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—Full Paper—

Ethylene Glycol-supplemented Calcium-free Media Improve Zona Penetration of Vitrified Rat Oocytes by Sperm Cells

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Abstract. Cryopreservation of matured oocytes is a useful technique because the oocytes can be used for some assisted reproductive technologies after warming. Even though rats, like mice, have been used in various research fields including reproductive technology, information about cryopreservation of rat oocytes is limited. The objective of the present study was to improve the vitrification protocol for matured rat oocytes. To determine the optimal equilibration time, oocytes were equilibrated in 7.5% ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) + 20% fetal calf serum (FCS) for 1, 4, 7 or 10 min at 24 C and then 15% EG + 15% DMSO + 0.5 M sucrose + 20% FCS for 1 min at 24 C before being plunged into liquid nitrogen on Cryotops. Oocytes exposed to equilibration medium for 4 min showed higher survival and cleavage rates after artificial activation than those of oocytes exposed for 1, 7 or 10 min. The survival and cleavage rates of vitrified oocytes after activation were 98.3 and 78.4%, respectively. However, the perivitelline spaces of most of the vitrified/warmed oocytes (6/168, 3.6%) could not be penetrated by sperm after *in vitro* fertilization, and cortical granule exocytosis (CGE) was observed in the oocytes. Therefore, the inhibitory effect of calcium and cryoprotectants in vitrification medium on CGE was examined. In most of the oocytes vitrified in calcium-free media, CGE was strongly suppressed independent of cryoprotectants. Oocytes vitrified in EG-supplemented calcium-free media showed high survival rates after warming (79.4%). After artificial activation, the cleavage and blastocyst formation rates of the oocytes were also high (72.8 and 23.1%, respectively). The zona penetration rate of vitrified/warmed oocytes was dramatically improved by using EG-supplemented calcium-free media after *in vitro* fertilization (111/155, 63.9%). Thus, our data suggest that EG-supplemented calcium-free media improve zona penetration of vitrified rat oocytes by sperm cells.

Key words: Cryopreservation, Oocytes, Rat, Vitrification

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Cryopreservation of germ cells and reproductive organs is a useful and important technology for efficient production and maintenance of transgenic, mutant, knock-out and other animals. In particular, embryo and sperm cryopreservation has been routinely used for not only efficient production of experimental animals but also clinical medicine. On the other hand, the most desired germ cells for freezing or vitrification are matured oocytes because successfully cryopreserved oocytes can be used for *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) or somatic-cell nuclear transfer (SCNT) [1, 2]. Although successful freezing or vitrification of oocytes has been reported in several mammalian species [3–6], the survival is generally low. Especially in rats, a high rate of successful vitrification of early-stage embryos has not been obtained until recent years, possibly because rat germ cells seem to be sensitive to cryopreservation [7, 8].

The cryoprotective agent (CPA) is the key to the success of cryopreservation. It is understood that the CPA affects survival and development of the preserved oocytes. Moreover, not only the kind of CPA but also its concentration in cryopreservation media is important for the success of oocyte vitrification. In general, use of high concentrations of CPAs potentially increases the toxicity to oocytes. In addition, increasing the volume of the vitrification solution interferes with the survival of vitrified-warmed oocytes [9]. Therefore, a smaller volume of vitrification solution in the container is key to obtaining higher success rates of vitrification. Numerous devices and methods have been developed to achieve small volumes of vitrification solution. An electron microscope grid [10], gel-loading tip [11], open pulled straws (OPS) [12], CryoLoop [13], solid surface vitrification [14], microdrops [15] and nylon mesh [16] have been developed to minimize the volume of vitrification solution.

The Cryotop (a container for oocytes and embryo vitrification) is an alternative device has a thin strip of plastic film [17]. Since almost all medium can be removed before plunging oocytes or embryos into LN_2, the oocytes or embryos are only covered with a very thin solution layer, and the capped Cryotop is then directly plunged into liquid nitrogen [18]. In rabbit embryos, it has been shown that vitrification with a Cryotop yields higher post-warming

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survival than either the gel-loading tip or the CryoLoop [11]. In pigs, it has been reported that the Cryotop method is superior to the OPS technique for vitrification of matured porcine oocytes [19]. We pioneered use of the Cryotop for vitrification of pronuclear rat embryos at a high success rate [8]. To date, Cryotop methods have been used for vitrification of oocytes and/or embryos in various other species including rabbits [11], humans [20], cattle [21], minke whales [22], pigs [23], buffalo [24], cats [25], horses [26] and sheep [27, 28]. These reports strongly suggest that the Cryotop is one of the most powerful devices for vitrification of mammalian oocytes and embryos. However, the Cryotop method has not been applied to vitrification of unfertilized oocytes in rats.

On the other hand, Larman *et al*. [9] demonstrated that exposure to vitrification solution containing CPAs causes a rise of intracellular calcium in mouse oocytes. A rise of intracellular calcium in oocytes is involved in exocytosis of cortical granules, resulting in the occurrence of zona hardening known as the zona reaction [29]. The zona reaction is an obstacle to penetration of sperm into perivitelline spaces of oocytes [29]. Therefore, suppression of intracellular calcium rise may improve the fertility and developmental ability of vitrified oocytes. In this present study, we attempted to optimize the conditions of vitrification for rat oocytes. In particular, the effect of calcium and cryoprotectants in vitrification medium on cortical granules exocytosis (CGE) was examined.

Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All procedures for handling and treatment of animals adhered to the guidelines established by the Animal Research Committee of Azabu University.

Oocyte preparation

Specific-pathogen-free Crj:Wistar female rats (4 to 5 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The rats were housed in an environmentally controlled room with a 12-h dark/12-h light cycle at a temperature of 23 ± 2 C and humidity of 55 ± 5% with free access to laboratory diet and filtered water. The females were superovulated by intraperitoneal injections of 300 IU/kg equine chorionic gonadotropin (eCG; Nippon Zenyaku Kogyo, Tokyo, Japan) and 300 IU/kg human chorionic gonadotropin (hCG; Asuka Pharmaceutical, Tokyo, Japan) at 48-h intervals, as previously reported by Seita *et al*. [30]. At 18 h after the hCG injection, cumulus-oocyte complexes were collected from the oviductal ampullae of the donor females with modified Dulbecco's phosphate buffered saline (PB1) [31] supplemented with 0.1% hyaluronidase. In some experiments, calcium-free PB1 [PB1(–)] was used for collection. After the cumulus cells were removed, the denuded oocytes were washed 3 times with PB1 and kept in the same medium at 37 C until being subjected to treatments. In some experiments, oocytes were used for *in vitro* fertilization (IVF).

Experimental design

Vitrification was carried out by the Cryotop method as previously described [8] with some modifications. First, the optimal exposure time in equilibration solution for vitrification was evaluated. Oocytes were placed in equilibration solution composed of 7.5% (v/v) ethylene glycol (EG) + 7.5% (v/v) DMSO + 20% (v/v) fetal calf serum (FCS) in PB1 for 1, 4, 7 or 10 min at 24 C. After exposure to equilibration solution, the oocytes were transferred into vitrification solution composed of 15% EG + 15% DMSO + 0.5 M sucrose + 20% FCS in PB1 at 24 C. The oocytes were placed on the top of a Cryotop (Kitazato Supply, Shizuoka, Japan) in a small volume of vitrification solution at 24 C. The Cryotop was plunged into liquid nitrogen when the oocytes were exposed to the vitrification solution for 1 min and stored for at least 1 week. The oocytes were warmed by immersing the Cryotop into warming solution composed of 0.5 M sucrose + 20% FCS in PB1 at 37.5 C for 5 min and then exposed to PB1 supplemented with 20% FCS for 5 min. After warming, the surviving oocytes were morphologically evaluated and then used for artificial activation. The oocytes were activated by the following protocol, and the survival and cleavage rates were evaluated at 0 and 24 h after artificial activation. Artificial activation was carried out by a combined treatment with ethanol and 6-dimethylaminopurine (6-DMAP) according to our previous report [32]. In brief, oocytes were put in a fertilization medium (modified Rat 1-cell Embryo Culture Medium (mR1ECM) containing 110 mM NaCl and 4 mg/ml bovine serum albumin (BSA) and omitting polyvinyl alcohol (PVA) [33]) supplemented with 7% (v/v) ethanol (Kanto Chemical, Tokyo, Japan) for 3 min. After being washed 3 times in mR1ECM supplemented with 1 mg/ml PVA [34], oocytes were cultured in mR1ECM supplemented with 2 mM 6-DMAP for 4 h to form diploids and further cultured in mR1ECM without 6-DMAP.

The effect of vitrification on development of the oocytes was then compared. As the vitrification group, oocytes were exposed to equilibration solution for 4 min and then vitrified as described above. After warming, the oocytes were activated as described above. In the fresh group, oocytes were treated with ethanol and 6-DMAP as mentioned above. In the exposure group, oocytes were placed in equilibration solution for 4 min and then vitrification solution for 1 min. After exposure to vitrification solution, oocytes were transferred to warming solution and treated in the same manner as vitrified oocytes. After several washings in fertilization medium, oocytes were treated with ethanol and 6-DMAP. At the end of 6-DMAP treatment, survival of the oocytes was evaluated. The oocytes were further cultured for 18 h, and cleavage of the oocytes was examined.

To clarify the developmental ability of vitrified oocytes after fertilization, IVF was carried out as previously reported [30]. In brief, cauda epididymides were collected from male rats (12–24 weeks old) at 24 C. Epididymal sperm were counted by hemacytometer, and sperm motility and viability were evaluated. More precisely, the sperm motility was assessed visually and determined by direct observation at 37.5 C under light microscopy at 100 ×. Sperm was directly added to a 200-μl paraffin-oil-covered drop of mR1ECM (final sperm concentration was 5×10^5 sperm/ml) and then cultured for 5 h. Collected oocytes were transferred into drops containing the sperm and co-cultured at 37.5 C under 5% CO_2 in air for 10 h. After culture, oocytes were transferred into a 100 μl drop of mR1ECM and then evaluated using an inverted phase-contrast microscope (Olympus, IX70). Oocytes having accessory sperm

Table 1. Effect of duration of exposure to equilibration medium on survival of virtified-warmed rat oocytes

Exposure duration (min)	No. examined	No. surviving (%)	No. surviving after activation (%)	No. cleaved (%)
1	73	50 (71.2 ± 10.1)[a]	46 (66.9 ± 10.3)[ab]	38 (56.7 ± 10.9)[ab]
4	71	69 (97.8 ± 2.2)[b]	64 (89.9 ± 4.6)[a]	55 (74.4 ± 12.4)[a]
7	83	64 (76.0 ± 7.2)[a]	58 (69.1 ± 5.9)[ab]	39 (46.9 ± 2.1)[ab]
10	73	46 (61.8 ± 9.5)[a]	43 (58.1 ± 9.5)[b]	16 (21.8 ± 2.0)[b]

[a,b] Different superscripts within columns denote significant differences (P<0.05).

Table 2. Development of vitrified-warmed rat oocytes after artificial activation

Treatment	No. examined	No. surviving (%)	No. surviving after activation (%)	No. cleaved (%)
Control (Fresh)	90	90 (100)	90 (100)	82 (91.1 ± 1.3)
Exposure	94	94 (100)	88 (93.6 ± 6.5)	78 (88.6 ± 6.5)
Vitrification	86	84 (98.3 ± 1.7)	79 (91.9 ±3.3)	69 (78.4 ± 8.4)

There were no significant differences among any groups (P<0.05).

inside the zona pellucida were determined to be penetrated. Oocytes having two pronuclei (PN) were also determined to have formed PN.

The effect of the vitrification procedure on CGE was evaluated. The CGE of fresh, activated and vitrified oocytes was examined according to a previous study [29] with some modifications. In brief, oocytes were fixed in 3% (v/v) paraformaldehyde, 0.2% (v/v) Triton-X 100 and 0.1% (w/v) PVA in Dulbecco's phosphate-buffered saline (DPBS; Nissui Pharmaceutical, Tokyo, Japan) for 30 min at 24 C. After washing three times in DPBS supplemented with 0.1% (w/v) PVA (PVA-DPBS), the oocytes were blocked with 1% (w/v) BSA and 10% (v/v) normal goat serum (Cedarlane Laboratories, Hornby, ON, Canada) in PVA-DPBS for 40 min at 24 C. After blocking, the oocytes were treated with fluorescein isothiocyanate (FITC)-labeled Lens culinaris agglutinin (LCA) (1:100) and 1% (w/w) BSA in PVA-DPBS for 30 min at 24 C. The stained oocytes were washed three times in PVA-DPBS and then mounted using the Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were obtained using a confocal laser scanning microscope (TCS-E, Leica Microsystems, Tokyo, Japan). As the control, fresh oocytes were used for CGE staining. As the activation group, fresh oocytes were treated with ethanol and then used for CGE staining. Oocytes exposed to equilibration and vitrification solution only were defined as the exposure group. In each group, more than 30 oocytes were used for CGE staining, and almost all oocytes in each group showed similar levels of intensity.

The effects of calcium and CPAs on CGE and development of vitrified oocytes were examined. Oocytes were collected in PB1(–) and then vitrified in calcium-free media as described above. Warming of the vitrified oocytes was carried out as described above but using calcium-free warming solution. Three different groups were designed as follows: EG and DMSO group [ED(–)], EG alone group [E(–)] and DMSO alone group [D(–)]. Oocytes were also vitrified in a medium that contained calcium, EG and DMSO [ED(+)]. After warming, CGE in these oocytes was evaluated as described above. Development of the vitrified oocytes after artificial activation to the 2-cell stage and blastocyst stage was examined at 24 h and 120 h, respectively. More than 70 oocytes were used for each treatment group. Oocytes vitrified in EG-supplemented calcium-free medium were also used for IVF as described above. Fresh oocytes were used for IVF as the control.

Statistical analysis

Each experiment included at least three replicates. Statistical analysis of data except for IVF data, was carried out by Fisher's protected least significant difference (PLSD) procedure using the Statview program (Abacus Concepts, Berkeley, CA, USA). IVF data were statistically compared by Student's *t*-test using the Statview program. All percentage data were subjected to arcsine transformation before statistical analysis. P<0.05 was considered significant. Data are shown as means ± standard error of means (SEM).

Results

The effect of exposure time to the equilibration solution on survival and development after artificial activation of oocytes is shown in Table 1. Survival of oocytes exposed for 4 min (97.8 ± 2.2%) was significantly higher than those of oocytes exposed for 1, 7 or 10 min (71.2 ± 10.1, 76.0 ± 7.2 or 61.8 ± 9.5%, respectively; P<0.05). Survival after artificial activation in the 4-min group (89.9 ± 4.6%) was also higher than those in the 1-min (66.9 ± 10.3%), 7-min (69.1 ± 5.9%) and 10-min (58.1 ± 9.5%) groups. Moreover, the cleavage rate after artificial activation in the 4-min group (74.4 ± 12.4%) was higher than those in the 1-min (56.7 ± 10.9%), 7-min (46.9 ± 2.1%) and 10-min (21.8 ± 2.0%) groups. Therefore, 4 min was used as the optimal exposure time in the sub-

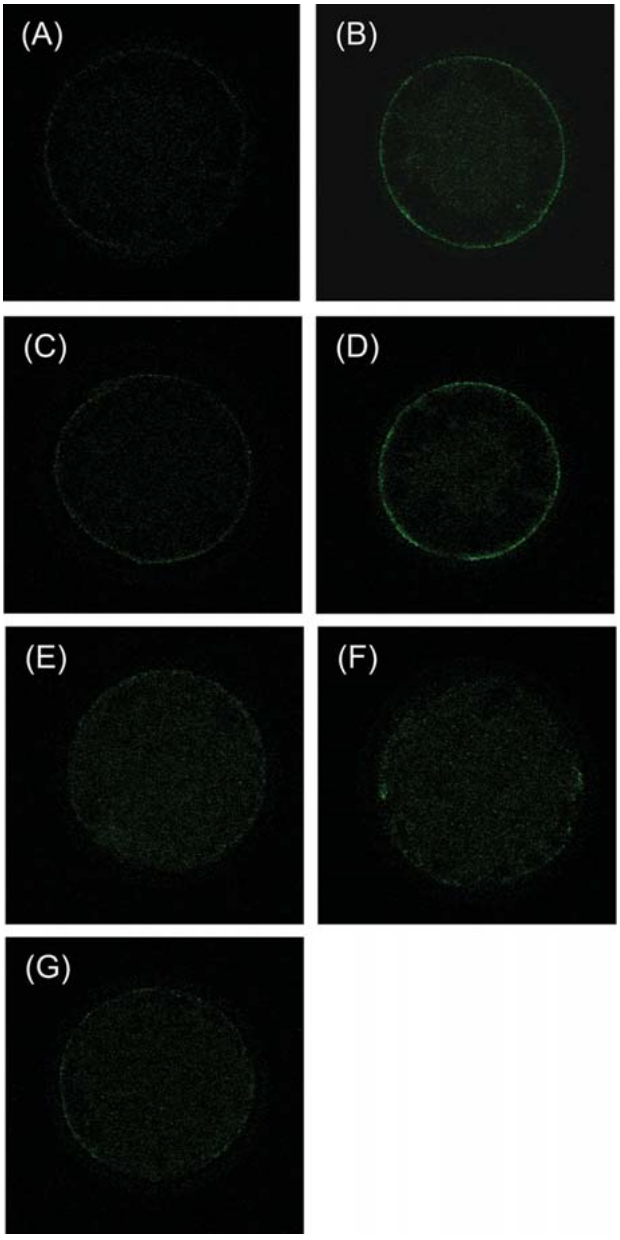

Fig. 1. The effect of vitrification on cortical granule exocytosis of rat oocytes. (A) Control: Fresh oocytes. (B) Activation: Oocytes treated with 7% ethanol for 3 min. (C) Exposure: Oocytes were exposed to vitrification medium. (D) Vitrification: Oocytes were vitrified in EG- and DMSO-supplemented medium. (E) EG(–): Oocytes were vitrified in calcium-free, EG- and DMSO-supplemented medium. (F) E(–): Oocytes were vitrified in calcium-free EG-supplemented medium (G) D(–): Oocytes were vitrified in calcium-free DMSO-supplemented medium. Green fluorescence shows FITC-LCA. In each group, more than 30 oocytes were used for CGE staining, and almost all oocytes in each group showed similar levels of intensity.

sequent experiments. The survival and development after artificial activation of vitrified oocytes are shown in Table 2. All control (fresh) and exposed oocytes survived. The control and exposed oocytes also showed high survival (100% and 93.6 ± 6.5%, respectively) and cleavage rates (91.1 ± 1.3 and 88.6 ± 6.5%, respectively). On the other hand, the survival and cleavage rates of vitrified oocytes were slightly lower (91.9 ± 3.3 and 78.4 ± 8.4%, respectively), although the differences compared with those of the control and exposed oocytes were not significant. Although vitrified oocytes were used for IVF, only 6 of 168 oocytes were penetrated by sperm (3.6%), and none of them formed pronuclei.

CGE of the oocytes is shown in Fig. 1. In the control groups, fluorescence indicating CGE was not observed (Fig. 1A). On the other hand, activated oocytes exhibited strong fluorescence intensity (Fig. 1B). When oocytes were exposed to vitrification solution but not vitrified, the fluorescence intensity was very weak in the oocytes (Fig. 1C). However, vitrified oocytes exhibited strong fluorescence intensity as did activated oocytes (Fig. 1D). On the other hand, vitrification of calcium-free medium reduced the fluorescence intensity of CGE in the ED(–) group (Fig. 1E), E(–) group (Fig. 1F) and D(–) group (Fig. 1G). The intensity was similar level to that in the exposed oocytes.

The survival and developmental abilities of vitrified oocytes using calcium-free medium are shown in Fig. 2. Except for the D(–) group (23.6 ± 9.7%), high survival rates were observed in the groups [ED(+), 85.8 ± 4.6%; ED(–), 90.7 ± 3.2%; D(–), 79.4 ± 4.3%] (Fig. 2A). Most of the fresh oocytes were cleaved (96.8 ± 2.2%; Fig. 2B). On the other hand, more than half of the oocytes in the ED(+), ED(–) and E(–) groups were also cleaved (55.8 ± 9.5, 77.0 ± 3.9 and 72.8 ± 4.0%, respectively). The cleavage rate of the D(–) group was significantly low (14.9 ± 2.0%). The rate of development to blastocysts in the fresh groups was significantly higher than those in the other groups (76.1 ± 5.2%; Fig. 2C). The rate of development to blastocysts in the E(–) group (23.1 ± 4.2%) tended to be higher than those in the ED(+) and D(–) groups.

IVF was carried out using oocytes vitrified in calcium-free and EG-supplemented medium. The data was shown in Table 3. The zona penetration rates of the control and vitrification groups were 81.7 ± 17.5 and 63.9 ± 9.9%, respectively. There was no significant difference between groups ($P>0.05$). However, the PN formation rate in vitrification group was significantly lower (0%) than t hat in the control group (40.5 ± 10.4%; $P<0.05$).

Discussion

Cryopreservation of germ cells, especially female gametes prior to fertilization (oocytes), is one of the most important technologies, not only in reproductive biology but also in human obstetrics and gynecology because unfertilized oocytes can be used for IVF, ICSI or SCNT [1, 2]. Even though many researchers are trying to improve oocyte cryopreservation, the fertilization and developmental abilities of oocytes after thawing or warming are generally compromised. Very recently, successful cryopreservation of mouse oocytes by injection of trehalose into the oocytes has been reported [6]. Since this protocol requires a great deal of skill and cannot be used to cryopreserve many oocytes at the same time, a simpler and more general-purpose protocol is in demand. The aim of the present study was to improve the survival and embryonic development of vitrified rat oocytes.

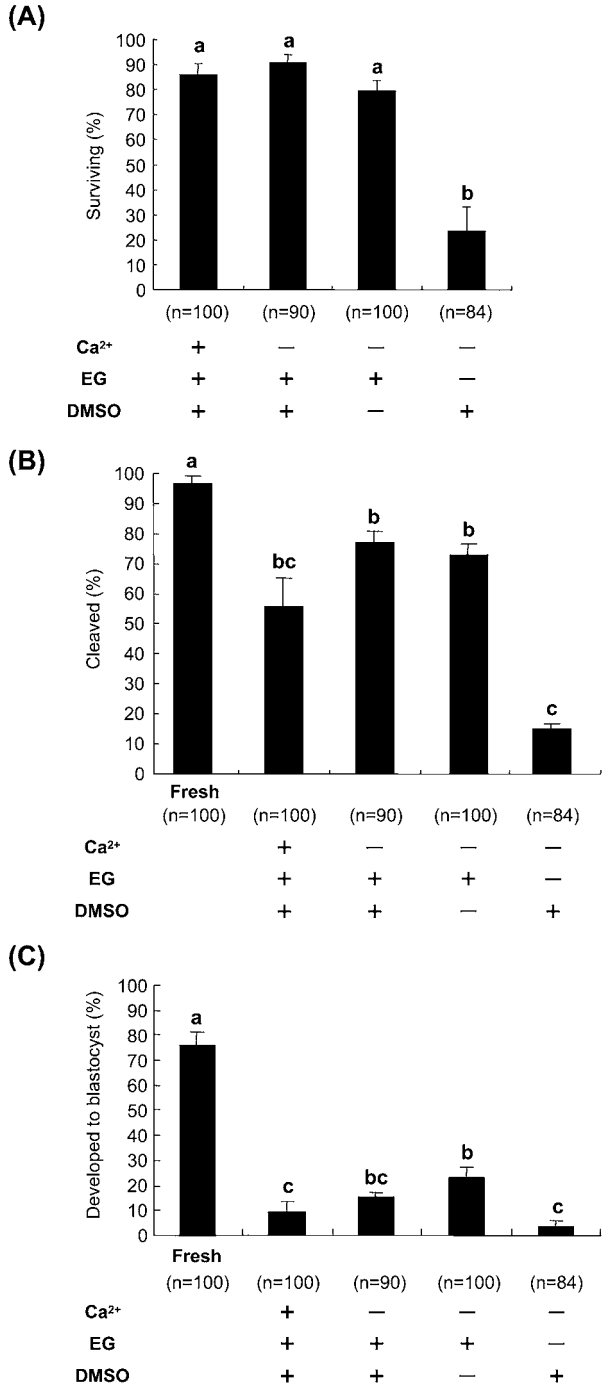

Fig. 2. The survival and developmental ability of rat oocytes vitrified using different cryoprotectants. (A) Percentage of surviving oocytes. (B) Percentage of cleaved oocytes after artificial activation. (C) Percentage of blastocysts after artificial activation. Data are shown as means ± SEM. Different superscripts denote significant differences among treatments ($P<0.05$).

Larman *et al*. [9] demonstrated that exposure to vitrification solution containing CPAs (DMSO or EG) causes a rise of intracellular calcium in mouse oocytes. Our present study showed that, at the very least, rat oocytes exposed to vitrification solution did not seem to display a rise of intracellular calcium because CGE was only slightly observed (Fig. 1C). On the other hand, strong intensity of fluorescence indicating CGE was observed in vitrified oocytes (Fig. 1D). Since CGE is induced by the rise of calcium, our present result suggests the possibility that the vitrification procedure *per se* induces CGE through an increase of intracellular calcium. In rats, cooling induces oocyte activation as manifested by an increase of intracellular calcium and second polar body extrusion [35]. The same study also reported that the pattern of calcium dynamics is similar to that observed in oocytes activated by sperm [35]. Therefore, exposure to low temperatures during the process of vitrification seems to cause an increase of intracellular calcium in vitrified rat oocytes. The increase of intracellular calcium in mouse oocytes exposed to CPAs can be reduced by vitrification using calcium-free media [9]. In order to inhibit the increase in calcium, collection and vitrification medium from which calcium had been removed was used for vitrification. In rat oocytes, the use of calcium-free medium improved CGE independent of cryoprotectants, probably by reducing the increase in calcium (Fig. 1E–G). These results suggest that CGE during the process of vitrification seems to be caused not by release from stored intracellular calcium but by uptake of extracellular calcium. Therefore, we succeeded in improving CGE in vitrified oocytes using calcium-free medium. Since CGE was improved using calcium-free media, the optimal CPA for vitrification of rat oocytes was evaluated. Embryonic development of vitrified oocytes to the blastocyst stage was low overall, especially using DMSO-supplemented medium. On the other hand, oocytes vitrified in calcium-free and EG-supplemented medium showed higher developmental ability compared with those of oocytes vitrified in DMSO alone or EG- and DMSO-supplemented medium. In oocyte and embryo vitrification in other species, EG would be the ideal CPA [36] because the permeability of EG is higher than those of other CPAs [37] and EG is less toxic than other permeable CPAs [38, 39]. Therefore, a vitrification medium supplemented with EG alone would be suitable for vitrification of rat oocytes, although further improvements will be required.

Even though the zona penetration rate of the vitrified oocytes was dramatically improved using calcium-free and EG-supplemented media, none of the oocytes formed pronuclei. This result suggests that calcium-free and EG-supplemented media could not improve vitelline block (membrane block) in vitrified oocytes. Our preliminary study demonstrated that some of vitrified rat oocytes could develop to term using ICSI (Fujiwara *et al*., unpublished data), suggesting that at least the developmental ability of vitrified oocytes was maintained after vitrification/warming. In mammalian species, the factors involved in vitelline block are not well defined. However, it is well known that the vitrification procedure exerts a detrimental effect(s) on oocytes; for example, the most serious damage after vitrification is disorganization of spindles potentially caused by microtubule disassembly in vitrified oocytes [40]. Recently, paclitaxel treatment prior to vitrification has been shown

Table 3. Development of vitrified-warmed rat oocytes after *in vitro* fertilization

Treatments	No. examined	No. surviving (%)	No. zona-penerated (%)	No. with PN formation (%)
Control (Fresh)*	163	163 (100)[a]	116 (81.7 ± 17.5)	47 (40.5 ± 10.4)[a]
Vitrification[#]	155	111 (66.6 ± 5.8)[b]	71 (63.9 ± 9.9)	0 (0)[b]

[ab] Different superscripts within columns denote significant differences ($P<0.05$). *Fresh (denuded) oocytes were used for IVF. [#]Oocytes were vitrified in EG-supplemented calucium-free media and then used for IVF after warming.

to improve the developmental ability of mouse [41], human [42], bovine [43] and porcine [44] oocytes. Paclitaxel is a chemical that promotes formation of highly stable microtubules that resist depolymerization, thus preventing normal cell division and arresting the cell cycle at the M-phase in somatic cells [45, 46]. It has been reported that rat oocytes are easily activated, undergoing so-called "spontaneous activation" [47]. Moreover, exposure of rat oocytes to low temperatures induces chromosome segregation and results in the extrusion of a second polar body [35]. Therefore, a treatment for spindle stability during vitrification, such as by paclitaxel, seems to be required. Alternatively, $p34^{cdc2}$ kinase, which is an M-phase-specific serine/threonine kinase, is inactivated during spontaneous activation in rat oocytes [48, 49]. It has been demonstrated that low $p34^{cdc2}$ kinase activity in matured oocytes is involved in abnormal development after artificial activation [50]. We previously reported that treatment with a proteasome inhibitor, MG132, dramatically improved the decrease of $p34^{cdc2}$ kinase activity and inhibited spontaneous activation in rat oocytes [49]. Thus, treatments that maintain high $p34^{cdc2}$ kinase activity during vitrification, such as treatment with MG132, may also be effective for further improvement of pronuclear formation in vitrified oocytes by suppressing vitelline block after *in vitro* fertilization.

In the present study, we attempted to improve the vitrification protocol for rat oocytes, and our results revealed that calcium-free, EG-supplemented media were effective, at least for inhibition of CGE of the vitrified oocytes. Although further improvements of the vitrification protocol are required for application to IVF, the vitrified oocytes will be able to be used for ICSI and as recipients for SCNT.

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