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Successful Production of Blastocysts Following Ultrarapid Vitrification with Step-Wise Equilibration of Germinal Vesicle-Stage Mouse Oocytes

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Abstract. The purpose of this study was to evaluate the viability and subsequent developmental ability of murine germinal vesicle (GV) oocytes ultrarapidly vitrified after step-wise exposure to cryoprotectants (CPAs). Oocytes were transferred to a vitrification solution composed of 15% ethylene glycol, 15% dimethyl sulfoxide and 0.5 M sucrose in a direct manner (non-preequilibrium) or in a step-wise manner (single-, two-, or ten-step preequilibrium). After ultrarapid vitrification and storage in liquid nitrogen, the oocytes were thawed, washed by diluting the CPAs in five steps, and then subjected to *in vitro* maturation, fertilization and culture. In the non-preequilibrium group, the rates of post-thawed oocytes surviving, maturing to metaphase-II, developing to blastocysts and to hatching/hatched blastocysts were 91.8, 87.1, 15.9 and 2.3%, respectively. In the single- and two-step groups, the corresponding rates were 97.0–98.2%, 92.2–95.0%, 22.0–29.4% and 8.8–15.6%, whereas in the ten-step group they were 98.2, 91.8, 38.6 and 22.8%, respectively. In the non-vitrified control group, the rates of oocytes maturing to metaphase-II, developing to blastocysts and to hatching/hatched blastocysts were 90.2, 75.2 and 51.5%, respectively. The present study shows that the ultrarapid vitrification of murine GV oocytes by a step-wise manner involving 10 steps preequilibrium may have an advantage in maintaining the viability and subsequent production of blastocysts.

Key words: Cryopreservation, Immature oocyte, Germinal vesicle (GV), Step-wise, Vitrification
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The storage of oocytes is important for advances in reproductive biology and infertility treatment. In a clinical context, the cryopreservation of human oocytes is important for storage of oocytes from patients who risk losing ovarian function because of extirpative therapy, chemotherapy, or radiation. Although successful procedures for cryopreservation of human

metaphase II (MII) oocytes have been reported [1, 2], their results have been shown to be unsatisfactory, and they appear to require still development. The microtubular spindles of MII oocytes to which the chromosome is attached are sensitive to temperature changes, and hence chromatid disjunctions occur during cooling, resulting in aneuploidy after fertilization [3–5]. On the other hand, germinal vesicle (GV) stage oocytes do not have a microtubular spindle. For these reasons, the cryopreservation of GV oocytes may be

an alternative approach to the storage of gametes. Successful cryopreservation of GV oocytes has been reported [6–8], but low survival rates, fertilization and developmental capacity following cryopreservation of GV oocytes have also been shown [9–12].

Oocytes can be cryopreserved using slow cooling or vitrification methods. Although slow cooling is the widespread method, the cryopreservation of MII oocytes by the vitrification method has shown better results than that by the slow cooling method [13, 14]. In addition, ultrarapid vitrification, characterized by more rapid cooling and warming rates than conventional vitrification, needs smaller amounts of vitrification solution for suspending the oocytes. The oocytes are plunged directly into liquid nitrogen (LN_2), using cryodevices such as an open pulled straw [15, 16], electron microscopy grid [17], cryoloop [18], nylon mesh [19] and plastic sheet [20, 21].

In this study, we examined the viability and subsequent developmental ability of murine GV oocytes by ultrarapid vitrification in combination with a step-wise preequilibrium method.

Material and Methods

Animals and collection of GV oocytes

All investigations were performed in accordance with the Guide for Care and Use of Laboratory Animals of the Graduate School of Agricultural Science, Tohoku University.

Female C57BL/6J × CBA/Ca virgin mice of 3–4 weeks-old were injected with 5IU of pregnant mare serum gonadotropin (PMSG, Serotropin, Teikokuzohki, Tokyo) and sacrificed 48 h after PMSG administration. GV-oocytes were obtained by puncture of antral follicles in Leibovitz L-15 medium (Invitrogen Corp, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 0.1 mg/ml dibutyryl cyclic AMP (dbcAMP; Sigma Chemical Co., St. Louis, MO, USA) (holding medium). In all experiments, only full-grown cumulus-oocyte complexes (COC) with compact and dense cumulus cell layers were used and partly or completely naked oocytes were discarded. The GV oocytes were randomly allocated to treatment groups.

Vitrification and thawing

The GV oocytes were cryopreserved by ultrarapid vitrification using the MVC method [20, 21]. For preequilibrium before vitrification, the GV oocytes were treated by four different manners of exposure to cryoprotectants (CPAs) in six experimental groups. In the non-preequilibrium treatment, the GV oocytes were directly transferred to vitrification solution. In the single step preequilibrium treatment, the GV oocytes were treated with one of three solutions, each of which was composed of 0.5% ethylene glycol (EG; Sigma) plus 0.5% dimethyl sulfoxide (DMSO; Sigma), or 2.5% EG plus 2.5% DMSO, or 5% EG plus 5% DMSO in the holding medium at 37°C for 5 min. In the two steps preequilibrium treatment, the GV oocytes were treated with 0.5% EG plus 0.5% DMSO and 2.5% EG plus 2.5% DMSO in the holding medium at 37°C for 5 min each. In the ten steps preequilibrium treatment, the GV oocytes were transferred sequentially into increasing concentrations of EG (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, and 2.5%) plus DMSO (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, and 2.5%) in the holding medium at 37°C in 1 min intervals. The GV oocytes were then equilibrated for 40–60 sec in the final vitrification solution composed of 15% EG, 15% DMSO and 0.5 mol/L sucrose in the holding medium at room temperature, and using a fine pipette, were loaded into a very small volume drop (<1 μl) on the inner surface of the tip of an obliquely cut 0.25 ml plastic straw (IMV, L'Aigle, France), then plunged into LN_2 . The vitrified GV oocytes were thawed by directly placing in 0.5 mol/L sucrose in the holding medium at 37°C for 15 sec. Then, the GV oocytes were transferred to 0.2, 0.1 and 0.05 mol/L sucrose in the holding medium for 30, 30 and 60 sec, respectively, and washed three times in Leibovitz L-15 medium supplemented with 5% FBS.

Maturation of GV oocytes

The vitrified-thawed GV oocytes or fresh GV oocytes (non-vitrified control group) were cultured in 100 μl droplets of Waymouth's MB752/1 medium [22] supplemented with 5% FBS, 0.23 mM sodium pyruvate (Sigma), 10 ng/ml epidermal growth factor (Sigma), 75 $\mu\text{g}/\text{ml}$ penicillin G-K salts (Sigma) and 50 $\mu\text{g}/\text{ml}$ streptomycin sulphate (Sigma) under liquid paraffin oil at 37°C in an atmosphere of 5% CO_2 in humidified air. Eighteen

to 20 h after culture, the oocytes were denuded and assessed for the stage of meiotic maturation: germinal vesicle breakdown (GVBD) or metaphase II (presence of the first polar body).

In vitro fertilization and development

Spermatozoa from male C57BL/6J × CBA/Ca mice of 8 to 12 weeks old were released by epididymal puncture into human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, CA, USA) supplemented with 30 mg/ml bovine serum albumin (BSA; Sigma), and kept for 1.5–2 h to allow capacitation. Vitrified and non-vitrified control oocytes were cultured in HTF medium containing approximately 5×10^5 motile sperm / ml for 6–8 h, then washed three times in HTF, and cultured in 100 μ l droplets of HTF supplemented with 4 mg/ml BSA under liquid paraffin oil at 37 °C in an atmosphere of 5% CO₂ in humidified air. The oocytes cleaving 24 h post-insemination were judged to be fertilized. The number of embryos developing to the blastocyst stage was recorded 120 h post-insemination.

Statistical analysis

Data were compared by χ^2 analysis. Differences were considered significant at a level of P<0.05.

Results

Survival and maturation of vitrified GV oocytes

The morphological survival and maturation rates in vitrified GV oocytes and non-vitrified GV

oocytes are shown in Table 1. The morphological survival rate in the single step preequilibrium treatment with 0.5% EG plus 0.5% DMSO (97.0%) was not significantly different from that of the non-preequilibrium group (91.8%), but those of others vitrified with preequilibrium treatments (98.1 to 98.2%) were significantly higher. Although the maturation rate to the MII stage in the non-preequilibrium group (87.1%) was significantly lower than that of the single step preequilibrium group with 2.5% EG plus 2.5% DMSO (95.0%), the rates between the control group (90.2%) and each of the vitrified groups (87.1 to 95.0%) were not significantly different.

In vitro fertilization and development of vitrified GV oocytes

In vitro development after IVMF of vitrified GV oocytes and non-vitrified GV oocytes is shown in Table 2. The rates of fertilization and development to blastocysts and hatching/hatched blastocysts in the control group were significantly higher than all of the vitrified groups. Among the vitrified groups, the fertilization rate in the single step preequilibrium treatment with 5.0% EG plus 5.0% DMSO (58.5%) was significantly lower than those of the single step preequilibrium treatment with 0.5% EG plus 0.5% DMSO (72.5%) and the ten steps preequilibrium treatment (73.3%). Although the development rate to blastocysts in the ten steps preequilibrium treatment (38.6%) (Fig. 1) was not significantly different from the two steps preequilibrium treatment (29.4%), both of them were significantly higher than those of the non-

Table 1. Survival and *in vitro* maturation (IVM) of murine GV oocytes vitrified with preequilibrium

Groups	Condition of preequilibrium	No. of oocytes vitrified	No.(%) of oocytes survived	No. of oocytes cultured	No. (%) of oocytes matured to		
					GV	GVBD	MII
Control (non-vitrified)	–	–	–	121	0	11 (9.8)	101 (90.2) ^{ab}
Non-preequilibrium	–	110	101 (91.8) ^a	101	1 (1.0)	12 (11.9)	88 (87.1) ^a
Single step	0.5%EG/0.5%DMSO	100	97 (97.0) ^{ab}	97	0	6 (6.2)	91 (93.8) ^{ab}
	2.5%EG/2.5%DMSO	103	101 (98.1) ^b	101	0	5 (5.0)	96 (95.0) ^b
	5.0%EG/5.0%DMSO	104	102 (98.1) ^b	102	0	8 (7.8)	94 (92.2) ^{ab}
Two steps	0.5%EG/0.5%DMSO and 2.5%EG/2.5%DMSO ^{*1}	112	110 (98.2) ^b	110	1 (0.9)	7 (6.4)	102 (92.7) ^{ab}
	0.25%EG/0.25%DMSO to 2.5%EG/2.5%DMSO ^{*2}	112	110 (98.2) ^b	110	0	9 (8.2)	101 (91.8) ^{ab}

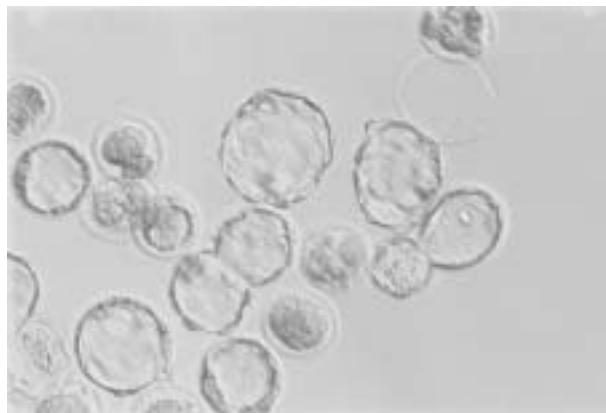
^{*1} The GV oocytes were treated with 0.5%EG/0.5%DMSO followed by 2.5%EG/2.5%DMSO for 5 min, respectively.

^{*2} The GV oocytes were treated in a step-wise manner with increasing concentrations from 0.25 to 2.5% for 1 min, respectively.

^{a,b} Values with different superscripts within each column are significantly different, P<0.05.

Table 2. *In vitro* development after IVMF in murine GV oocytes vitrified

Groups	Condition of preequilibrium	No. of oocytes examined	No. (%) of oocytes developed to		
			Cleavage (2-cell)	Blastocysts	Hatching/Hatched blastocysts
Control (non-vitrified)	–	101	95 (94.1) ^a	76 (75.2) ^a	52 (51.5) ^a
Non-preequilibrium	–	88	61 (69.3) ^{bc}	14 (15.9) ^d	2 (2.3) ^d
Single step	0.5%EG/0.5%DMSO	91	66 (72.5) ^b	20 (22.0) ^{cd}	8 (8.8) ^{cd}
	2.5%EG/2.5%DMSO	96	69 (71.9) ^{bc}	24 (25.0) ^{cd}	15 (15.6) ^{bc}
	5.0%EG/5.0%DMSO	94	55 (58.5) ^c	22 (23.4) ^{cd}	13 (13.8) ^{bc}
Two steps	0.5%EG/0.5%DMSO and 2.5%EG/2.5%DMSO ^{*1}	102	71 (69.6) ^{bc}	30 (29.4) ^{bc}	15 (14.7) ^{bc}
Ten steps	0.25%EG/0.25%DMSO to 2.5%EG/2.5%DMSO ^{*2}	101	74 (73.3) ^b	39 (38.6) ^b	23 (22.8) ^b

^{*1, *2} See Table 1 footnote.^{a-d} Values with different superscripts within each column are significantly different, P<0.05.**Fig. 1.** Mouse blastocysts derived from GV oocytes vitrified with ten step-wise preequilibrium treatments.

preequilibrium treatment (15.9%). Furthermore, in the ten steps preequilibrium treatment the rate was significantly higher than those of all the single step preequilibrium treatments (22.0 to 25.0%).

Discussion

In this study, we showed that the cryopreservation of murine GV oocytes by ultrarapid vitrification enabled oocytes to survive, mature, fertilize and develop to blastocysts. Successful cryopreservation of GV oocytes has been reported in several species [6–8], but there were low rates of survival, fertilization and developmental capacity after cryopreservation [9–12].

The cryopreservation of murine immature

oocytes has been reported using a slow cooling method [6, 9, 23, 24], but with limited success. These studies have indicated lower embryo development rates in immature oocytes than in mature oocytes [6, 9]. Some studies have reported a loss of cumulus cells from the COC following slow cooling and thawing, after which cumulus cells were no longer connected to the oocyte [23, 24]. The connection between cumulus cells and oocyte is important for completion of normal maturation *in vitro* [25], and the cumulus cells of the COC may be disrupted physically by damage due to ice formation in the slow cooling method. In order to maintain developmental potency after cryopreservation of immature oocytes, minimization of such disruption by ice formation is important. Vitrification circumvents the formation of potentially damaging intracellular and extracellular ice, although osmotic injury caused by the higher concentrations of CPAs in vitrification could occur more frequently than in the slow cooling method. It has been reported that egg yolk provides further beneficial effects on both the oocyte and cumulus cell integrity, and high survival and meiotic normality [26]. However, its effect on subsequent fertilization and development has not been reported. Cryopreservation by vitrification of immature oocytes of mice has been reported [10, 11, 27] with low resultant rates of fertilization and development.

In the present study, for the first time as far as we know, we applied ultrarapid vitrification to cryopreserve GV oocytes of mice. We found that high rates of survival and IVM were followed by efficient production of blastocysts when the oocytes

were vitrified after increasing the number of steps of preequilibration to CPAs. These results suggest that the process of gradual equilibrium conversion of CPA seems to adjust permeability of the plasma membrane, which may contribute to maintaining the connection between the oocyte and cumulus cells, and/or may decrease rapid changes in osmotic pressure. Kuwayama *et al.* [28] reported that vitrification of bovine blastocysts following a 16-step equilibration resulted in minimization of ultrastructural damage to the plasma membrane, suggesting that the integrity of the normal

characteristics of plasma membrane especially in cryopreservation of GV stage oocytes may be important.

In conclusion, the ultrarapid vitrification accompanied with step-wise equilibration in murine GV oocytes showed improved results for viability and production of blastocysts, suggesting that this method may be a useful strategy for the cryopreservation of immature oocytes. Further study of the *in vivo* developmental potential of vitrified GV oocytes after transfer to recipients will be required.

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