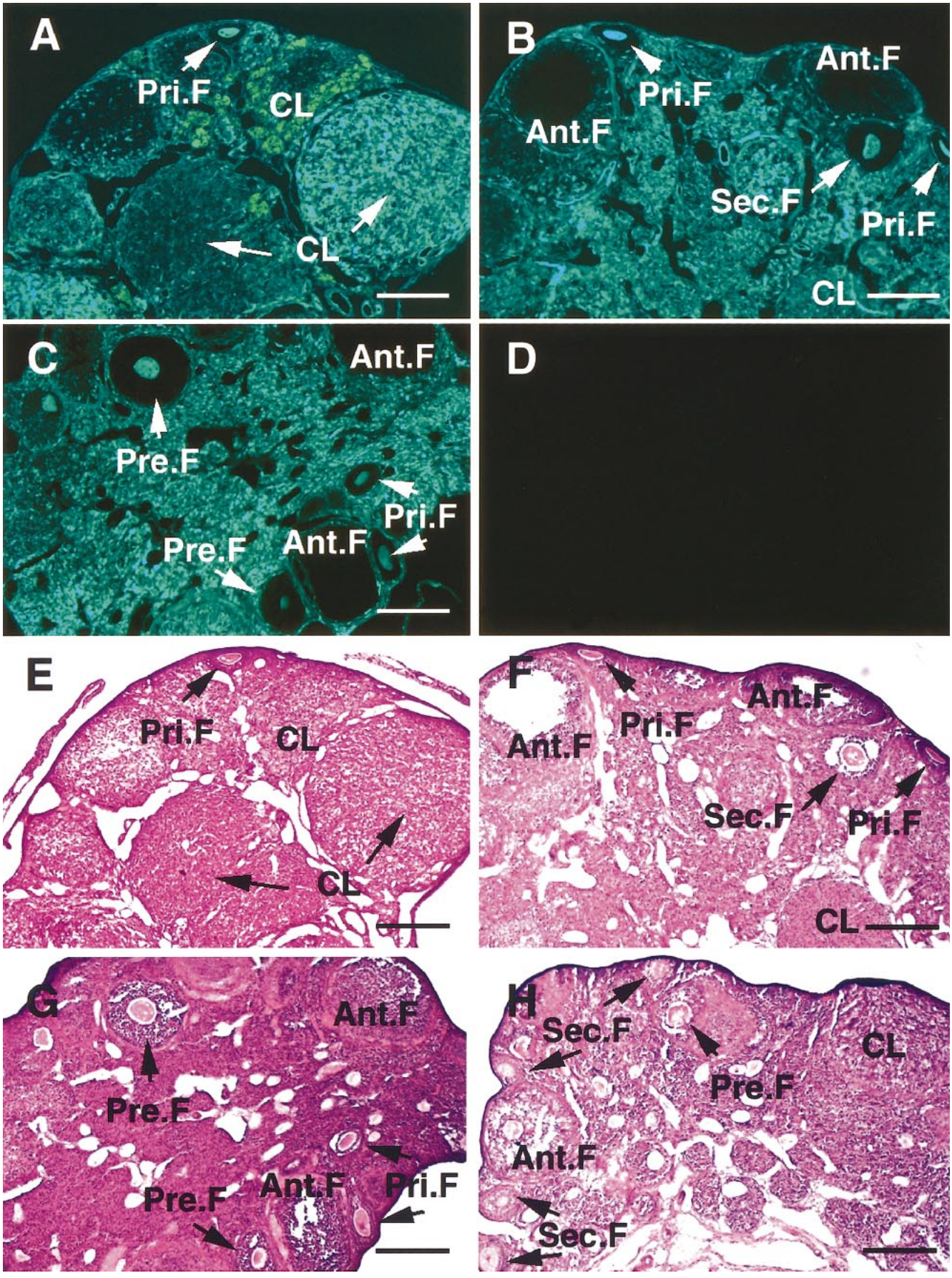

TABLE 2. Fecundity of the recipients transplanted with fresh or frozen-thawed ovarian grafts examined by IVF-ET.

	Fresh ovaries*	Frozen-thawed ovaries†
Number of recipients‡	3	7
Total number of collected oocytes	68	49
Oocytes/recipient (mean ± SEM)	22.7 ± 3.2	7.0 ± 1.7§
Number of GFP+ oocytes among collected oocytes	39 (57.4%)	26 (53.1%)‖
Fertilized GFP+ two-cell embryos	35 (90.0%)	10 (38.5%)#
Number of embryos transferred**	35	10
GFP+ pups born	16 (45.7%)	6 (60.0%)‖

* Freshly isolated ovaries were directly transplanted.
† Ovaries were frozen by vitrification and thawed immediately before transplantation as described in *Materials and Methods.*
‡ Two ovaries were transplanted into a recipient, one graft into each ovarian bursa on both sides.
§ Significant different ($P < 0.05$) by Mann-Whitney *U*-test.
‖ No significant difference ($P > 0.05$) by chi-square analysis.
Significant difference ($P < 0.05$) by chi-square analysis.
** Fertilized GFP+ two-cell embryos were transplanted into the oviducts of ICR mice prepared as peudopregnant recipients.

TABLE 3. Number of oocytes in maximum cross-sectional area of fresh and frozen-thawed ovarian grafts.

	Fresh ovaries	Frozen-thawed ovaries
Number of ovaries examined	4	4
Total number of GFP+ oocytes	49	16
GFP+ oocytes/graft (mean ± SEM)	12.3 ± 1.3	4.0 ± 0.4*

* Significant difference ($P < 0.05$) by Mann-Whitney *U*-test.

to the concentration and temperature of the vitrification solution as well as the exposure time of the sample to the solution [24].

We improved various procedures of vitrification for better cryopreservation of whole ovaries. First, the ovaries were suspended in the PBI medium containing 1 M DMSO for 5 min at 0°C before adding DAP213. This procedure may decrease the toxic action of DAP213 so that DMSO can penetrate into the ovaries [14]. Second, all of the procedures were conducted while maintaining the ovarian tissue at 0°C except for the pretreatment of ovaries with the PBI medium containing 1 M DMSO at room temperature and thawing of the cryopreserved ovaries (see *Materials and Methods*). It is critical to handle tissues at a low temperature to minimize their damage by decreasing oxygen demand and diminishing the production of toxic chemical mediators.

Another cause of damage of cryopreserved ovaries is devitrification that may occur during the thawing procedure. In order to avoid this effect, it is necessary to increase the thawing rate to the highest extent by either using small ovaries or cutting the ovaries into smaller pieces. In the present series of experiments, we chose ovaries of 10-day-old mice that seemed sufficiently small to minimize devitrification during the thawing procedure. For the vitrification of large-sized ovaries, it is probably necessary to cut the tissue into small pieces before freezing.

Orthotopic transplantation is an extremely important technique. However, only a limited number of reports on the ovarian tissue transplantation by this technique have been published compared with exotopic transplantation, probably due to the technical difficulties such as bleeding and suturing the ovarian bursa. We could minimize bleeding using diluted epinephrine. We transplanted the grafts of 10-day-old mice into the ovarian bursa of 4-wk-old recipients. This enabled the rapid and reliable transplantation and the smaller size of excision of the bursa, which can be closed easily without suturing. Furthermore, we left approximately 10% of the recipient ovaries in situ to facilitate sufficient regeneration of blood supply to the grafts. As a result, we succeeded in orthotopic transplantation and obtained GFP+ pups from 75% of recipients of fresh ovarian grafts and 70% of recipients of cryopreserved grafts. Fecundity of the ovarian graft was expressed as the total number of live GFP+ pups/total number of grafted ovaries. The values were 0.8 and 2.0 for the cryopreserved and fresh ovarian graft, respectively (Table 1). Despite the partial reduction in fecundity, the present result unequivocally indicates that the cryopreserved ovaries by vitrification were functional and fertile after orthotopic transplantation into the recipients.

There were no apparent abnormalities in GFP+ oocytes, which were obtained from the recipients of the experimental group by superovulation, with respect to the morphological criteria. However, the fertilization rate of these GFP+ oocytes was significantly lower than that of GFP+ oocytes from the control group. Nevertheless, the rate of birth (number of GFP+ pups born/number of two-cell embryos transferred) was similar between the two groups (Table 2). It is thus suggested that, once the fertilized oocytes have grown to two-cell embryos, they would grow normally, although the cryopreserved ovaries by vitrification may have certain problems in the fertilization. Moreover, the number of collected oocytes per mouse in the experimental group was approximately one third of that in the control group, while the percentage of GFP+ oocytes among collected oocytes were not statistically different between the two groups (Table 2). This suggests that the cryopreservation procedure may have some negative effects even on the residual recipient's ovary as well as on the ovarian graft. The cryoprotectant remaining in the transplanted graft might be one of the factors that have such effects.

The present study indicates the effectiveness of vitrification for cryopreservation of ovaries and also the possibility for other solid tissues. Nevertheless, there appears to be a limitation in the size of the frozen tissue. Therefore, it will probably be necessary to cut large-sized tissues into small pieces before freezing. Another problem observed from this study is the possible tissue toxicity of the cryoprotectant remaining within the thawed tissue. This problem might be overcome by culturing the thawed tissue for a certain period before transplantation. Further studies along these lines are necessary to improve cryopreservation by vitrification for various organs, including the ovary.

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REFERENCES

1. Parrot DM. The fertility of mice with orthotopic ovarian grafts derived from frozen tissue. J Reprod Fertil 1960; 1:230–241.
2. Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196 °C. Hum Reprod 1994; 9:597–603.

3. Harp R, Leibach J, Black J, Keldahl C, Karow A. Cryopreservation of murine ovarian tissue. Cryobiology 1994; 31:336–343.
4. Candy CJ, Wood MJ, Whittingham DG. Follicular development in cryopreservation marmoset ovarian tissue after transplantation. Hum Reprod 1995; 10:2334–2338.
5. Hovatta O, Silye R, Krausz T, Abir R, Margara R, Trew G, Lass A, Winston RM. Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants. Hum Reprod 1996; 11:1268–1272.
6. Newton H, Aubard Y, Rutherford A, Sharma V, Gosden R. Low temperature storage and grafting of human ovarian tissue. Hum Reprod 1996; 11:1487–1491.
7. Gook DA, Edgar DH, Stern C. Effect of cooling rate and dehydration regimen on the histological appearance of human ovarian cortex following cryopreservation in 1,2-propanediol. Hum Reprod 1999; 14: 2061–2068.
8. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984; 21:407–426.
9. Pomp D, Eisen EJ. Genetic control of survival of frozen mouse embryos. Biol Reprod 1990; 42:775–786.
10. Nakagata N. High survival rate of unfertilized mouse oocytes after vitrification. J Reprod Fertil 1989; 87:479–483.
11. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimura Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 1997; 407: 313–319.
12. Toyoda Y, Yokoyama M, Hoshi T. Studies on the fertilization of mouse eggs in vitro fertilization of eggs by fresh epididymal sperm [in Japanese]. Jpn J Anim Reprod 1972; 16:147–151.
13. Whitten WK, Biggers JD. Complete development in vitro of the preimplantation stages of the mouse in a simple chemically defined medium. J Reprod Fertil 1968; 17:399–401.
14. Nakao K, Nakagata N, Katsuki M. Simple and efficient vitrification procedure for cryopreservation of mouse embryos. Exp Anim 1997; 46:231–234.
15. Whittingham DG. Embryo banks in the future of developmental genetics. Genetics 1974; 78:395–402.
16. Richard Behringer. Transferring ovaries. In: Brigid H, Rosa B, Frank C, Elizabeth L (eds.), Manipulating the Mouse Embryo, 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1994: 185–186.
17. Gunasena KT, Villines PM, Crister ES, Crister JK. Live births after autologous transplant of cryopreserved mouse ovaries. Hum Reprod 1997; 12:101–106.
18. Sztein J, Sweet H, Farley J, Mobraaten L. Cryopreservation and orthotopic transplantation of mouse ovaries: new approach in gamete banking. Biol Reprod 1998; 58:1071–1074.
19. Candy CJ, Wood MJ, Whittingham DG. Effect of cryoprotectant on the survival of follicles in frozen mouse ovaries. J Reprod Fertil 1997; 110:11–19.
20. Gosden RG. Low temperature storage and grafting of human ovarian tissue. Mol Cell Endocrinol 2000; 163:125–129.
21. Takahashi E, Miyoshi I, Nagasu T. Rescue of a transgenic mouse line by transplantation of a frozen-thawed ovary obtained postmortem. Contemp Top Lab Anim Sci 2001; 40:28–31.
22. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984; 21:407–426.
23. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at −196 °C by vitrification. Nature 1985; 313:573–575.
24. Rall WF. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology 1987; 24:387–402.