Production of Donor-Derived Offspring from Cryopreserved Ovarian Tissue in Japanese Quail (Coturnix japonica)¹

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ABSTRACT

Cryopreservation of avian ova and embryos is challenging because of the yolky structure of the egg. As an alternative, with the development of effective cryopreservation protocols, ovarian tissue cryopreservation could be used for cryobanking for birds. Pieces of ovarian tissue of week-old Japanese quail (Coturnix japonica) were frozen at 0.5°C/min in a programmable freezer or vitrified by immersion in liquid nitrogen. Straws containing slow-frozen samples were thawed in ice water, and vitrified samples were removed from the vials and transferred into sucrose, with the concentration lowered in sequence at room temperature. Cell viability of tissue was estimated by trypan blue assay, and tissue histology was examined by light microscopy. Frozen-thawed or vitrified-warmed tissue from WB (recessive plumage color) chicks was transplanted into week-old ovariectomy-mized QO (wild-type plumage) chicks, with some chicks receiving fresh tissue as a control group. At sexual maturity, QO recipients were mated to WB males, and the production of WB offspring demonstrated successful cryopreservation and transplantation. Donor-derived offspring were obtained from the ovarian tissue that had been cryopreserved by either slow-freezing or vitrification. The vitrification protocol used in this study showed better outcomes at each level of evaluation. This study demonstrated that the function of ovarian tissue in avian species can be successfully preserved at subzero temperatures and recovered by transplantation. The vitrification protocol is recommended because of high efficiency and overall simplicity.

cryopreservation, Japanese quail, ovary, transplantation, vitrification

INTRODUCTION

Cryopreservation of ovarian tissue has been used in mammals, including humans, to preserve female reproductive potential. Live offspring from cryopreserved ovarian tissue or whole ovaries have been reported in various mammalian models [1–3]. In human clinical practice, ovarian cryopreservation is used as a treatment to restore female patients' fertility threatened by cancer therapies [4]. There are currently two main approaches to cryopreserving ovarian tissues. One is slow-freezing (also known as slow-rate freezing or equilibrium freezing), in which tissues are usually frozen in a programmable freezer at controlled slow cooling rates and cryoprotective agents (CPAs) are required. This method has been used to preserve embryos, oocytes, ovarian tissues, and whole ovaries in previous studies [3, 5–7]. As an alternative method, vitrification preserves tissues by achieving a vitreous state of the whole tissue, which can be obtained by a high cooling rate and a high concentration of CPA. Vitrification does not require special devices and is usually very fast. It may also be preferable for cryopreservation of multicellular structures such as tissues or entire organs [8, 9]. Exploration and applications of vitrification in ovary cryopreservation have been documented [10–12].

In combination with transplantation, ovarian cryopreservation can be used as a means of germplasm banking [13–15], especially for those species in which the cryopreservation of germplasm is challenging such as avian species. Avian oocytes cannot be cryopreserved because of the presence of the yolk, which diminishes the protective effectiveness of existing cryopreservation methods, leaving oocytes vulnerable to the challenges induced by freezing protocols in the microenvironment [16, 17]. Though germline chimeras can be generated from cryopreserved early embryonic germ cells [18–20], low efficiency and inconsistency in the outcomes make them impractical when applied to avian genetic resource conservation [16, 21].

Recent studies demonstrated techniques of orthotopic ovarian transplantation in newly hatched chickens [22] and in week-old Japanese quail (Coturnix japonica) [23]. Donor-derived offspring from fresh transplants were obtained in both species, suggesting the possibility of combining cryopreservation and transplantation as a universal and practical approach to female fertility preservation in avian species. The objectives of the present study were two-fold. First, using the Japanese quail as a model, we evaluated slow-freezing and vitrification cryopreservation protocols for use in birds. Second, we evaluated whether the reproductive function of cryopreserved ovarian tissue in quail could be recovered by transplantation.

MATERIALS AND METHODS

Birds and Tissue Preparation

Week-old female chicks of (recessive) white-breasted (WB) [24] and wild-type (QO) [25] Japanese quail maintained at the Agassiz Research Centre were used as donors and recipients, respectively. All methods were approved by the Animal Care Committee of the Agassiz Research Centre and followed principles described by the Canadian Council on Animal Care [26]. All chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) unless otherwise indicated.

Ovaries were removed immediately after killing by cervical dislocation and immersed in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) on ice. The surrounding connective tissue and fat were then gently trimmed off, and each ovary was cut into two approximately equal pieces under a dissecting microscope. All tissue pieces were kept on ice before further treatment within 4 h.
Ovarian Tissue Cryopreservation

**Slow-freezing.** Two pieces of ovarian tissue were loaded in each 0.5-ml CBSTM High Security Straw (Cryo Bio System, Paris, France) filled with DMEM containing 10% (v/v) dimethyl sulphoxide (DMSO) and 10% (v/v) FBS. Straws were then put on ice to allow the tissue pieces to equilibrate for at least 20 min. The freezing procedure was carried out using a programmable freezer (Kryo 360–1.7; Planer PLC, Middlesex, England) according to the protocol described by Silversides et al. [27]. Briefly, straws were placed in the freezing chamber, which was cooled to 10°C. The temperature was then lowered to –7°C at the rate of 0.5°C/min. After manual seeding at this temperature, the straws were held at –7°C for 10 min and then further cooled to –55°C at 0.5°C/min. Finally, the straws were plunged into liquid nitrogen and stored for at least 72 h.

Thawing was similar to that used for cryopreserved chicken testicular tissue [28], with slight modifications. The straws were removed from liquid nitrogen and quickly plunged into ice water until the ice in the straws melted. The contents of each straw were then emptied into Petri dishes containing DMEM supplemented with 10% FBS. The CPA was removed by gently shaking the dishes plus repeated rinsing for at least three times with media. The tissue pieces were then suspended in media on ice before further use.

**Vitrification.** The procedure used in this study was based on two methods, direct cover vitrification [11] and needle immersed vitrification [29], which have been applied in mouse ovarian tissue vitrification. Four tissue pieces were put on each acupuncture needle (Cloud & Dragon Medical Device Co. Ltd., Jiangsu, China) that had been manually modified to fit the cryovials (Fisher Scientific, Edmonton, AB, Canada) mounted on a microscope (Olympus BX51; Olympus Corp., Tokyo, Japan). In this study, follicles with the following characteristics as shown in Figure 1 were defined as morphologically normal: 1) intact follicular epithelium, 2) intact ooplasm with a visible germinal vesicle, and 3) widest diameter larger than 20 μm. The number of morphologically normal follicles was counted from five different sections of each sample.

Cell Viability Estimation

Cell viability after freezing-thawing or vitrification-warming procedures was estimated by trypan blue assay [30, 31]. Fresh tissue was used as control. Tissue pieces were finely chopped using two scalpels in 0.25% trypsin-edetic acid solution and 1.5 mg/ml of collagenase. The fine fragmented were then incubated at 37°C and were gently pipetted every 10 min to promote the digestive reaction. After 30 min, 50% FBS was added to inhibit the enzyme activity, and the digestive products were centrifuged at 500 × g for 10 min. The precipitate of each sample (two tissue pieces) was resuspended thoroughly in 20 μl of DMEM. Samples were stained by 0.4% trypan blue at RT. The cell viability was examined under a light microscope using a hemocytometer (Hauser Scientific, Horsham, PA); dead cells were stained blue, and living cells were not stained.

Histological Examination

Fresh and frozen-thawed or vitrified-warmed tissue samples were fixed in Bouin solution for 24 h, dehydrated in alcohol, and subsequently embedded in paraffin. The embedded samples were cut into 7-μm serial sections, mounted on slides, and stained with hematoxylin-eosin. Images were captured and examined using a digital camera (1300R; Qimaging Corp., Burnaby, BC, Canada) mounted on a microscope (Olympus BX51; Olympus Corp., Tokyo, Japan).

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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample size</th>
<th>Normal follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>26.8 ± 4.5a</td>
</tr>
<tr>
<td>Slow-freezing</td>
<td>8</td>
<td>14.3 ± 2.9b</td>
</tr>
<tr>
<td>Vitrification</td>
<td>8</td>
<td>24.6 ± 1.4c</td>
</tr>
</tbody>
</table>

a, b Values with different superscripts are significantly different (P < 0.05).

TABLE 1. Cell viability (mean ± SEM) of cryopreserved ovarian tissues.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample size</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>81.9 ± 0.9a</td>
</tr>
<tr>
<td>Slow-freezing</td>
<td>15</td>
<td>70.0 ± 0.9b</td>
</tr>
<tr>
<td>Vitrification</td>
<td>9</td>
<td>77.1 ± 1.9c</td>
</tr>
</tbody>
</table>

a–c Values with different superscripts are significantly different (P < 0.05).
TABLE 3. Egg production to 20 wk, fertility and age at first egg (mean ± SEM) of quail receiving ovarian transplants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Recipients producing eggs</th>
<th>Egg production*</th>
<th>Fertility (%)*</th>
<th>Age at the first egg (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>38.5 ± 10.2</td>
<td>58.8 ± 10.4</td>
<td>76.5 ± 6.4</td>
</tr>
<tr>
<td>Slow-freezing</td>
<td>11</td>
<td>42.0 ± 7.0</td>
<td>64.5 ± 9.2</td>
<td>61.9 ± 5.1</td>
</tr>
<tr>
<td>Vitrification</td>
<td>7</td>
<td>56.9 ± 5.3</td>
<td>63.6 ± 6.6</td>
<td>66.9 ± 3.1</td>
</tr>
</tbody>
</table>

a No significant difference was seen between any two treatments.

of the recipient’s ovary and were covered by the remains of the greater abdominal air sac. Four or five interrupted sutures were used to close the skin. Fresh tissue pieces were transplanted using the same technique for use as a control treatment. An immunosuppressant (CellCept; Hoffmann-LaRoche Ltd., Mississauga, ON, Canada) was given orally to all the operated birds at a dose of 100 mg/kg of body weight every day for 2 wk after the operation.

**Progeny Test**

To assess the function of the grafts, a progeny test was conducted using plumage color as a genetic marker [24]. The white-breasted trait carried by the WB quail is determined by a homozygous recessive genotype of a single autosomal wb gene (wb/wb), and the QO line has wild-type (+/+ ) coloration that is a dark plumage color [24, 25]. Thus, heterozygous offspring (+/wb) from the cross of the WB and QO lines would be phenotypically wild type. In our study, after the QO recipients obtained their sexual maturity at the age of 6 wk, each was paired with a fertile WB male bird. Eggs were collected and incubated, and most were opened after 12–14 days of incubation. Some chicks were allowed to hatch. The feather color of the embryos and chicks indicated their maternal origin; that is, the white-breasted embryos (wb/wb) were derived from the WB donor ovarian tissue, whereas the dark-colored embryos (+/wb) originated from regenerated ovarian tissue of the host QO recipients. The test was continued until the recipients were 20 wk old.

**Statistical Analysis**

All statistical analyses were conducted with SASBatch 4.1.1 (SAS Institute, Cary, NC). Cell viability, egg production, fertility, and age at first egg were analyzed using the general linear model. ANOVA was used to test the difference in the means of follicle counts among treatments. Analyses in the FREQ procedure were used to compare the differences in the numbers of surviving recipients, recipients producing fertile eggs, recipients producing only host-derived offspring, recipients producing only donor-derived offspring, and recipients producing both host-derived and donor-derived offspring. Statistical significance was set as P ≤ 0.05.

**RESULTS**

**Cell Viability Estimation**

Cell viability of samples (Table 1) that had been treated with either the slow-freezing or vitrification protocol was significantly lower than that of the control group (81.9%). It was significantly higher in the vitrification group (77.1%) than in the slow-freezing group (70.0%).

**Histological Examination**

Table 2 summarizes the results of histological examination. No significant differences were seen in the mean number of morphologically normal follicles between the control group (26.8 ± 4.5) and the vitrification group (24.6 ± 1.4), but both of these groups had significantly more morphologically normal follicles than those in the slow-freezing group (14.3 ± 2.9).

**Surgical Transplantation and Progeny Test**

The results of the surgical transplantation and progeny test are given in Table 3 and Table 4. Egg production, fertility, and age at first egg of recipients that produced at least one egg were not influenced by the treatment (Table 3). The recipients that survived surgery and lived until the end of the test at 20 wk were considered to be surviving recipients (Table 4). There was no significant difference between groups in surviving recipients among recipients receiving surgery. Likewise, the number of recipients producing fertile eggs of surviving recipients was comparable among groups. Of recipients that produced at least one offspring, one of six in the control group and two of seven in the vitrification group produced only host-derived offspring, while this ratio was significantly greater in the slow-freezing group (seven of nine). No significant difference was seen in the number of recipients that produced only donor-derived offspring for the control, slow-freezing, and vitrification groups. Four of six recipients in the control group and five of seven recipients in the vitrification group produced both donor-derived and host-derived offspring, while no recipients in the slow-freezing group produced both.

**DISCUSSION**

Ovarian tissue cryopreservation and transplantation enable us to preserve female germplasm. Current applications are exclusively in laboratory mammals, farm mammals, and human clinical cases. It is urgent to develop a practical and reliable means of female fertility preservation in birds. The present study demonstrated that the fecundity of ovarian tissue in an avian species could be cryopreserved in a simple and inexpensive manner and reconstituted satisfactorily by surgical transplantation. Donor-derived offspring were obtained from transplanted ovarian tissue that had been cryopreserved by either slow-freezing or vitrification. Vitrification had greater efficiency as measured by cell viability, tissue morphology, and overall reproductive performance of the cryopreserved transplants.

Ovarian tissues cryopreserved by slow-freezing in the present study had lower cell viability, fewer morphologically normal follicles, and decreased efficiency in producing donor-derived offspring compared with their vitrified counterparts. Our protocol originated from protocols used for mammalian ovarian tissue cryopreservation [32]; large differences may exist in how tissues from different species respond to the freezing protocol. Therefore, more refinements may be needed such as rate of freezing/thawing, type and concentration of the cryoprotectants, length of equilibration, and seeding temperature. A more important reason is that to date slow-freezing protocols were mostly based on those for cell or embryo
cryopreservation. To protect cells from lethal intracellular ice formation, the efflux of the supercooled intracellular water is facilitated by inducing extracellular ice nucleation (i.e., seeding). This might be detrimental to the extracellular matrix of ovarian tissue, which is of great importance in ovarian follicle development [33] and thus to the reestablishment of fertility. In the future, instead of using a single penetrating cryoprotectant such as DMSO, a combination of both penetrating and nonpenetrating cryoprotectants such as polymers may help to reduce or eliminate the adverse effects of extracellular ice crystallization [34].

In contrast to conventional slow-freezing, a vitrification protocol could theoretically preserve tissues both intracellularly and extracellularly through ice-free solidification. This could be achieved by a high cooling/warming rate plus a high concentration of CPAs. The concentration of CPAs could be lowered to a small extent to reduce the toxicity as long as the cooling/warming rate is enhanced correspondingly, making use of the reciprocal relationship of the two [35].

A practical way to gain a higher cooling rate is to let the sample contact liquid nitrogen directly, which has been facilitated by special devices such as cryoloops [36] and cryotops [37] that have been exploited in the study of oocyte or embryo vitrification. Unfortunately, these devices are not ready for tissue vitrification. Chen and colleagues [11] have developed a new method called direct cover vitrification in which liquid nitrogen is applied directly onto the mouse ovaries contained in cryovials. In this way, ovaries could be effectively vitrified by using cryoprotectants with relatively lower concentration, hence lower toxicity. However, this method may not be appropriate for a large number of samples because it requires too much manipulation, and the resultant prolonged time of exposure to CPAs may lead to damage. Wang and colleagues [29] improved the method by introducing acupuncture needles as carriers to hold multiple samples and immersing the samples into liquid nitrogen in the process they called needle immersed vitrification. The method ensured the homogeneity and was faster for a large number of samples. In the present study, we used acupuncture needles as carriers and stored samples in cryovials to meet the requirement of routine use.

Furthermore, it is easier to extrapolate vitrification protocols to preserve the ovarian tissue of local stocks and commercial lines in the poultry industry and endangered wild avian species because there are fewer variables. These variables include the type and concentration of the CPAs and the length of time that samples are exposed to equilibration and vitrification solution.

Apart from the cryopreservation strategy, failure to retain reproductive potential of grafts may also be associated with the transplantation procedure itself (e.g., ischemia-reperfusion injury and failure of the reestablishment of hormonal cycling) [38, 39]. In the prenyon test in this study, only two of nine recipients in the slow-freezing group produced donor-derived offspring, and the offspring from both of them were only donor derived, indicating complete removal of host ovaries but a low rate of recovery of the grafts. In the vitrification group, five of seven recipients produced donor-derived offspring, and all of them produced host-derived offspring as well, indicating incomplete ovarioctomy and regeneration of host ovarian tissue but better recovery of the grafts.

As in rodents, the fragments of quail ovarian tissue for grafting are small, and angiogenesis cannot easily be obtained by surgical reanastomosis (as in the transplantation of larger organs) but merely by the process of revascularization [40]. The wound healing of the host ovary may induce an endogenous process that benefits rebuilding in the vasculature of the grafts, thereby reducing the loss of the follicle pool due to ischemia-reperfusion injury. On the other hand, it has long been known that the ovary is an active endocrine organ, and its activities (including follicle development and ovulation) are dependent on both intraovarian and extraovarian hormones or other signal molecules. The grafts may benefit from the host endocrine products (e.g., growth factors and signal receptors) [41, 42], enabling them to recover faster and more effectively. These postulations require further investigation.

In future practice, instead of removing the entire host ovary, incomplete ovarioctomy could be used to avoid excessive bleeding, which is the primary cause of death during surgery in very small birds [22]. Although this allows the production of host-derived offspring, it may be compensated for by a higher recovery rate. Future studies could attempt to promote revascularization and survival of the grafts by implantation into angiogenic granulose tissue generated by a prepared injury site as suggested by Isrealy et al. [40]. Details such as where and how to create the injury site need to be determined.

In conclusion, our production of live donor-derived offspring from cryopreserved ovarian tissue demonstrated that cryopreservation and transplantation of ovarian tissue can be used as a practical and reliable way to preserve female germplasm in birds. This represents a major step toward the genetic resource conservation of endangered commercial lines of poultry and endangered wild avian species.

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