Optimal vitrification protocol for mouse ovarian tissue cryopreservation: effect of cryoprotective agents and in vitro culture on vitrified–warmed ovarian tissue survival

Hye Won Youm1,2†, Jung Ryeol Lee1,2†, Jaewang Lee1,2, Byung Chul Jee1,2, Chang Suk Suh1,2*, and Seok Hyun Kim2

1Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital, 300 Gumi-dong, Bundang-gu, Seongnam 463-707, Korea 2Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul 110-744, Korea

*Correspondence address. Tel: +82-31-787-7251; Fax: +82-31-787-4054; E-mail: suhcs@snu.ac.kr

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STUDY QUESTION: What is the optimal vitrification protocol according to the cryoprotective agent (CPA) for ovarian tissue (OT) cryopreservation?

SUMMARY ANSWER: The two-step protocol with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 10 min then 20% EG, 20% DMSO and 0.5 M sucrose for 5 min showed the best results in mouse OT vitrification.

WHAT IS KNOWN ALREADY: Establishing the optimal cryopreservation protocol is one of the most important steps to improve OT survival. However, only a few studies have compared vitrification protocols with different CPAs and investigated the effect of in vitro culture (IVC) on vitrified–warmed OT survival. Some recent papers proposed that a combination of CPAs has less toxicity than one type of CPA. However, the efficacy of different types and concentrations of CPA are not yet well documented.

STUDY DESIGN, SIZE, DURATION: A total of 644 ovaries were collected from 4-week-old BDF1 mice, of which 571 ovaries were randomly assigned to 8 groups and vitrified using different protocols according to CPA composition and the remaining 73 ovaries were used as controls. After warming, each of the eight groups of ovaries was further randomly divided into four subgroups and in vitro cultured for 0, 0.5, 2 and 4 h, respectively. Ovaries of the best two groups among the eight groups were autotransplanted after IVC.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The CPA solutions for the eight groups were composed of EDS, ES, ED, EPS, EF, EFS, E and EP, respectively (E, EG; D, DMSO; P, propanediol; S, sucrose; F, Ficoll). The IVC medium was composed of α-minimal essential medium, 10% fetal bovine serum and 10 mIU/ml follicle-stimulating hormone (FSH). Autotransplantation of vitrified–warmed OTs after IVC (0 to 4 h) using the EDS or ES protocol was performed, and the grafts were recovered after 3 weeks. Ovarian follicles were assessed for morphology, apoptosis, proliferation and FSH level.

MAIN RESULTS AND THE ROLE OF CHANCE: The percentages of the morphologically intact (G1) and apoptotic follicles in each group at 0, 0.5, 2 and 4 h of IVC were compared. For G1 follicles at 0 and 4 h of IVC, the EDS group showed the best results at 63.8 and 46.6%, respectively, whereas the EP group showed the worst results at 42.2 and 12.8%, respectively. The apoptotic follicle ratio was lowest in the EDS group at 0 h (8.1%) and 0.5 h (12.7%) of IVC. All of the eight groups showed significant decreases in G1 follicles and increases in apoptotic follicles as IVC duration progressed. After autotransplantation, the EDS 0 h group showed a significantly higher G1 percentage (84.9%) than did the other groups (42.4–58.8%), while only the E5 4 h group showed a significant decrease in the number of proliferative cells (80.6%, 87.6–92.9%). However, no significant differences in apoptotic rates and FSH levels were observed between the groups after autotransplantation.

LIMITATIONS, REASONS FOR CAUTION: The limitation of this study was the absence of in vitro fertilization using oocytes obtained from OT grafts, which should be performed to confirm the outcomes of ovarian cryopreservation and transplantation.

† The first two authors contributed equally to this work.

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**WIDER IMPLICATIONS OF THE FINDINGS:** We compared eight vitrification protocols according to CPA composition and found the EDS protocol to be the optimal method among them. The data presented herein will help improve OT cryopreservation protocols for humans or other animals.

**STUDY FUNDING/COMPETING INTEREST(S):** This study was supported by a grant (No. A120080) from the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea.

**Key words:** ovarian tissue / vitrification / in vitro culture / transplantation / fertility preservation

### Introduction

Recently, the prevalence of cancer has been increasing remarkably, as has the growing population of survivors (Byrne et al., 1987). However, the treatment of cancer using radiotherapy or chemotherapy can cause the depletion of ovarian follicles, premature ovarian failure, and occasionally, permanent infertility in female patients. Thus, the long-term quality of life of cancer survivors has become a major concern. For women with cancer who are at the risk of infertility, cryopreservation of oocytes or embryos is a possible option for fertility preservation, but it is inappropriate for children, adolescents or women whose clinical course does not provide adequate time. Therefore, cryopreservation of ovarian tissues (OTs) became a potential alternative for preserving fertility in women (Donnez and Bassil, 1998). Fertility among female patients can be restored by transplantation of cryopreserved-thawed OT to the women after survival from the cancer (Broecke et al., 2001).

Cryopreservation of OT has been widely studied in various species, including human (Gook et al., 2005), porcine (Imhof et al., 2003), bovine (Lucci et al., 2004) and murine (Newton and Illingworth, 2001). Cryopreservation can be performed using either slow freezing or vitrification. So far, slow freezing has been mainly used to cryopreserve OT, with several reported successful cases (Amorim et al., 2003). Meanwhile, vitrification has been recently used in humans and other animals, with many reports of success (Kagawa et al., 2009; Jee et al., 2010; Chang et al., 2011). In terms of disadvantages, the ice crystal formation in the course of slow freezing harms cells, destroys cell interactions and leads to cell death, whereas ice crystal formation is not required in vitrification because the vitrification solution is ultrarapidly cooled and transformed into a glassy state. However, vitrification requires high cryoprotective agent (CPA) concentrations and cooling rates, which can cause fatal cellular damage and osmotic stress (Zhang et al., 2009), therefore warranting vitrification protocol optimization.

Vitrification has been used as an effective, simple and inexpensive protocol for cryopreservation of sperm (Endo et al., 2012), oocytes (Cai et al., 2005) and embryos (Ali and Shelton, 1993). Although cryopreservation of OT is still relatively at an experimental stage, it is currently considered as a promising and future method for fertility preservation (Isachenko et al., 2003; Hasegawa et al., 2004). Recent reports indicate that follicle morphological integrity and hormone levels do not significantly differ between conventional and rapid freezing (Li et al., 2007). In addition, a high survival rate of oocytes in human tissue was obtained after vitrification (Kagawa et al., 2009). Keros et al. (2009) demonstrated that the ovarian stroma showed a significantly better morphological integrity after vitrification than after slow freezing. However, live birth after transplantation of vitrified/warmed human OT has not yet been reported. In contrast, a worldwide series of 24 live births achieved through the slow freezing procedure has been reported by Donnez et al. (2013).

The types of CPA commonly used in OT vitrification are dimethyl sulfoxide (DMSO), propanediol (PrOH) and ethylene glycol (EG) as permeating CPAs, and sucrose, trehalose and Ficoll as non-permeating CPAs. CPA permeation into the center of the OT predisposes the surface tissue to the risk of excessive toxicity. Therefore, establishing the optimal vitrification protocol is important, with consideration of the CPA type, CPA concentration and equilibration time to suit various types of OT. In an attempt to define an optimal cryopreservation protocol for OT, various CPA compositions have been investigated (Candy et al., 1997; Rodrigues et al., 2004; Luz et al., 2009; Sheikh et al., 2011). Some recent studies proposed that the combination of two types of CPAs is less toxic and more effective than a single type of CPA (Edyta and Ali, 2011). However, the efficacy of CPA compositions and concentrations for OT vitrification are not yet well documented.

The aim of this study was to determine the optimal vitrification protocols for mouse OTs by comparing eight different CPA compositions. In addition, to evaluate the damage caused by vitrification, the vitrified/warmed OTs were in vitro cultured for a short-term period (∼4 h) because an analysis performed immediately after warming may not reveal the actual ovarian survival and function.

### Materials and Methods

#### Experimental animals

Four-week-old BDF1 female mice (Orient Co., Seoul, South Korea) were housed under a 12-h light/dark cycle at 22 °C and fed ad libitum. The experimental protocols and animal handling procedures were performed with the approval of the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital.

#### Vitrification solutions

Whole ovaries were dissected from the BDF1 female mice. Because of the small dimension (∼2 × 2 × 2 mm³) of the mouse ovary, we vitrified whole organs instead of OT slices. Then, the ovaries were randomly assigned to eight groups and vitrified using eight different protocols according to CPA type. The compositions of the CPAs for each group were EDS, ES, ED, EPS, EF, EFS, E and EP, respectively (E, EG; D, DMSO; P, PrOH; S, sucrose; F, Ficoll). The base medium used was Dulbecco’s phosphate-buffered saline (DPBS; Gibco, Paisley, UK) supplemented with 20% fetal bovine serum (FBS; Gibco, Paisley, UK). The experiment protocols are presented in Table I.

#### Vitrification and warming procedures

Intact ovaries were exposed to the equilibration solutions before vitrification. The CPA compositions and exposure times are described in Table I. The
vitrification protocols in this study were based on those previously described in published reports, which we modified in terms of equilibration and vitrification times and treatment temperature to compare the different CPA solutions under the same condition (Isachenko et al., 2003; Choi et al., 2007; Kagawa et al., 2009; Amorim et al., 2011). The ovaries were placed on top of transmission electron microscope copper grids (JEOL, Tokyo, Japan), and the droplet of vitrification solution on the grid was removed through the underlying sterilized filter paper. We put the grids into 1.5-ml cryovials and the droplet of vitrification solution on the grid was removed through the underlying sterilized filter paper. We put the grids into 1.5-ml cryovials and the droplet of vitrification solution on the grid was removed through the underlying sterilized filter paper. We put the grids into 1.5-ml cryovials and the droplet of vitrification solution on the grid was removed through the underlying sterilized filter paper.

**Table 1** Experimental protocols.

<table>
<thead>
<tr>
<th>CPA</th>
<th>EDS</th>
<th>ES</th>
<th>ED</th>
<th>EPS</th>
<th>EF</th>
<th>EFS</th>
<th>E</th>
<th>EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrification protocols</td>
<td>7.5% EG + 7.5% DMSO: 10 min</td>
<td>20% EG + 20% DMSO + 0.5 M sucrose: 5 min</td>
<td>38% EG + 0.5 M sucrose: 5 min</td>
<td>7.5% EG + 7.5% DMSO: 10 min</td>
<td>20% EG + 20% DMSO: 5 min</td>
<td>10% EG + 10% PrOH: 10 min</td>
<td>20% EG + 20% PrOH + 0.5 M sucrose: 5 min</td>
<td>20% EG for 10 min</td>
</tr>
<tr>
<td>CPA, cryoprotective agents; E, ethylene glycol; D, dimethyl sulfoxide (DMSO); P, propanediol (PrOH); S, sucrose; F, Ficoll.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**In vitro culture**

After warming, the ovaries in each group were further randomly divided into four subgroups and in vitro cultured for 0, 0.5, 2 or 4 h, respectively. We regarded the end-point of OT warming as 0 h of in vitro culture (IVC). After collecting fresh ovaries, the ovaries in the control group were cultured in the same manner as described for the eight experimental groups cultured for 0, 0.5, 2 or 4 h, respectively. All the surgical procedures were performed under a laminar flow hood and aseptic conditions.

Using only normal-appearing intact ovaries, follicular normality and apoptosis were assessed by hematoxylin–eosin (H&E) staining and TUNEL assay. The follicle proliferation and endocrine function recovery of OT grafts were evaluated by immunohistochemistry (IHC) for Ki67 and enzyme-linked immunosorbent assay (ELISA) for serum FSH.

**Follicle classification and morphological analysis**

All the OTs that were in vitro cultured and/or autotransplanted were recovered and fixed in 4% buffered paraformaldehyde. Each group of ovaries was embedded in a paraffin block and cut into serial sections of 4- to 5-μm thicknesses. Each paraffin section was stained with Mayer’s H&E solution (Merck, Darmstadt, Germany) to prepare it for assessment and evaluation under light microscopy (Nikon, Tokyo, Japan) at ×400 magnification.

Each follicle type was classified according to the following categories (Lundy et al., 1999):

(i) Primordial: single layer of flattened pre-granulosa cells.
(ii) Primary: single layer of granulosa cells, one or more being cuboidal cells.
(iii) Secondary: two or more layers of cuboidal granulosa cells, with the antrum absent.
(iv) Antral: multiple layers of cuboidal granulosa cells, with the antrum present.

The morphological integrity of each follicle was evaluated using the following criteria (Gandolfi et al., 2006):

(i) Primordial/primary follicle: G1, spherical with even distribution of the granulosa cells; G2, granulosa cells pulled away from the edge of the follicle but with the oocytes still spherical; G3, pyknotic nuclei, misshapen oocytes or vacuolation.
(ii) Secondary/antral follicle: G1, intact spherical follicle with evenly distributed granulosa and theca cells, small space and spherical oocytes; G2, intact theca cells, disrupted granulosa cells and spherical oocytes; G3, disruption and loss of granulosa and theca cells, pyknotic nuclei and missing oocyte.

Figure 1 shows the follicle type and morphological integrity together as the basis of mouse follicular development. To avoid miscounting, the follicles were analyzed in only one section per ovary when they contained oocytes.

**Analysis of apoptosis**

The paraffin sections of the apoptotic OTs were assessed using a TUNEL assay. In brief, after deparaffinization and rehydration, the sections were treated with 0.1% Triton X-100 (Amresco, Solon, OH, USA) in citrate buffer for 15 min at RT and then incubated with a TUNEL reaction mixture (enzyme-to-label solution, 1:9) for 1 h at 37°C in a humidified chamber in the dark, followed by rinsing with DPBS. After incubation, the slides were mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and examined under an inverted Zeiss AX10 microscope (Carl Zeiss, Oberkochen, Germany).

Green fluorescence was visible in the cells that tested positive by TUNEL assay, by using an excitation wavelength ranging from 450 to 500 nm and detection capability ranging from 515 to 565 nm. DAPI reached excitation at ≏360 nm and emitted blue fluorescence in all the nuclei at ≏460 nm when bound to DNA. When apoptosis was observed in more than 30% cells in a follicle, the follicle was classified as apoptotic.

**Auto-transplantation**

The ovaries that were vitrified with the EDS or ES protocol and in vitro cultured for 0, 2 and 4 h, respectively, were autotransplanted beneath the bilateral kidney capsule at 1 week after ovariectomy. The mice were anesthetized with an intraperitoneal injection of a solution containing ketamine (0.15 mg/g of body weight) and xylazine (0.016 mg/g of body weight). The animals were killed via cervical dislocation at 3 weeks after transplantation. All the surgical procedures were performed under a laminar flow hood and aseptic conditions.

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Measurement of FSH level

Whole blood samples were obtained from the OT transplanted mice, and serum was separated by centrifugation. Serum FSH concentrations were measured, for evaluation of endocrine function after recovery of the OT grafts, using an ELISA kit (Endocrine Technologies, Newark, USA), according to the manufacturer’s instructions. The intra- and inter-assay coefficients of variation were 6.35 and 5.88%, respectively, with a sensitivity of 0.5 ng/ml.

Proliferation assay

The paraffin sections were assessed using Ki67, a nuclear antigen associated with cell proliferation (late G1, S, G2 and M phases, but not G0 phase), to detect the proliferating follicles. All reagents were purchased from Dako (Glostrup, Denmark), except the anti-Ki67 antibody (ab15580; Abcam, Cambridge, UK). The paraffin sections were deparaffinized, rehydrated and pretreated using microwave-mediated Target Retrieval Solution (pH 6) for 20 min. After antigen retrieval, the sections were treated with a peroxidase-blocking solution for 5 min at RT, followed by incubation with anti-Ki67 antibody at a dilution of 1:1000 for 1 h at RT. After rinsing three times with wash buffer, the sections were treated with the EnVision + horseradish peroxidase for 30 min and then treated with liquid 3,3′-diaminobenzidine tetrahydrochloride + substrate for 10 min at RT. All the sections were counterstained with hematoxylin and dehydrated. Finally, the slides were mounted with a mounting medium and examined under an

Table II
The numbers and proportions of morphologically intact follicles (G1) according to the vitrification protocols and IVC durations.

<table>
<thead>
<tr>
<th>IVC duration</th>
<th>CPA</th>
<th>Follicle No.</th>
<th>G1 No.</th>
<th>G1%</th>
<th>Follicle No.</th>
<th>G1 No.</th>
<th>G1%</th>
<th>Follicle No.</th>
<th>G1 No.</th>
<th>G1%</th>
<th>Follicle No.</th>
<th>G1 No.</th>
<th>G1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td></td>
<td>362</td>
<td>265</td>
<td>73.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>309</td>
<td>190</td>
<td>61.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>292</td>
<td>158</td>
<td>54.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>239</td>
<td>102</td>
<td>42.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5 h</td>
<td></td>
<td>323</td>
<td>206</td>
<td>63.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>295</td>
<td>173</td>
<td>58.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>314</td>
<td>172</td>
<td>54.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>305</td>
<td>142</td>
<td>46.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 h</td>
<td></td>
<td>333</td>
<td>210</td>
<td>63.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>311</td>
<td>131</td>
<td>42.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>299</td>
<td>110</td>
<td>36.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>267</td>
<td>77</td>
<td>28.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 h</td>
<td></td>
<td>275</td>
<td>159</td>
<td>57.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>285</td>
<td>114</td>
<td>40.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>258</td>
<td>104</td>
<td>40.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>238</td>
<td>53</td>
<td>22.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPA</td>
<td>361</td>
<td>203</td>
<td>56.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>328</td>
<td>118</td>
<td>36.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>292</td>
<td>75</td>
<td>25.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>328</td>
<td>47</td>
<td>14.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td></td>
<td>211</td>
<td>114</td>
<td>54.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>223</td>
<td>83</td>
<td>37.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>248</td>
<td>67</td>
<td>27.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>322</td>
<td>85</td>
<td>26.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 h</td>
<td></td>
<td>268</td>
<td>143</td>
<td>53.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>325</td>
<td>144</td>
<td>44.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>278</td>
<td>75</td>
<td>27.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>242</td>
<td>51</td>
<td>21.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>E</td>
<td>221</td>
<td>103</td>
<td>46.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>220</td>
<td>56</td>
<td>25.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>326</td>
<td>72</td>
<td>22.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>179</td>
<td>37</td>
<td>20.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td></td>
<td>223</td>
<td>94</td>
<td>42.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>326</td>
<td>118</td>
<td>36.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>299</td>
<td>96</td>
<td>32.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>148</td>
<td>19</td>
<td>12.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The ovaries in the control group were in vitro cultured without vitrification. IVC, in vitro culture; CPA, cryoprotective agents; E, ethylene glycol; D, dimethyl sulfoxide (DMSO); P, propanediol (PrOH); S, sucrose; F, Ficoll.

Different superscript letters indicate statistically significant differences (P < 0.05) and the superscripts were used for each group separately.

Figure 1
Morphological classification of mouse ovarian follicles. Horizontal axis represents the developmental stage of follicles. Vertical axis represents the quality of follicles. G1, G2 and G3 mean good, fair and bad, respectively.
inverted Zeiss AX10 microscope (Carl Zeiss, Oberkochen, Germany). The sections were analyzed by two technologists. The follicles showing at least one Ki67-positive granulosa cell were regarded as proliferating (Dolmans et al., 2007).

**Statistical analyses**

The proportions of the follicle stages and normality in each sample were calculated for each group. Data were analyzed using the SPSS version 12.0 software (SPSS, Inc., Chicago, IL, USA). The proportions were compared using the $\chi^2$ test, and values were considered significant when $P < 0.05$.

## Results

### Morphological analysis of the vitrified and in vitro cultured mouse OTs

A total of 10,073 follicles from the control and eight experimental groups were morphologically evaluated by histological analysis. Table II shows the number and proportion of the morphologically intact follicles (G1) according to the vitrification protocol and IVC duration. The stromal tissue at 0 h of IVC had a relatively normal structure and preserved morphological integrity, without a significant sign of pyknotic nuclei and

![Figure 2](http://example.com/figure2.png)

**Figure 2** Histology of vitrified and in vitro cultured ovaries (× 100). Horizontal axis represents the IVC durations. Vertical axis represents the control and eight vitrification protocols [IVC, in vitro culture; E, ethylene glycol; D, dimethyl sulfoxide (DMSO); P, propanediol (PrOH); S, sucrose and F, Ficoll].
edema. However, in all of the eight groups, the numbers of morphologically intact follicles decreased and the prevalence of stromal cell damage increased, accompanied with cell losses, vacuoles, granulated oocytes and follicular detachment, as the IVC duration increased (Fig. 2).

In all the groups, the G1 ratios decreased as the IVC duration increased. Among the eight experimental groups, the EDS group had the highest G1 proportion across all the IVC durations (0 h: 63.8%, 0.5 h: 58.6%, 2 h: 54.8% and 4 h: 46.6%; Fig. 3). The decrease in G1 follicle ratio in the EDS group was not significant until 4 h of IVC but was significant at 0.5 h of IVC in the other groups, except the EP group. In the EP group, the decrease in the G1 follicle ratio became significant at 2 h of IVC. The most significant depletion of intact follicle was observed in the EP group between 0 h (42.2%) and 4 h (12.8%), and in the E group at 0.5 h (25.5%) and 2 h (22.1%).

**Apoptosis in the vitrified and in vitro cultured mouse OTs**

A total of 8486 follicles from the control and eight experimental groups were evaluated for apoptosis by TUNEL assay. Table III shows the number and proportion of apoptotic follicles after vitrification and/or IVC. In all the groups, the proportion of apoptotic follicles that were stained green by the TUNEL assay increased (Fig. 4), and the apoptotic follicle percentages were significantly increased (Fig. 5), with the IVC duration. Among the eight vitrification groups, the EDS group had the lowest apoptotic follicle percentages across all the IVC durations (0 h: 8.1%, 0.5 h: 12.7%, 2 h: 30.9% and 4 h: 56.2%), indicating coherence with the morphological analysis results presented in Figs 2 and 3.

**Autotransplantation of the vitrified and in vitro cultured mouse OTs**

The OTs vitrified using the EDS or ES protocol were autotransplanted after 0, 2 and 4 h of IVC, respectively. The mean number of follicles per section was not significantly different between all the groups (EDS

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**Table III**

The numbers and proportions of apoptotic [TUNEL (+)] follicles according to the vitrification protocols and IVC durations.

<table>
<thead>
<tr>
<th>IVC duration</th>
<th>CPA Follicle No.</th>
<th>TUNEL (+) No.</th>
<th>TUNEL (+) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>147</td>
<td>6</td>
<td>4.2%</td>
</tr>
<tr>
<td>0.5 h</td>
<td>254</td>
<td>19</td>
<td>7.5%</td>
</tr>
<tr>
<td>2 h</td>
<td>265</td>
<td>31</td>
<td>11.9%</td>
</tr>
<tr>
<td>4 h</td>
<td>273</td>
<td>39</td>
<td>14.3%</td>
</tr>
</tbody>
</table>

*Different superscript letters indicate statistically significant differences (P < 0.05).* and the superscripts were used for each group separately.
however, the proportion of intact follicles (G1) in the EDS 0 h group (84.9%) was highest when compared with those in the other groups (EDS 2 h: 47.6% and 4 h: 51.6%; ES 0 h: 58.8%, 2 h: 49.7% and 4 h: 49.4%; Fig. 6). No significant difference in the proportions of apoptotic follicles was found between all the groups (EDS 0 h: 2.98%, 2 h: 1.72% and 4 h: 3.51%; ES 0 h: 1.16%, 2 h: 2.08% and 4 h: 1.71%). Follicular proliferation of OT grafts was measured by evaluating the portion of follicles that were stained brown by Ki67 IHC (Fig. 7a). In all the groups, the proportion of proliferating follicles was high, but a significant decrease in Ki67-positive follicles was only observed in the ES 4 h group (EDS 0 h: 92.9%, 2 h: 89.0% and 4 h: 87.6%; ES 0 h: 88.4%, 2 h: 87.6% and 4 h: 80.6%; Fig. 7b). There were no significant differences in FSH levels among the groups (EDS 0 h: 1.64 ± 0.34, 2 h: 1.80 ± 0.33, 4 h: 1.71 ± 0.27; ES 0 h: 1.69 ± 0.30, 2 h: 1.65 ± 0.22, 4 h: 1.36 ± 0.23 ng/ml).

**Discussion**

In the present study, eight vitrification solutions containing different CPAs (EG, PrOH and DMSO), with or without sucrose or Ficoll, were used to vitrify mouse OTs. They were compared in terms of normality.
of mouse follicles based on morphological changes and apoptosis ratios after vitrification and short-term IVC. We found that the solutions containing EG, DMSO and sucrose retained follicular morphological integrity most effectively and that after 4 h of IVC, only the OTs vitrified with the EDS solutions maintained a percentage of intact follicles, which was comparable with the cultured fresh control OTs that were cultured but not vitrified.

The most common approaches to minimize CPA toxicity are modifying CPA composition and combining different permeable CPAs (Pegg, 2005). Combining different CPAs at low individual concentrations to achieve a tolerable total CPA concentration can decrease the specific toxicity of each CPA (Wusteman et al., 2004). Each CPA has its own specific properties. EG is the most commonly used CPA for vitrification because of its low toxicity but high diffusivity into cells (Bautista and Kanczuga, 1998). In addition, EG (62.07 kDa) has a lower molecular weight than DMSO (78.13 kDa) and PrOH (76.09 kDa), which in turn facilitates better permeation into OT. Recently, Mochida et al. (2013) demonstrated that a very high osmolality of a vitrification solution caused embryos to float near the surface of the solution. This phenomenon is common to other EG-based vitrification methods and could be overcome by using a solution containing DMSO to reduce its viscosity. Similarly, our results indicate that the use of a solution containing EG, DMSO and sucrose with a stepwise increase in their concentrations can achieve successful OT dehydration and vitrification.

The CPA is a critical determining factor of successful cryopreservation. Membrane-permeating CPAs (e.g. EG, DMSO and PrOH) decrease the freezing point of a solution and prevent damage from high electrolyte concentrations (Rall et al., 1984). Membrane non-permeating CPAs (e.g. sucrose and Ficoll) act as an osmotic buffer against the stress incurred during addition and removal of penetrating CPAs (Mandelbaum et al., 1988). Santos et al. (2006) reported that all cryopreservation treatments significantly reduced the percentage of viable pre-antral follicles when compared with the control (92%) and that the highest percentage of viable frozen/thawed pre-antral follicles was found after cryopreservation of OT in a combination of sucrose and EG (72%). Several studies proposed that the addition of an optimal concentration of sucrose to a vitrification medium enhances the morphological features of frozen OTs (Marsella et al., 2008). However, sucrose addition at high concentrations conferred great osmotic stress and increased damage in OTs, in accordance with the proposal of Zhang et al. (2009) that the addition of 0.4 or 0.8 M sucrose to a vitrification solution can be beneficial to vitrified OTs, whereas 1.6 M sucrose proves detrimental. In the present study, the intact and apoptotic follicle ratios in the EDS, ES and EPS groups were better compared with those in the ED, E and EP groups, respectively. Furthermore, the intact and apoptotic follicle ratios in the ES group were better compared with those in the EFS group. These results confirm that sucrose addition can improve the outcome of vitrification and that the addition of non-permeating CPAs at high concentrations can cause harmful effects, as demonstrated in the previous studies.

Sufficient diffusion of CPAs through the stroma and granulosa cells to the oocytes is important because stromal cells play a critical role in the proliferation and differentiation of granulosa cells and oocytes (Hovatta et al., 1999; Liu et al., 2000). However, an analysis performed immediately after warming may not accurately reveal the actual cryogenic damage. Several authors have reported that short-term IVC can be used as a valuable tool to verify the developmental ability of and toxicity in cryopreserved follicles (Rodrigues et al., 2005; Choi et al., 2008). Rodrigues et al. (2006) reported that there was a reduction in the percentages of viable follicles after 1- and 5-day IVC of cryopreserved OTs and pre-antral follicles. Carvalho et al. (2011) showed that 24 h of IVC reduced the percentages of morphologically normal follicles in fresh or vitrified ovarian fragments, relative to non-cultured fresh samples. The optimal protocol for OT vitrification should maintain follicular structure and provide a higher capacity to survive throughout the subsequent IVC. Thus, we used short-term IVC as an additional tool to precisely evaluate the vitrification procedures. In all of the fresh control and eight experimental groups, follicular loss and stromal damage worsened as the IVC...
duration was extended, in concordance with the result of the previous study (Oskam et al., 2011). However, we observed that when the OTs were vitrified in a combination of EDS solutions, their morphological integrities were maintained for at least 2 h of IVC and the proportion of intact follicle in the EDS group was highest among those of all the groups across all IVC durations. These results support the vitrification-warming results, which indicate that EDS was the best among the eight protocols. Moreover, our results altogether suggest that the most effective protocol for OT vitrification was EDS.

We cultured whole intact ovaries instead of ovarian follicles in the culture medium containing α-MEM, 10% FBS and 10 mIU/ml recombinant FSH. This condition was simple but not ideal for the culture whole ovaries because our purpose for the short-term IVC was to compare the extent of damage and function recovery ability of vitrified–warmed OTs between the eight experimental groups. In other studies on follicular survival after cryopreservation, IVC was performed for 7 days (Faustino et al., 2010), 5 days (Rodrigues et al., 2005), 24 h (Santos et al., 2006) and 2 h (Borges et al., 2009). Oskam et al. (2011) reported no significant difference between the 4-h or 24-h IVC of frozen-thawed OTs, similar to our preliminary data. Our preliminary study showed that the proportions of G1 follicles were 51.0% (n = 210) at 4 h IVC and 39.0% (n = 121) at 24 h IVC, indicating no significant difference (P = 0.088). Therefore, we regarded that 0- to 4-h IVC was an efficient assessment duration; accordingly, instead of long-term IVC, OT transplantation was performed after short-term IVC in this study. Many follicles were damaged after vitrification and IVC, but some intact follicles remained, with the best potential to survive. Therefore, OT transplantation was necessary to observe the outcomes of intact follicles after vitrification and IVC. In this study, we performed autotransplantation using vitrified–warmed and in vitro cultured OTs from either the EDS or ES protocol because they showed the best follicular normality and morphological integrity after vitrification and IVC. Similar to the IVC result, the proportion of intact follicles in the EDS 0 h group after autotransplantation was significantly higher compared with the proportions in all the other groups, suggesting that the EDS protocol was better than the ES protocol and that IVC had a detrimental effect on follicle survival. The poor follicular status after IVC was not restored after grafting. In addition, to confirm normal ovarian graft growth and function, we measured the proliferation ratios of follicles (Ki67 IHC) and FSH levels. The Ki67-positive proliferation ratio of the follicles ranged from 80.6 to 92.9%, and the FSH levels in all groups were ~2 ng/ml (normal limit: lower than 10 ng/ml (Shikanov et al., 2011)). These results indicate that the transplanted OTs were growing and recovered their function.

The EDS protocol showed the best results after vitrification, IVC and transplantation. In addition, these results could be interpreted as indicating that the EDS protocol had the best tolerability to cryo-injury, ischemic damage, oxidative stress and the other possible damages caused by vitrification and transplantation.

The present study was performed using mice ovaries; however, its findings could not be automatically applied to other mammals, as doing so would require modification of the CPA combinations and exposure times according to species. Nevertheless, the results of the present study may be considered useful as a basic protocol for obtaining an optimal CPA composition because it dealt with various CPA solutions under the same conditions. To assess the quality of the vitrified OTs, we used H&E staining for morphological examination and a TUNEL assay for apoptosis, as previous studies had proven that these methods are useful to evaluate and compare the outcomes of different cryopreservation protocols for OTs (Rimon et al., 2005; Fauqe et al., 2007; Martinez et al., 2007). In addition, we performed Ki67 IHC and ELISA for serum FSH to assess ovarian function and follicular growth. We did not use any additional assessment tools to verify follicle quality because these methods already provided a sufficient baseline evaluation.

In conclusion, the findings of the present study suggest that the combination of EG, DMSO and sucrose (the EDS protocol) is one of the most effective protocols for mouse OT preservation. Moreover, short-term IVC and transplantation are essential to precisely evaluate the quality and function of vitrified OTs. However, further research is necessary.
to modify this protocol for OT vitrification in other species such as bovine and human.

**Authors’ roles**

The authors were responsible for the following roles in the study. H.W.Y.: conception and design, experiments, data interpretation, drafting and revision of the article; J.R.L.: conception and design, data interpretation, drafting and revision of the article; J.L.: conception and design, experiments, data analysis and interpretation, revision of the article; B.C.J.: conception and design, data analysis and interpretation, revision of the article; C.S.S.: conception and design, data analysis and interpretation, revision of the article; S.H.K.: conception and design, data analysis and interpretation, revision of the article.

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**Conflict of interest**

The authors have no conflict of interest to declare.

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