Cryopreservation of Gonadal Germ Cells (GGCs) from the Domestic Chicken Using Vitrification

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We cryopreserved gonadal germ cells (GGCs) collected from 7-day-old chick embryos using the vitrification method. Gonadal germ cells were suspended in vitrification medium and plunged directly into liquid nitrogen. The recovery rates of GGCs after freezing via vitrification and slow-freezing methods were 36.8 ± 1.5 and $56.7 \pm 3.0\%$, respectively. The survival rates of GGCs in the unfrozen control, vitrified and slow-frozen cells were 98.0 ± 0.2 , 85.8 ± 1.2 and $91.2 \pm 2.8\%$, respectively. The recovery and survival rates were significantly lower for vitrified GGCs than for slow-frozen GGCs ($p < 0.05$). To evaluate the migratory ability of cryopreserved GGCs, frozen/thawed GGCs were labeled with PKH2 fluorescent dye, and 20 GGCs were transferred into 2-day-old chick embryos. After incubating for three days, the embryonic gonads were collected and the number of PKH2-labeled GGCs per chick was counted. These values for the control, vitrified and slow-frozen cells were 96.5 ± 23.9 , 102.0 ± 39.0 and 90.0 ± 34.2 cells, respectively. No significant differences were observed among treatments ($p > 0.05$). These results demonstrate that it is possible to cryopreserve GGCs using vitrification, although the recovery and survival rates are lower than those observed using the slow-freezing method.

Key words: chicken, cryopreservation, gonadal germ cells, migratory ability, vitrification

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Introduction

As the number of endangered avian species increases in both wild (IUCN, 2006) and domesticated populations (Hodges, 2006), it is increasingly important to develop methods to conserve avian genetic resources. In addition, avian genetic resources developed for various research purposes need to be conserved for our future use. Furthermore, the genetic material of commercial parent stocks needs to be preserved to reduce the risk of losses as a result of unexpected disease outbreaks and accidents.

In avian species, the cryopreservation of semen has already been achieved in the chicken (Polge, 1951), turkey (Bakst and Sexton, 1979), duck (Maeda *et al.*, 1984) and some non-domesticated avian species such as the Sandhill Crane (Gee *et al.*, 1985). In contrast, an effective method for the cryopreservation of avian oocytes and embryos has not yet been developed. Because of the unique physiological and anatomical characteristics of the ovum, a method other than freezing is required for the preservation of oocytes.

Primordial germ cells (PGCs) are undifferentiated germ cells that subsequently differentiate into spermatogonia in

the testis or into oogonia in the ovary. PGCs originate from the epiblast (Eyal-Giladi *et al.*, 1981) and appear in the area pellucida (Swift, 1914). After the development of the vascular system, PGCs circulate along with embryonic blood cells and finally migrate into the embryonic gonads (Ukeshima and Fujimoto, 1984; Kuwana, 1993; Kuwana and Rogulska, 1999). After migration, PGCs are called gonadal germ cells (GGCs). Tajima *et al.* (1993) produced germline chimeras by transferring PGCs from White Leghorn into Rhode Island Red chickens, and *vice versa*. Naito *et al.* (1994) produced germline chimeras by transplanting cryopreserved PGCs. Tajima *et al.* (1998) also produced germline chimeras using cryopreserved GGCs collected from 5-day-old embryos. All of these studies used the slow-freezing method and the Bicell bio-freezing vessel to cryopreserve germ cells. To optimize the freezing rate in this method, the Bicell container needs to be placed in a deep-freeze maintained at -80°C .

Rall and Fahy (1985) demonstrated the cryopreservation of mouse embryos via vitrification. Vitrification is an ultra-rapid cryopreservation technique that uses high concentrations of cryoprotectants that inhibit the formation of ice crystals. Nakao *et al.* (1997) developed a simplified vitrification method that uses a modified freezing solution; this method successfully produced young mice from vitrified embryos. However, the vitrification of chick germ cells has not been reported until now. Therefore, we attempted to cryopreserve avian GGCs using the vitrific-

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ation method.

Materials and Methods

Experiment 1: Vitrification of GGCs

All equipment that came into direct contact with germ cells was coated with silicone (Sigma Coat SL-2; Sigma, St. Louis, MO, USA). Fertilized Y3-strain White Leghorn (WL) eggs were incubated at 37.8°C for 7 days. This strain was obtained from the Takikawa Animal Experiment Station (Takikawa, Hokkaido, Japan) and maintained at the Agricultural and Forestry Research Center at the University of Tsukuba, Japan. After incubation, the embryos were dissected off the egg yolks and washed with phosphate-buffered saline that did not contain Mg^{2+} and Ca^{2+} (PBS(−), 05913; Nissui Pharmaceutical Co., Tokyo, Japan). The head and tail of each embryo were fixed with needles, and the left embryonic gonad was collected under an Olympus dissecting microscope (SZH; Tokyo, Japan). We used five embryos.

The collected gonads were sliced using the tip of a 30G dental needle (DN-3021K; Terumo, Tokyo, Japan) in 30 μ l of PBS(−). The sliced gonads were placed into 1.5-ml centrifuge tubes containing 0.05% trypsin (203-11302; Wako, Osaka, Japan). The tubes were incubated at 37.8°C for 20 minutes.

After the trypsin treatment, an equal volume of bovine serum (B-0771; Sigma, St. Louis, MO, USA) was added to the centrifuge tubes and agitated to form a cell suspension containing GGCs. To remove the large cell clusters and undigested tissue fragments, the cell suspension was filtered through a 20- μ m nylon mesh filter (CMN-20-D; Small Parts Inc, Florida, USA) and centrifuged at 200g for 10 minutes.

After centrifugation, the supernatant was removed, and an equal volume of minimum essential medium (MEM, M0769; GIBCO BRL, New York, USA) containing 10% bovine serum was added. The cell suspension was allocated into the one of the three experimental treatments: vitrification, slow-freezing, or unfrozen control.

a) Vitrification method

Vitrification was performed according to the method described by Nakao *et al.* (1997), with some modifications. The germ cell suspension was centrifuged at 500g at 4°C for 5 minutes, and the supernatant was replaced with 20 μ l of PB1 medium (Nakao *et al.*, 1997) containing 10% DMSO. This step was repeated twice. The sample was then incubated in ice water for 5 minutes, after which 10 μ l of the cell suspension was placed into a sterile cryogenic vial (5000-0012; Nalgene Co., NY, USA), followed by the slow addition of 90 μ l of DAP213 medium (Nakao *et al.*, 1997). The vial was incubated in ice water for 5 minutes and then plunged into liquid nitrogen (−196°C). After storing the sample for 1–3 weeks, the sample was thawed by adding 500 μ l of PB1 medium containing 0.25M sucrose and warmed to 37°C.

The frozen sample was then incubated in a water bath maintained at 37°C, after which it was transferred to a 1.5

ml centrifuge tube and centrifuged at 800g for 5 minutes at 37.8°C. The supernatant was removed, and 50 μ l of PB 1 medium containing 0.25M sucrose was added.

b) Slow-freezing Method

Slow-freezing was conducted according to the method described by Tajima *et al.* (1998), with some modifications. The sample was centrifuged at 500g at 4°C for 5 minutes. The supernatant was removed, and 20 μ l of MEM supplemented 10% DMSO (MEM-D) was added. This step was repeated twice.

The cell suspension was incubated in ice water for 5 minutes, after which 20 μ l of the cell suspension was placed into a sterile cryogenic vial. The vial was placed into a biofreezing vessel (Bicell; Nihon Freezer Co., Tokyo, Japan) and then into a deep-freezer (ULT 1785-5-D12; Revco, Inc., NC, USA) at −80°C for at least 2 h. After freezing, the cryogenic vial was removed from the freezing vessel, placed in liquid nitrogen (−196°C) and stored for 1–3 weeks. The sample was thawed at room temperature and diluted stepwise by adding 20, 40 and 80 μ l of MEM. The diluted samples were pipetted into 1.5 ml centrifuge tubes and centrifuged at 800g at 37.8°C for 5 minutes. After centrifugation, the supernatant was replaced with 200 μ l of MEM.

c) Control

In the control, the samples were centrifuged at 800g at 4°C for 5 minutes, after which the supernatant was replaced with 20 μ l of MEM. This step was repeated twice. A 10 μ l aliquot of sample was then diluted in 90 μ l of MEM.

Observation

The viability of GGCs was evaluated three times for each sample using trypan blue staining. The recovery rate (the number of GGCs after freezing divided by number of GGCs before freezing) was calculated in triplicate for each sample. Gonadal germ cells were identified by the same morphological criteria as circulating PGCs, i.e., large granulated cells with large nuclei (Minematsu *et al.*, 2004).

Statistical Analyses

The data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests.

Experiment 2: The Migratory ability of vitrified GGCs

Approximately 40 frozen/thawed GGCs were collected using a fine glass pipette and incubated in medium containing PKH2 fluorescent dye (ZPKH2-GL; Zynaxis, PA, USA) at 37.8°C for 5 minutes. After staining, 20 GGCs were collected and injected through a window into the dorsal aorta of stage 13–14 embryos (Hamburger and Hamilton, 1951). Eggshell windows were prepared 1–2 hours prior to germ cell injection; during this time, approximately 2.5–4 μ l of blood was removed from the embryo using a fine glass pipette.

After germ cell injection, the eggshell window was sealed with plastic tape, and the embryo was allowed to develop in an incubator for three days. The collection of

GGCs from embryonic gonads was conducted according to the method described by Minematsu *et al.* (2004), with some modifications. After incubation, the gonads were collected from the GGC recipient embryos under a dis-

Fig. 1. Morphological characteristics of intact GGCs and trypan blue stained GGCs after cryopreservation.

A: PGCs collected from dorsal aorta of 2-day-old embryos. B: GGCs collected from gonad of 7-day-old embryo. C: Frozen-thawed GGCs with Trypan blue staining. Black arrow: trypan blue unstained live GGC; White arrow: trypan blue stained dead GGC. Black arrowhead: somatic cell.

PGCs: Primordial Germ Cells, GGCs: Gonadal Germ Cells.

secting microscope and placed into 30 μ l of PBS (–). After slicing the gonads, an equal volume of 0.05% trypsin was added into the PBS (–) containing gonadal suspension, which was then incubated at 37.8°C for 10 minutes.

The suspension was diluted and mixed with an equal volume of bovine serum. The suspension was placed on the HTCS and observed under a fluorescence microscope (IMT2RFC; Olympus, Tokyo, Japan) using a 490-nm excitation filter to count the number of fluorescent GGCs.

The migratory ability of frozen/thawed GGCs was determined by counting the total number of fluorescent GGCs in the recipient gonads. The data were analyzed using Chi-square (χ^2) tests. The proliferation rate of GGCs was estimated by calculating the ratio of the number of fluorescent GGCs to the number of transferred GGCs.

Results

Experiment 1

As shown in Figure 1, the morphology of GGCs was similar to circulating PGCs. The survival of cryopreserved GGCs was determined using trypan blue staining (Fig. 1) and the survival rates of GGCs in the control, vitrified and slow frozen cells were 98.0 ± 0.2 , 85.8 ± 1.2 and $91.2 \pm 2.8\%$, respectively (Table 1); there was no significant difference among treatments ($p < 0.05$). The recovery rates of vitrified and slow-frozen GGCs were 36.8 ± 1.5 and $56.7 \pm 3.0\%$, respectively. The recovery rate of vitrified GGCs was significantly lower than that of the slow-frozen cells ($p < 0.05$).

Experiment 2

For the migratory ability of transferred GGCs (Table 2), the proportion of recipient embryos with fluorescent GGCs from the control, vitrified and slow-frozen samples were 80.0, 80.0 and 60.0%, respectively.

The number of fluorescent GGCs per embryo from the control, vitrified and slow-frozen samples were 96.5 ± 23.9 , 102.0 ± 39.0 and 90.0 ± 34.2 cells, respectively ($p > 0.05$). No significant difference in proliferation rate was observed among the treatment groups; the values for the control, vitrified and slow-frozen GGCs were 4.8, 5.1 and 4.5 times, respectively.

Table 1. Survival and recovery rates for GGCs after cryopreservation

	Survival rate (%)	Recovery rate (%)
Control	98.0 ± 0.2^a	—
Vitrification	85.8 ± 1.2^c	36.8 ± 1.5^b
Slow-freezing	91.2 ± 2.8^b	56.7 ± 3.0^a

Recovery rate; (the rate of frozen GGCs/unfrozen GGCs) $\times$ 100.

^{a-c}: Values with different letters within columns are significantly different ($P < 0.05$). GGCs: Gonadal germ cells.

Table 2. Migratory ability of transferred GGCs

	Recipient embryo with fluorescent GGCs	Number of fluorescent GGCs per embryo	Proliferation rate (%) ¹
Control	80% (4/5)	96.5±23.9	4.8
Vitrification	80% (4/5)	102.0±39.0	5.1
Slow- freezing	60% (3/5)	90.0±34.2	4.5

Proliferation rate; (the ratio of the number of fluorescent GGCs to the number of transferred GGCs) × 100. GGCs: Gonadal germ cells.

¹) No significant difference among groups was detected using a χ^2 test ($P > 0.05$).

Discussion

Even though the survival rate of vitrified GGCs was significantly lower than that of slow-frozen cells ($p < 0.05$), it was still possible to retrieve viable GGCs that could successfully migrate to the embryonic gonad after they were transferred into the blood stream of 2-day-old embryos.

We encountered a significant disadvantage of vitrification: the recovery rate of vitrified cells was significantly lower than that of slow-frozen cells. However, because the method that we used was optimized for the vitrification of mouse embryos, it may be possible to improve the recovery rate of avian germ cells through a similar optimization process.

Several cryoprotectants have been used in freezing mammalian germ cells, e.g., ethylene glycol (EG) and polyvinyl-pyrrolidone for mouse oocytes and embryos (Leibo and Oda, 1993). EG, Ficoll and sucrose have been used to cryopreserve mouse embryos (Kasai *et al.*, 1990) and bovine embryos (Tachikawa *et al.*, 1993). A combination of EG and DMSO has been used to cryopreserve bovine oocytes (Vajta *et al.*, 1998). DMSO has also been used in combination with acetamide and propylene glycol to cryopreserve mouse embryos (Nakao *et al.*, 1997). An extensive study into the selection and concentrations of cryoprotectants is required to optimize the vitrification methods for avian germ cells.

The superficial dimensions of the freezing vessel strongly affect the cooling rate, making this an important factor in the optimization of vitrification. Recent studies have investigated the effects of several freezing vessels in working with mouse oocytes and embryos, including cryotubes (Nakao *et al.*, 1997), glass micropipettes (Kong *et al.*, 2000), the open pulled straw (Kong *et al.*, 2000) and the closed pulled straw (Chen *et al.*, 2001). The low recovery rates that we observed after vitrification could be improved by experimenting with different freezing vessels.

Shaw *et al.* (1992) studied the effect of exposure time to cryoprotective agents prior to vitrification on post-thaw survival and developmental ability in mouse oocytes. They found that shorter exposure times before vitrification corresponded to increased survival rates and developmental ability.

In Experiment 2, PKH2-positive GGCs, which we as-

sumed were the injected GGCs, were observed in all treatments. No significant differences in migratory ability were observed among treatments. In addition, the number of fluorescent GGCs in all treatments was approximately 90–100 cells, which is 4.5–5 times the number of injected cells. These results suggest that vitrified GGCs have normal migratory ability, as well as the ability to proliferate in the embryonic gonads.

In conclusion, vitrification is a simple and inexpensive method that can be used to cryopreserve avian genetic material in the field. Future studies are required to determine whether progeny can be produced from vitrified GGCs.

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