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Comparison of the microdrop and minimum volume cooling methods for vitrification of porcine *in vitro*-produced zygotes and blastocysts after equilibration in low concentrations of cryoprotectant agents

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Abstract. We compared the efficacy of the microdrop and minimum volume cooling (MVC) methods for the vitrification of *in vitro*-produced porcine zygotes and blastocysts after equilibration in low concentrations of cryoprotectant agents. Zygotes and blastocysts were equilibrated in 2% (v/v) ethylene glycol and 2% (v/v) propylene glycol for 13–15 min. Then, they were vitrified in a medium comprised of 17.5% ethylene glycol, 17.5% propylene glycol, 0.3 M sucrose, and 50 mg/ml polyvinylpyrrolidone either by either dropping them directly into liquid nitrogen (microdrop method) or placing them on Cryotop sheets in a minimum volume of medium and plunging into liquid nitrogen (MVC method). Both zygotes and blastocysts were successfully vitrified. For the vitrification of zygotes, the MVC and microdrop methods were equally effective; however, for blastocyst vitrification, MVC was superior. For both methods, the vitrification of zygotes produced higher-quality embryos than the vitrification of blastocysts.
Key words: Blastocyst, *In vitro* embryo production, Porcine, Vitrification, Zygote

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In vitro embryo production systems serve an important role in gene banking for pigs [1]. However, the cryopreservation of the resultant embryos is a challenging task because porcine embryos show higher sensitivity to cryopreservation procedures than other species [2]. Furthermore, the viability of porcine *in vitro*-produced (IVP) embryos is inferior to the viability of those developed *in vivo* [3]. Application of sophisticated vitrification techniques on porcine IVP embryos such as the minimum volume cooling (MVC) method at the blastocyst stage [4, 5] or the Hollow Fiber Vitrification at the morula stage [6] result in survival rates over 70%. These techniques require specific devices as carriers, which increase the cost of the procedure. However, mammalian oocytes and embryos can be vitrified without a carrier by directly dropping them into liquid nitrogen (LN) [7, 8] or onto a solid metal surface cooled with LN (also known as Solid Surface Vitrification (SSV)) [9]. Previously, porcine blastocysts produced *in vitro* were vitrified using the SSV method, with approximately 30% survival [10]; however, offspring could not be obtained after transfer of surviving embryos. Interestingly, when IVP porcine zygotes were vitrified using the same method, the survival rates were over 90%, with a high rate of survivors developing to blastocysts and to term [11, 12]. Thus, the debate arose whether vitrification at the zygote stage is more advantageous than that at the blastocyst stage in porcine IVP systems. Recently, we optimized the SSV method specifically for porcine immature oocytes, which resulted in increased survival and embryo developmental competence [13–15]. Our results revealed that, for the vitrification of immature porcine oocytes, the combination of ethylene glycol (EG) and propylene glycol (PG) is superior to a single cryoprotectant agent (CPA) or to the combination of EG with dimethyl sulfoxide (DMSO). In addition, equilibration in a low (4%) concentration of total permeable CPA was superior to that in a higher (15%) total CPA concentration [13–15]. Nevertheless, it remained unclear whether this protocol is effective for the vitrification of IVP porcine zygotes and blastocysts. In addition, it remained unknown whether IVP porcine zygotes and blastocysts can be effectively vitrified in microdrops (MDs) directly dropped into LN instead of dropping onto a solid metal surface. Vitrification of MDs directly in LN can be advantageous when air-humidity is high, which is typical, for instance, in Vietnam and Japan during the wet season. Under such conditions, a layer of ice crystals forms quickly on the dry metal surface cooled by LN, which reduces the efficacy of vitrification and the handling of vitrified droplets. Therefore, the aims of the present study were to: 1) assess post-thaw survival and subsequent *in vitro* development of IVP porcine zygotes and blastocysts after vitrification, using the CPA treatment and warming protocols previously developed for

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Table 1. Retrieval, survival, and development of *in vitro* produced porcine zygotes vitrified using the microdrop (MD) or minimum volume cooling (MVC) methods

Group	Total	Retrieved (% total)	Surviving (% retrieved)	Cleaved (% retrieved)	Blastocyst on Day 6		No. total cells in blastocysts
					(% retrieved)	(% total)	
Control	145	145 (100) [a]	145 (100) [a]	120 (81.4 ± 2.5)	47 (34.6 ± 4.3)	(34.6 ± 4.3)	46.6 ± 1.9
Vitrified/MD	182	163 (89.7 ± 0.5) [b]	161 (98.5 ± 0.7) [ab]	127 (78.0 ± 0.4)	34 (21.1 ± 1.5)	(18.9 ± 1.3)	44.4 ± 2.2
Vitrified/MVC	144	144 (100) [a]	132 (90.1 ± 5.8) [b]	93 (62.0 ± 9.4)	29 (19.1 ± 4.1)	(19.1 ± 4.1)	46.1 ± 6.4

Three biological replicates were performed. Percentages are presented as mean ± SEM. a and b in the same column indicate significant differences. ($P < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparisons test). Day 0 = the day of IVF.

Table 2. Retrieval and survival of *in vitro* produced porcine blastocysts vitrified using the microdrop (MD) or minimum volume cooling (MVC) methods

Group	Total vitrified	Retrieved (% total)	Re-expanded (% retrieved)	Hatched (% retrieved)
Vitrified/MD	128	94 (69.7 ± 9.8)	25 (26.8 ± 2.7) [a]	1 (2.5 ± 2.5)
Vitrified/MVC	126	125 (98.7 ± 1.2)	63 (52.2 ± 6.1) [b]	3 (2.3 ± 1.6)

Three biological replicates were performed. Percentages are presented as mean ± SEM. a and b indicate significant differences at $P < 0.05$ (Welch's *t*-test). Vitrification/warming was performed on Day 6 (Day 0 = the day of IVF). Re-expansion and hatching were recorded after 24 h of post-warming culture on Day 7.

immature porcine oocytes; 2) compare the efficacy of vitrification either using the MVC method with Cryotop sheets as carriers or inserting MDs of vitrification solution containing embryos directly in LN; and 3) clarify whether the vitrification at the blastocyst or the zygote stage is more advantageous in a porcine IVP system, using this CPA treatment and warming protocol.

In *Experiment 1*, when zygotes were vitrified, there was no difference in the survival rate and subsequent development to the blastocyst stage between the MVC and MD groups (Table 1). Using both methods, the survival rate measured by morphology was over 90% and the development rate to the blastocyst stage was approximately 20%. Furthermore, the blastocyst quality in terms of total cell numbers in both vitrified groups was similar to that of the non-vitrified control. These results demonstrate that for the vitrification of zygotes, cooling, storage, and warming using the MD and MVC methods are equally effective for post-warming survival and subsequent embryo development. Irrespective of the cooling method used, developmental competence of vitrified zygotes to the blastocyst stage was not statistically different from that of the control group, corroborating our previous results [12]. Previously, we reported similar survival and blastocyst development rates when IVP porcine zygotes were vitrified using the SSV method with EG as the only permeating CPA [11, 12]. In those studies, zygotes were dropped onto a dry aluminum surface cooled by LN; however, in the present study the zygotes were dropped directly into LN. The similarity in the results suggests that 1) the efficacy of cooling may be similar when 1–2 µl droplets of our vitrification medium are placed either on the surface of aluminum foil cooled by LN or directly in LN, and 2) the combination of EG + PG is as effective as EG alone for the vitrification of porcine zygotes.

In *Experiment 2*, when IVP blastocysts were vitrified on Day 6, MVC was superior to the MD method in terms of the survival of embryos measured by their ability to re-expand (Table 2). Although there was no statistically significant difference between the groups in the retrieval rates, approximately 30% of the blastocysts were lost during the MD vitrification because of adhesion to the glass pipette, which is clearly a disadvantage of this method. The higher survival of blastocysts by the MVC method could be explained by the high cooling/warming rate provided by this method [16]. Nevertheless, the survival rates obtained after blastocyst vitrification using the same device for cooling show a great variation among studies. In the present study, 52.2% of porcine IVP blastocysts re-expanded within 24 h of the warming process using the MVC method. This rate is higher than those reported previously by Li *et al*. [17] and Castillo-Martin *et al*. [18,19], similar to that reported by Esaki *et al*. [4], and lower than that reported by Mito *et al*. [5], using the same device on Day 6 porcine IVP blastocysts without manipulation. In the present study, when blastocysts were vitrified using the MD method, the re-expansion rate was 26.8%. However, previously we reported 57.1% survival after MD vitrification of Day 6 porcine IVP blastocysts generated from vitrified oocytes using the same method [20]. Such variation in efficacy among studies using the same device indicates that several factors such as the quality of available materials, the IVP protocol used for embryo production, the vitrification/warming protocol, and the experience of the technician performing the vitrification may affect embryo survival after vitrification and warming.

Experiment 3 demonstrated that after vitrification by either method, the quality of surviving blastocysts is inferior to that of

the non-vitrified control in both morphology (Fig. 1) and cell number (Table 3). Irrespective of the cooling method, total cell numbers in blastocysts that survived vitrification and warming on Day 6 were significantly lower than in the non-vitrified control after subsequent culture for 24 h in a serum supplemented medium (Table 3). On the other hand, in all groups, total cell numbers in Day 7 blastocysts were higher than those in Day 6 blastocysts in *Experiment 1,* irrespective of treatment group. This suggests that cell proliferation occurred in both vitrified and non-vitrified blastocysts during IVC from Day 6 to Day 7. In a previous study, vitrification reduced the number of viable cells in porcine parthenogenetic blastocysts via membrane damage of blastomeres [17]. However, in the present study, the number and percentage of blastomeres with membrane damage did not differ significantly among surviving vitrified and non-vitrified blastocysts. This suggests that in the present study, the reduction of cell numbers in surviving vitrified blastocysts did not occur because of direct damage to blastomere cell membranes, but rather because of hampered proliferation of membrane intact embryonic cells after warming. Recent studies reported altered expression of developmentally important genes in vitrified porcine IVP blastocysts [21, 22], which might explain the reduced cell proliferation in vitrified blastocysts. Further studies are necessary to clarify the impact of such alterations in vitrified blastocysts on their ability to develop to term.

Thus, irrespective of the cooling method used, IVP porcine zygotes survived vitrification at high rates and developed to blastocysts with similar cell numbers comparable to those in non-vitrified zygotes. On the other hand, less than 60% of the Day 6 blastocysts survived vitrification and the surviving embryos had reduced cell numbers compared with those in control embryos. This suggests that irrespective of the device used, vitrification at the zygote stage was more advantageous than vitrification at the blastocyst stage for the quality of live blastocysts obtained after the procedure. This suggestion was confirmed in *Experiment 4,* which directly compared the efficacy of MVC vitrification in zygotes and Day 6 blastocysts obtained from the same batch of fertilized oocytes after *in vitro* fertilization (IVF) in terms of live blastocyst yield on Day 7 and quality measured via cell number. After warming and subsequent culture to Day 7, the percentage of live blastocysts did not differ significantly between vitrified zygotes and Day 6 blastocysts (Table 4). However, compared with that in the control, total cell numbers were significantly lower in vitrified blastocysts without an increase in the number of membrane-damaged cells, corroborating the results of *Experiment 3*. On the other hand, blastocysts developing from vitrified zygotes had a cell number similar to that of the control (Table 4). Based on these results, it is likely that vitrification at the zygote and the blastocyst stages affects proliferation of embryonic cells differently. Further research will be necessary to clarify the mechanism behind this phenomenon.

Regarding the optimum device for the vitrification of IVP porcine blastocysts, the MVC method employing a Cryotop sheet as a carrier was superior to the MD method regarding both the higher retrieval and survival rates. However, for the vitrification of zygotes, although the retrieval rate was slightly higher with the MVC method, the overall efficacy of blastocyst production was similar between the MVC and the MD methods because of the high survival rates in the MD

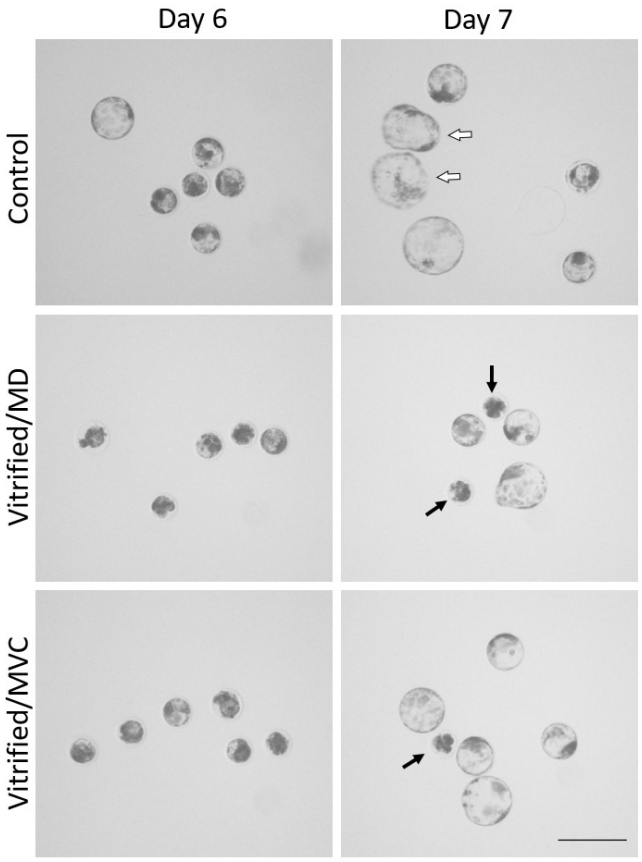

Fig. 1. Morphology of *in vitro* produced porcine blastocysts without (control) or after vitrification and warming on Days 6 and 7 (Day 0 = IVF). Note large hatching or hatched blastocysts (white arrows) in the control group and dead embryos in the vitrified groups (black arrows). Morphology of vitrified embryos was investigated immediately after warming (Day 6) and after 24 h of culture (Day 7). MD = microdrop. MVC = minimum volume cooling. Scale bar represents 500 µm.

Table 3. Cell numbers and the extent of membrane damage in re-expanded vitrified/warmed and non-vitrified (control) *in vitro* produced porcine blastocyst embryos after 24 h of post-warming culture on Day 7

Group	Total embryos	No. total cells	No. membrane damaged cells	% membrane damaged cells
Control	17	103.0 ± 7.0 [a]	0.34 ± 0.07	0.54 ± 0.29
Vitrified/MD	15	59.3 ± 5.3 [b]	1.44 ± 0.61	2.53 ± 1.00
Vitrified/MVC	17	69.9 ± 8.0 [b]	1.59 ± 0.53	2.68 ± 0.95

Three biological replicates were performed. Percentages are presented as mean ± SEM. a and b in the same column indicate significant differences at $P < 0.05$ (Kruskal-Wallis test followed by Dunn's multiple comparisons test). Vitrification/warming was performed on Day 6 (Day 0 = the day of IVF).

Table 4. The efficiency of zygote and blastocyst vitrification using the minimum volume cooling (MVC) method in a porcine *in vitro* production system

Group	Total presumptive zygotes	Number of blastocysts on Day 6	Live blastocysts on Day 7			
			Number (%)	No. total cells	No. membrane damaged cells	% membrane damaged cells
Control	120	22	22 (18.6 ± 3.4)	83.8 ± 3.1 [a]	0.89 ± 0.55	1.21 ± 0.87
Vitrified as zygote	149	20	20 (13.4 ± 3.2)	77.8 ± 2.4 [ab]	0.36 ± 0.23	0.71 ± 0.49
Vitrified as blastocyst	139	29	22 (15.7 ± 0.6)	55.4 ± 5.1 [b]	1.40 ± 0.34	3.91 ± 1.13

After *in vitro* fertilization (IVF), presumptive zygotes of the same batch were either vitrified/warmed at the zygote stage on Day 0 (the day of IVF) and cultured to Day 7 or were cultured and vitrified/warmed as blastocysts on Day 6 and cultured for an additional 24 h (Day 7). In the control group, presumptive zygotes were cultured to Day 7 without vitrification at any stage. In all groups, from Day 6 to Day 7, IVC2 medium was supplemented with 10% FBS. Cell numbers and the extent of membrane damage in live blastocysts were assayed on Day 7. Three biological replicates were performed. Results are presented as mean ± SEM. a and b in the same column indicate significant differences at $P < 0.05$ (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

method. Vitrification in MDs has an advantage over MVC because it enables the preservation of large groups (up to 120) of zygotes in only 20 min, whereas the MVC method is more time-consuming because the zygotes are processed in groups of 10. Therefore, when zygotes are vitrified, the number of embryos might define which method is expedient for preservation.

In conclusion, porcine IVP embryos at both the zygote and blastocyst stages were successfully vitrified using a CPA treatment and warming regimen previously developed for immature oocytes. The low concentration (4%) of permeable CPA during equilibration in this protocol might be advantageous because low toxicity; however, further investigations will be necessary to verify this point. For zygote vitrification, the MVC and MD methods were equally effective; however, for blastocyst vitrification, MVC was superior. Using the present protocol in our IVP system, vitrification at the zygote stage produced higher-quality embryos than those at the blastocyst stage.

Methods

Oocyte collection and in vitro maturation (IVM)

The oocyte collection and IVM procedures were performed using the method of Kikuchi *et al*. [23]. Ovaries from prepubertal crossbred gilts (Landrace × Large White) were collected at a local slaughterhouse and carried to the laboratory within 1 h in Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical, Tokyo, Japan) at 35−37°C. Cumulus-oocyte complexes (COCs) were collected by scraping follicles 3−6 mm in diameter into a collection medium consisting of medium 199 (with Hank's salts; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% (v/v) fetal bovine serum (FBS) (Gibco; Invitrogen, Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), 100 units/ml penicillin G potassium (Sigma-Aldrich), and 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich). The maturation culture medium was a modified NCSU-37 solution [24] supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 μM β-mercaptoethanol, 1 mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan), and 10 IU/ml hCG (500 units; Puberogen, Novartis Animal Health, Tokyo, Japan). IVM was performed in 4-well dishes (Nunc MultiDishes, Thomas Scientific, Swedesboro, NJ, USA) in 500-μl droplets of maturation medium without oil coverage for 22 h in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 39°C. The COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for an additional 22 h in the same atmosphere. Thirty to fifty COCs were cultured in each droplet.

In vitro fertilization (IVF) and embryo culture (IVC)

The IVF and IVC procedures were performed using the method of Kikuchi *et al*. [23]. In brief, the medium used for IVF was a modified Pig-FM medium containing 10 mM HEPES, 2 mM caffeine, and 5 mg/ml bovine serum albumin (BSA). After three consecutive washings in IVF medium, all COCs were transferred into 90-μl IVF droplets (approximately 20 oocytes in each droplet) covered by paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan). Frozen-thawed epididymal spermatozoa from a Landrace boar were introduced to 7 ml of medium 199 (with Earle's salts, Gibco, pH adjusted to 7.8) centrifuged at 750 × *g* for 2 min. Then, the pellet was re-suspended in 100 μl of the same medium and preincubated at 37°C for 15 min to facilitate capacitation [25]. Next, the sperm suspension was further diluted to 1×10^6 cells/ml with preincubated IVF medium. To obtain the final sperm concentration (1×10^5 cells/ml), 10 μl of the sperm suspension was introduced into the IVF medium containing oocytes and co-incubated for 3 h at 39°C in 5% CO_2, 5% O_2, and 90% N_2. The day of IVF was defined as Day 0. At the end of IVF, cumulus cells and spermatozoa were removed from the surface of the zona pellucida of each presumptive zygote by gentle pipetting with a fine glass pipette. The presumptive zygotes were subjected to IVC, which was performed in 500-μl drops of a glucose-free NCSU-37 medium supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 4 mg/ml BSA, and 50 μM β-mercaptoethanol [23] (IVC1 medium) on Days 0 to 2. On Day 2, embryos were transferred to 500-μl drops of IVC2 medium (NCSU-37 medium containing 5.55 mM glucose, 4 mg/ml BSA (Fraction V, Sigma-Aldrich), and 50 μM β-mercaptoethanol) and were subsequently cultured until

Day 6 in 4-well dishes in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39°C.

Vitrification of zygotes and blastocysts

IVP zygotes and blastocysts were vitrified, according to the *Experimental design* (detailed below). The treatment protocol with CPAs was performed according to a previous report [13] with some modifications. In brief, zygotes and blastocysts were washed in a basic medium (BM) consisting of modified NCSU-37 [24] without glucose but supplemented with 20 mM HEPES, 50 µM β-mercaptoethanol, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, and 4 mg/ml BSA. Then, zygotes and blastocysts were transferred to an equilibration medium (BM supplemented with 2% (v/v) EG (E9129, Sigma-Aldrich) and 2% (v/v) PG (29218-35, Nacalai Tesque)) and kept at 35°C for 13−15 min.

The vitrification solution was BM supplemented with 50 mg/ml polyvinylpyrrolidone (P0930, Sigma-Aldrich), 0.3 M sucrose (196-00015, Wako Pure Chemical Industries, Osaka, Japan), 17.5% EG, and 17.5% PG. After equilibration, the zygotes and blastocysts were vitrified using either the MD or MVC method. For the MD method, zygotes and blastocysts were washed three times in 30-µl drops of vitrification solution at 35°C, pipetted into a glass capillary tube, and dropped in 1−2 µl MDs directly into LN kept on the surface of aluminum foil cooled by LN from below. The transparent vitrified droplets were transferred to 2-ml cryotubes (Iwaki 2732-002; AGC Techno Glass, Tokyo, Japan) partly immersed in LN and stored in LN until use. For the MVC method, zygotes and blastocysts were washed two times in 50-µl drops of vitrification solution at 35°C, placed on Cryotop sheets (Kitazato Biopharma, Shizuoka, Japan) in a minimum volume of the vitrification solution, and plunged in LN. In both methods, treatment of zygotes and blastocysts with vitrification medium before introducing them to LN did not exceed 40 sec. Warming of vitrified samples was performed by inserting MDs or Cryotop sheets into 2.5 ml of a warming solution (0.4 M sucrose in BM) in a 35-mm plastic dish (Falcon 351008, Thomas Scientific) kept on a hot plate at 42.0°C. One to two minutes later, zygotes and blastocysts were consecutively transferred for periods of 1 min (each) to 500-µl droplets of BM supplemented with 0.2, 0.1, and 0.05 M sucrose at 38.0°C. Finally, zygotes and blastocysts were washed in BM and subjected to further culture to assess survival and development, according to the *Experimental design* (detailed below).

Assessment of total cell numbers in blastocyst stage embryos

Blastocysts were fixed and stained in 25 µg/ml Hoechst 33342 (H33342, Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol and kept at 4°C overnight. After washing in 99.5% ethanol, the blastocysts were mounted on a glass slide in glycerol drops and flattened with cover slips. Evaluation was performed under UV light with an excitation wavelength of 330–385 nm using an epifluorescence microscope (BX-51, Olympus, Tokyo, Japan). A digital image of the blastocyst was taken and the number of cells labelled with H33342 was assessed using the NIH ImageJ (v. 1.49) software (https://imagej.nih.gov/ij/).

Assessment of membrane damage in blastocyst stage embryos

On Day 7 of IVC, 10 µl of 100 µg/ml aqueous propidium iodide (PI, Sigma-Aldrich) solution was added to each 500 µl culture well containing either non-vitrified or vitrified blastocysts according to the *Experimental design* (detailed below), mixed, and cultured for 20 min to label blastomeres with damaged membranes. Then blastocysts were fixed and stained in 25 µg/ml Hoechst 33342 dissolved in 99.5% ethanol at 4°C overnight. After washing in 99.5% ethanol, the blastocysts were mounted on a glass slide in glycerol drops and flattened with cover slips. Evaluation of total cell numbers labelled with H33342 (appearing blue) and membrane damaged cell numbers labelled with PI (appearing red) was performed under UV light with excitation wavelengths of 330−385 nm and 530−550, respectively, using an epifluorescence microscope. Digital images of each blastocyst were taken and the number of cells labelled with H33342 and PI were assessed using NIH ImageJ.

Experimental design

Experiment 1: Comparison of the efficacy of the MD and MVC methods for the vitrification of IVP zygotes

Twenty hours after IVF, presumptive zygotes were subjected to vitrification either in MDs or on Cryotop sheets (MD and MVC groups, respectively) as described above, using the same CPA treatment and warming regimens. During MD vitrification, zygotes were processed together in groups of 22−40. During MVC vitrification, zygotes were processed in groups of 7−10. Warmed zygotes were cultured in IVC1 and IVC2 media for an additional 5 days as described above. Percentages of zygote retrieval and cleavage were recorded immediately after warming and on Day 2, respectively. Blastocyst development was recorded on Day 6. Cell numbers in blastocysts were assayed on Day 6. Developmental parameters of zygotes in MD and MVC groups were compared to those of a non-vitrified control group. Three biological replicates were performed.

Experiment 2: Comparison of the efficacy of the MD and MVC methods for the vitrification of IVP blastocysts

Blastocyst stage embryos produced *in vitro* as detailed above were vitrified and warmed using either the MD or MVC method on Day 6 using the same CPA treatment and warming regimens as in *Experiment 1*. During MD vitrification, blastocysts were processed in groups of 10−12. During MVC vitrification, blastocysts were processed in groups of 2−5. Warmed embryos were cultured in IVC2 medium supplemented with 10% FBS for 24 h. Embryo retrieval percentages were recorded 0 h after warming. Re-expansion and hatching were recorded 24 h after warming. Three biological replicates were performed.

Experiment 3: Comparison of blastocyst quality after vitrification and warming using the MD and MVC methods.

Blastocyst stage embryos produced *in vitro* were vitrified and warmed using either the MD or MVC method on Day 6 and cultured for 24 h as described in *Experiment 2*. The total cell number, membrane damaged cell number, and the percentage of membrane damaged cells in re-expanded embryos in both vitrified groups were compared to each other and to those of non-vitrified blastocysts, which were also cultured in IVC2 medium supplemented with 10% FBS on Day 6 for 24 h. Three biological replicates were performed.

Experiment 4: Comparison of the efficacy of zygote and blastocyst vitrification.

After IVM and IVF, presumptive zygotes from the same batch

were either vitrified/warmed using the MVC method described above at 20 h after IVF (Day 0) and subsequently cultured until Day 7 or cultured until Day 6 and vitrified/warmed as blastocysts and subsequently cultured for an additional 24 h (Day 7). In the control group, presumptive zygotes were cultured to Day 7 without vitrification at any stage. In all groups, from Day 6 to Day 7, the IVC2 medium was supplemented with 10% FBS. Numbers and percentages of live blastocysts and the numbers of total and membrane damaged cells were recorded on Day 7. Three biological replicates were performed.

Statistical analysis

Data are expressed as mean ± SEM. Data of experiments employing more than 2 experimental groups (*Experiments 1, 3,* and *4*) were analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test using GraphPad Prism software (Ver 7.02 for Windows, GraphPad Software, La Jolla, California, USA). Data from *Experiment 2* were analyzed using the Welch's *t*-test using the KyPlot package (Ver. 2.0, KyensLab, Tokyo, Japan). $P < 0.05$ was defined as the significance level.

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