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Hollow fiber vitrification allows cryopreservation of embryos with compromised cryotolerance

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Abstract

Purpose: This study aims to demonstrate vitrification methods that provide reliable cryopreservation for embryos with compromised cryotolerance.

Methods: Two-cell stage mouse embryos and in vitro produced porcine embryos were vitrified using the hollow fiber vitrification (HFV) and Cryotop (CT) methods. The performance of these two methods was compared by the viability of the vitrified-rewarmed embryos.

Results: Regardless of the method used, 100% of the mouse 2-cell embryos developed successfully after vitrification-rewarming into the blastocyst stage, whereas vitrification tests using porcine morulae with the HFV method produced significantly better results. The developmental rates of vitrified porcine morula into the blastocyst stage, as well as blastocyst cell number, were 90.3% and 112.3 ± 6.9 in the HFV group compared with 63.4% and 89.5 ± 8.1 in the CT group ($P < .05$). Vitrification tests using 4- to 8-cell porcine embryos resulted in development into the blastocyst stage (45.5%) in the HFV group alone, demonstrating its better efficacy. The HFV method did not impair embryo viability, even after spontaneous rewarming at room temperature for vitrified embryos, which is generally considered a contraindication.

Conclusion: Vitrification test using embryos with compromised cryotolerance allows for more precise determining of effective cryopreservation methods and devices.

KEYWORDS

cryotolerance, embryo cryopreservation, hollow fiber vitrification (HFV), mouse embryos, porcine embryos

1 | INTRODUCTION

Cryopreservation technology for early-stage embryos plays an important role in the dissemination of valuable laboratory animals and livestock, as well as the conservation of genetic resources.[1,2] In human-assisted reproduction technology (ART), the cryopreservation of early-stage embryos is an indispensable technique for ensuring conception.

Various methods have been developed for the cryopreservation of early mammalian embryos, including humans (nylon loop,[3] OPS,[4]

solid surface,[5] Cryotop [6]) with some of these methods becoming standard in the fields of reproductive medicine, animal husbandry, and biomedical research.[7,8]

The success or failure of cryopreservation is decisive to the success of ART (ie, patient conception) and the production efficiency of animals. In other words, cryopreservation has critical effects on patient benefit, the economics of the animal industry, and the overall welfare of animals. Cryotolerance of mammalian embryos varies depending on the species,[9,10] developmental stages,[11-14] and culture environment.[15,16] It is therefore important to strictly evaluate the efficacy of a given method to maximize the number of individuals obtained from cryopreserved embryos. The selection of the cryopreservation method is especially critical when embryos with compromised cryotolerance are involved, such as with the embryos of older patients or with the embryos of rare, wild species.

In this study, we tested cryopreservation methods using embryos with compromised cryotolerance, allowing us to evaluate the methods with greater accuracy, thereby developing a more effective method.

2 | MATERIALS AND METHODS

2.1 | Animal care

Mice were housed under controlled conditions (24 ± 2°C, humidity 70%, 12-h light/12-h darkness photoperiod) with food (CE-2; CLEA Japan) and drinking water available ad libitum. All of the animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC16-0009).

2.2 | Chemicals

All chemicals were obtained from Sigma-Aldrich, unless otherwise indicated.

2.3 | Collection and culture of mouse embryos

BDF1 female mice (7-10 weeks old; CLEA Japan) were induced to superovulate via an intraperitoneal injection of 7.5 IU eCG (Asuka Pharmaceutical) and 8.25 IU hCG (Kyoritsu Seiyaku) administered 44-46 h apart. Females were paired with males of the same strain immediately after injection with hCG; mating was confirmed by the presence of a vaginal plug the following morning (day 1). Two-cell stage embryos were collected by oviductal flushing using H-CZB medium [17] 44 h post-hCG injection.

Embryos were cultured for 72-74 h after embryo collection (until day 5) at 37.5°C in a humidified atmosphere of 5% CO_2 in 20 µl drops of CZB medium [18] supplemented with 5 mg/mL BSA, 0.146 mg/mL glutamine, and 0.029 mg/mL Na pyruvate under mineral oil in a 35-mm plastic petri dish (Iwaki 1000-035; Asahi Techno Glass).

2.4 | Embryo transfer

Female ICR mice (10-12 weeks old; Japan SLC) in estrus were mated to vasectomized males of the same strain. The presence of a vaginal plug on the following day was used to verify pseudopregnancy. Two-cell stage embryos were transferred into the oviducts of pseudopregnant recipients on day 1 under anesthesia using avertin. A total of 20 embryos were transferred into each oviduct. Females showing signs of pregnancy were sacrificed on day 18 of gestation. The number of normal fetuses in each uterine horn was then counted.

2.5 | Preparation of porcine parthenogenetic embryos

The preparation of the in vitro matured (IVM) porcine oocytes was carried out as previously described.[19] Cumulus-oocyte complexes (COCs) were aspirated from the follicles of porcine ovaries collected at a local abattoir. COCs with at least three layers of compacted cumulus cells were cultured in NCSU23 medium[20] in a humidified atmosphere of 5% CO_2 and 95% air at 38.5°C. IVM oocytes with expanded cumulus cells were treated with hyaluronidase dissolved in Tyrode's lactose medium and then isolated from cumulus cells by gentle pipetting. Oocytes with an evenly granulated ooplasm and an extruded first polar body were selected for subsequent experiments.

Oocytes 45 h after in vitro maturation were washed in an activation solution before aligning between two wire electrodes (separated by 1.0 mm) in a drop of activation solution on a fusion chamber slide (CUY500G1; Nepa Gene). A single direct current pulse of 150 V/mm was applied for 100 µs using an electrical pulsing instrument (LF201; Nepa Gene). Activated oocytes were treated with cytochalasin B for 3 h to suppress the extrusion of the second polar body.

Porcine parthenogenetic embryos were cultured in vitro using porcine zygote medium-5 (PZM-5; Research Institute for the Functional Peptides Corporation). A microdrop (20-35 µL) of PZM-5 was placed on a plastic petri dish and covered with paraffin oil. The embryos were cultured under a condition of 5% CO_2/5% O_2/90% N_2 in humidified atmosphere at 38.5°C. The day of oocyte electrical activation was defined as day 0. Embryos at the 4- to 8-cell stage after 3 days in culture (day 3) and morulae stage after 4 days in culture (day 4) were subjected to the experiments. For embryos after day 4, 10% (v/v) fetal bovine serum (FBS) was added to PZM-5. The culture was continued until day 7.

2.6 | Embryo vitrification and rewarming

2.6.1 | Vitrification and rewarming solutions

The solutions used for the embryo vitrification procedures were prepared as described by Kuwayama et al.[21] The vitrification solution (VS) and rewarming solution (RS) used in this study were

prepared using HEPES (20 mM)-buffered tissue culture medium 199 (Nissui Pharmaceutical) supplemented with 20% calf serum as the basal solution, which was also used as the washing solution (WS). Basal solution containing 7.5% (v/v) dimethyl sulfoxide (DMSO; Nacalai Tesque) and 7.5% (v/v) ethylene glycol (EG; Nacalai Tesque) as a permeable cryoprotectant was used as the equilibration solution (ES). The VS was composed of basal solution with 15% DMSO, 15% EG, and 0.5 M sucrose. The basal solution containing 1 M sucrose was used as the RS. The RS temperature was set to 37.5°C for mouse embryos and 38.5°C for porcine embryos. A basal solution containing 0.5 M sucrose was used as the dilution solution (DS). ES, VS, RS, DS, and WS (4 mL each) were used in 35-mm plastic dishes. The immersion time of each of the embryos in each solution was 7, 1, 1, 3, and 5 min, regardless of the animal species or developmental stage. Each solution was used at room temperature (RT; 24-27°C), except for RS.

2.6.2 | Embryo vitrification and rewarming by the hollow fiber vitrification (HFV) method

Vitrification of mouse and porcine embryos by the HFV method was performed as previously reported.[22] The embryos were placed in ES and then aspirated into a hollow fiber (~25 mm long with inner and outer diameters of 185 and 215 μm, respectively) (FB-170FH; Nipro Corporation) in a 1.0 to 2.0 mm column of ES flanked with air bubbles. Following equilibration, the fiber was transferred into VS using forceps, during which time it was moved gently in the dish to ensure the dehydration of the embryos inside. The fiber holding the embryos was then immersed in liquid nitrogen (LN) while being held vertically with forceps. After cryopreservation in LN, the fiber was rewarmed by rapid immersion in RS, followed by DS, and finally in the first and second WS. In the second WS, the embryos were expelled from the hollow fiber by gently squeezing the fiber from one end to the other using forceps.

2.6.3 | Embryo vitrification and rewarming with the Cryotop method

The vitrification and rewarming of embryos using the Cryotop (CT, Kitazato Corporation) were performed according to a previous report.[6] Embryo vitrification experiments were conducted using the same methods, regardless of the developmental stage or animal species.

Briefly, the embryos were placed in ES in a 35-mm plastic dish for 7 min to equilibrate. Next, the embryos were transferred to VS and, after about 45 s, a capillary pipette was used to load embryos, together with a small amount of VS, onto the sheet at the tip of the CT. Next, the embryos were immediately immersed in LN. The overall time from the transfer of the embryos into VS to immersion into LN did not exceed 1min. When loading the embryos onto a CT device, one embryo was placed in each drop, with the minimum volume of VS around the embryo (<0.1 μL).

During rewarming, CT was quickly transferred from LN to RS (37.5°C for mouse embryos, 38.5°C for porcine embryos). Embryos released from the CT device in the RS were recovered after 1 min using a capillary pipette and transferred to DS. After placing the embryos in the DS for 3 min, the embryos were transferred to WS.

2.7 | Counting of blastocyst cell number

The blastocysts obtained (mouse: day 5, porcine: day 7) were mounted onto glass slides with DPBS counting 20% EG and 5 mg/mL Hoechst 33 342 and examined under a fluorescence microscope (TE 2000, Nikon) for cell counting.

2.8 | Statistical analysis

Statistical analyses were performed using SPSS software, version 25 (SPSS Inc). All percentages of values were subjected to arcsine transformation prior to analysis. All data were evaluated by one-way analysis of variance (ANOVA) followed by multiple comparisons by Tukey's test. The level of significance was set at $P < .05$.

2.9 | Experimental design

2.9.1 | Experiment 1: Comparison of the HFV and CT methods for vitrification of mouse 2-cell stage embryos

The purpose of this experiment was to compare two cryopreservation methods, HFV [22] and CT,[6] using BDF1 strain 2-cell stage mouse embryos which possess a high cryotolerance.

An HFV device was originally developed to permit vitrifying multiple embryos simultaneously [19,22,23] with a single device. The CT device, on the other hand, is usually used for single or very few eggs or embryos. In order to ensure an equal comparison between the HFV and CT, the number of embryos to be vitrified using a single device was set to three. The developmental rate of blastocysts obtained via in vitro culture after rewarming, as well as the number of blastocyst cells, was compared between the two methods.

2.9.2 | Experiment 2: Comparison of the HFV and CT methods for vitrification of in vitro produced (IVP) porcine embryos

The purpose of this experiment was to compare the HFV and CT methods using porcine embryos characterized by a lower cryotolerance[12,13] than the mouse embryos. Vitrification and rewarming of parthenogenetic porcine embryos at the morula and 4- to 8-cell stage were performed using the HFV and CT methods. The number of embryos to vitrify using a single device was set to three, respectively. The developmental rates to blastocysts and hatched blastocysts obtained via

in vitro culture after rewarming, and the cell numbers of blastocysts obtained, were compared between the two methods.

2.9.3 | Experiment 3: Verification of the effect of suboptimal rewarming conditions on mouse embryos vitrified by the HFV method

The purpose of this experiment was to verify the possible range of acceptable rewarming conditions for the embryos cryopreserved using the HFV method.

Two-cell stage mouse (BDF1) embryos were vitrified by the HFV method, as described above, and rewarmed using the following three protocols: (a) ultra-rapid rewarming method-1, the hollow fibers removed from LN were rapidly (~0.5 s) immersed in RS. This was the standard rewarming protocol for the HFV method [22,23]; (b) ultra-rapid rewarming method-2, the hollow fibers removed from LN were maintained on a 37.5°C warming plate for 3 s. After the VS inside the fibers had melted, the fibers were transferred to RS at RT for 1 min and then handled as described above; and (c) RT in-air rewarming (suboptimal rewarming protocol), the hollow fibers removed from LN were held for 5 s in RT air, then immersed in RS at RT for 1 min, and handled the same manner as in the standard method. The VS completely melted after 5 s in RT air following ice crystal formation (opaqueness), appearing in the fibers after approximately 2 s.

The survival of the vitrified embryos was evaluated by the in vitro development or development to fetuses after their transfer into pseudopregnant recipient mice. For each hollow fiber vitrification device, 10 mouse embryos were loaded.

3 | RESULTS

3.1 | Experiment 1: Survival of vitrified mouse embryos was the same between the HFV and CT methods

In this experiment, HFV and CT methods were used to vitrify 2-cell stage mouse embryos. Regardless of the method used, the rate of development into the blastocyst stage after the vitrification was 100%. The number of blastocysts obtained from the vitrified-rewarmed embryos was equal (24/24 vs 24/24) in both groups, and there was no significant difference between the average cell number of blastocysts in either group (74.5 ± 4.1 vs 70.1 ± 2.6). The results of these vitrified group were comparable to the non-vitrified control group (blastocysts formation rate: 100%, 24/24. Cell number: 75.9 ± 2.6) (Table 1, Figure 1).

3.2 | Experiment 2: Survival of vitrified porcine embryos differed between the HFV and CT methods

In Experiment 1, a high embryo viability (survival rate: 100%) was obtained when the cryopreservation of 2-cell stage mouse embryos was performed using either the HFV or CT methods. In Experiment 2, the viability of the embryos after vitrification and rewarming by both methods was compared using porcine morulae or 4- to 8-cell stage embryos, which have a lower cryotolerance than 2-cell stage mouse embryos.

In the vitrification of porcine morulae, the post-rewarm developmental rate and blastocyst quality differed depending on the

TABLE 1 Survival of mouse 2-cell stage embryos vitrified by the HFV or CT method

Treatments[†]	No. of embryos			
	Cultured	Developed into blastocysts (%)[‡]	Developed into hatched blastocysts (%)[‡]	Cell numbers in the blastocysts (mean ± SEM)[‡]
Non-vitrified	24	24 (100)[a]	14 (58.3)[a]	75.9 ± 2.6[a]
HFV	24	24 (100)[a]	12 (50.0)[a]	74.5 ± 4.1[a]
CT	24	24 (100)[a]	10 (41.7)[a]	70.1 ± 2.6[a]

[†]8 repeated experiments were carried out for each group.

[‡][a]No significant difference.

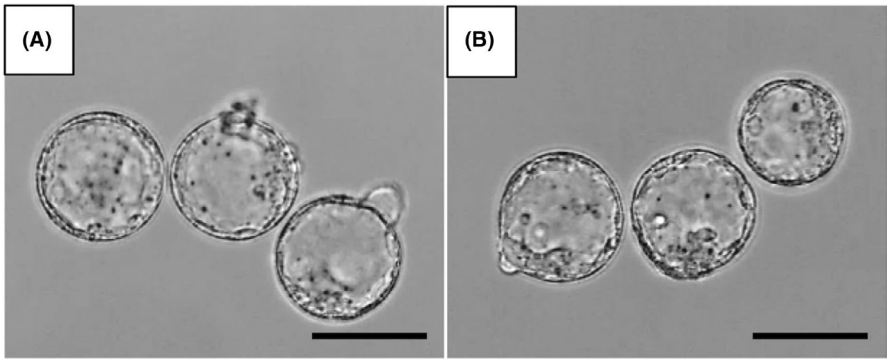

FIGURE 1 Development after rewarming 2-cell stage mouse embryos vitrified by the HFV and CT methods (day 5). (A) Blastocysts developed from 2-cell stage embryos vitrified by the HFV method. (B) Blastocysts developed from 2-cell stage embryos vitrified by CT method. Scale bar = 100 μm

method used. The developmental rate of embryos in the HFV group into the blastocyst stage and the cell number of blastocysts obtained were superior to those in the CT group (HFV: 90.3%, 112.3 ± 6.9 vs CT: 63.4%, 89.5 ± 8.1; $P < .05$). Furthermore, the results in the HFV group were equivalent to those obtained for the non-vitrified control group (93.5%, 130.9 ± 6.5) (Table 2, Figure 2).

Next, the results of both methods were compared using 4- to 8-cell stage porcine embryos, which have a higher cryosensitivity than morula. The difference in performance between the two methods was more prominent than in the comparative test using morulae. In the HFV group, 45.5% (15/33) of the embryos developed into blastocysts, although significantly fewer ($P < .05$) than in the non-vitrified control group. Conversely, blastocysts were not obtained from the vitrified embryos in the CT group (Table 3, Figure 3).

3.3 | Experiment 3: Mouse embryos vitrified by the HFV method permitted suboptimal rewarming conditions

The results of Experiment 2 showed an apparent difference in performance of vitrification devices when were applied to cryopreserve embryos with low cryotolerance. In other words, the HFV method was shown to be highly effective for vitrifying embryos with a high cryosensitivity, such as porcine embryos.

In order to further describe the characteristics of the HFV method, we confirmed the tolerable range of rewarming conditions. Using a procedure that reproduces spontaneous melting at room temperature (RT in-air rewarming), which is usually contraindicated when rewarming vitrified embryos, developmental rate of the vitrified embryos into the blastocyst stage did not decrease

TABLE 2 Survival of porcine morulae vitrified by the HFV or CT method

Treatments[†]	No. of embryos			
	Cultured	Developed into blastocysts (%)[‡]	Developed into hatched blastocysts (%)[‡]	Cell numbers in the blastocysts (mean ± SEM)[‡]
Non-vitrified	93	87 (93.5)[a]	64 (68.8)[a]	130.9 ± 6.5[a]
HFV	93	84 (90.3)[a]	38 (40.9)[c]	112.3 ± 6.9[ab]
CT	93	59 (63.4)[b]	14 (15.1)[b]	89.5 ± 8.1[b]

[†]Experiments were repeated 31 times for each group.

[‡a,b,c]Values with different superscripts differ significantly within each column ($P < .05$).

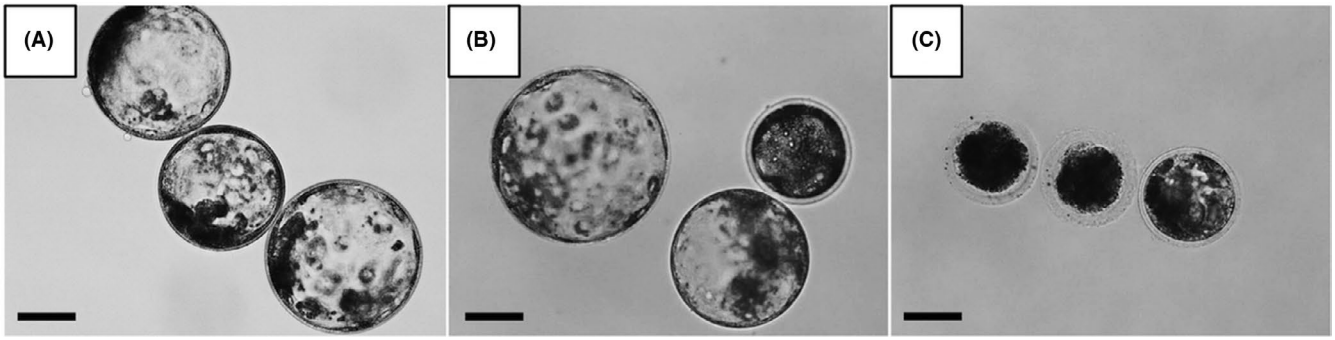

FIGURE 2 Development of parthenogenetic porcine morulae after vitrification and rewarming by the HFV and CT methods (day 6). (A) Blastocysts developed from non-vitrified morulae. (B) Blastocysts developed from morulae vitrified by the HFV method. (C) Blastocysts developed from morulae vitrified by the CT method. Scale bar = 100 μm

TABLE 3 Survival of porcine 4- to 8-cell stage embryos vitrified by the HFV or CT method

Treatments[†]	No. of embryos			
	Cultured	Developed into blastocysts (%)[‡]	Developed into hatched blastocysts (%)[‡]	Cell numbers in the blastocysts (mean ± SEM)[‡]
Non-vitrified	33	28 (84.8)[a]	20 (60.6)[a]	83.3 ± 5.8[a]
HFV	33	15 (45.5)[c]	4 (12.1)[c]	57.4 ± 9.7[b]
CT	33	0 (0)[b]	0 (0)[b]	-

[†]Experiments were repeated 11 times for each group.

[‡a,b,c]Values with different superscripts differ significantly within each column ($P < .05$).

(94.5%, 104/110). These results were similar to the rewarming results obtained using the ultra-rapid rewarming-1 (95.7%, 134/140) and ultra-rapid rewarming-2 (96.7%, 116/120) protocols (Table 4, Figure 4).

Spontaneous melting by the RT in-air rewarming did not affect the developmental rate of the cryopreserved embryos into fetuses. Developmental rates of the vitrified embryos into the fetal stage were equal (68.8%-70.0%) for each of the three groups (Table 5) compared in this experiment.

4 | DISCUSSION

In recent years, implementation of infertility treatments has increased in developed countries due to marriage and pregnancy later in life. Under such circumstances, embryo cryopreservation has become an established and indispensable technology for ART.[24-27] The quality of patient embryos can vary, with some exhibiting low cryotolerance. Minimizing the damage associated with embryo vitrification and rewarming will improve the efficiency of ART, benefiting the patients. Therefore, the development of improved embryo cryopreservation methods as well as the optimization of existing methods is one of the major issues of ART.

In Experiment 1, we vitrified BDF1 strain 2-cell stage mouse embryos, which are known to have a high cryotolerance.[28,29] The results clearly showed that a cryopreservation test using embryos with a high cryotolerance cannot manifest potential difference in performance of the methods. It is likely that methods other than HFV or CT may achieve the same results for vitrifying BDF1 mouse embryos. Mouse embryos have been used in the evaluation of various cryopreservation methods[30-35]; however, due to the intrinsic cryotolerance, they are not necessarily suitable for the detection of small differences in the performance of cryopreservation methods.

Experiment 2 demonstrated that the differences in the cryopreservation methods became significant when IVP porcine embryos with low cryotolerance were used. In other words, IVP porcine embryos with compromised cryotolerance were suitable for a stricter comparison of the cryopreservation methods. Early-stage porcine embryos are known to exhibit high species-specific cryosensitivity.[14,36,37] Notably, IVP embryos are affected by oxidative stress from in vitro culture, whereby cryotolerance is reduced to a greater extent than in vivo derived embryos.[16,38,39] Vitrification test of porcine IVP embryos with these traits showed that the HFV method could be used to cryopreserve embryos that are difficult to treat via other methods.

The quality of embryos is variable among patients undergoing infertility treatment. Embryo quality is greatly influenced by oocyte

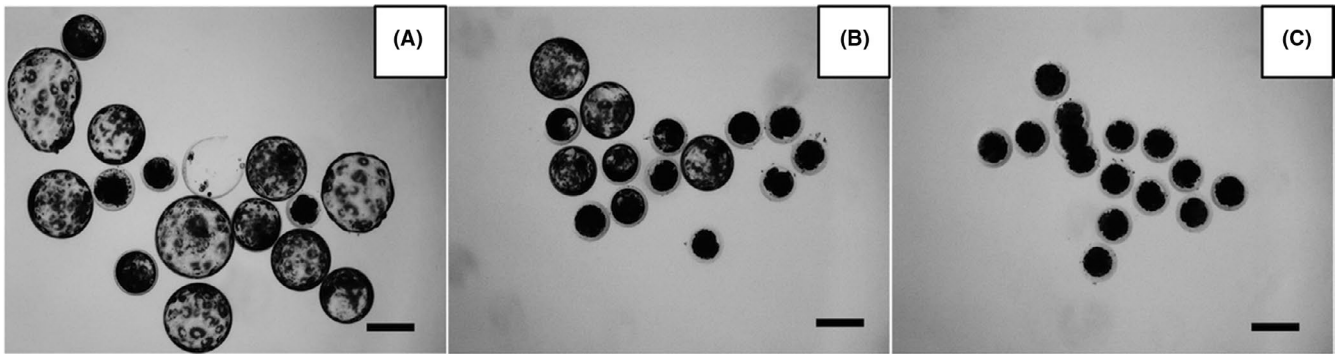

FIGURE 3 Development, after vitrification and rewarming, of 4- to 8-cell stage porcine embryos vitrified using the HFV and CT methods (day 6). (A) Blastocysts developed from non-vitrified embryos. (B) Approximately half of the embryos vitrified using the HFV method developed to the blastocyst stage. (C) All embryos vitrified by the CT method degenerated. Scale bar = 100 μm

TABLE 4 In vitro development of mouse 2-cell stage embryos vitrified by the HFV method using either the standard protocol or suboptimal rewarming conditions

Treatments	No. of embryos				
	Cultured[†]	Developed into blastocysts (%)[‡]	Developed into hatched blastocysts (%)[‡]	Degenerated (%)[‡]	Cell numbers in the blastocysts (mean ± SEM)[‡]
Non-vitrified	120	119 (99.2)[a]	77 (64.2)[a]	1 (0.9)[a]	77.1 ± 1.9[a]
Ultra-rapid rewarming-1	140	134 (95.7)[a]	56 (40.0)[ab]	6 (4.3)[a]	69.6 ± 2.1[a]
Ultra-rapid rewarming-2[§]	120	116 (96.7)[a]	65 (54.2)[ab]	4 (3.3)[a]	70.7 ± 2.6[a]
RT in-air rewarming[¶]	110	104 (94.5)[a]	35 (31.8)[b]	6 (5.5)[a]	70.1 ± 2.3[a]

[†]Experiments were repeated 11-14 times for each group.

[‡a,b]Values with different superscripts differ significantly within each column ($P < .05$).

[§]The hollow fibers removed from LN were maintained on the surface of a 37.5°C warming plate for 3 s.

[¶]The hollow fibers removed from LN were held for 5 s in RT air and then immersed in RS at RT for 1 min.

quality, which is influenced by various factors including physical health, lifestyle, and age [40-44] of a patient. In addition, retarded or subnormal in vitro development of embryos produced by in vitro fertilization or intracytoplasmic sperm injection (ICSI) is not uncommon.[45-47] Such low-grade embryos are susceptible to cryopreservation-related damage,[47,48] which is why ART should prioritize a cryopreservation method with a superior performance. Application of the HFV method to human ARTs is expected to improve the pregnancy rate of patients. In fact, we have confirmed the effectiveness of HFV method in IVP embryos of common marmosets, non-human primates (data not shown).

Although the actual reason for the efficacy of the HFV method is not clear, osmotic stress, a known stressor to embryo viability,[49-51] is a possible factor. During the vitrification and rewarming process, embryos are exposed to fluctuations in osmotic pressure though the sequential treatment with ES, VS, RS, DS, and WS. With the HFV

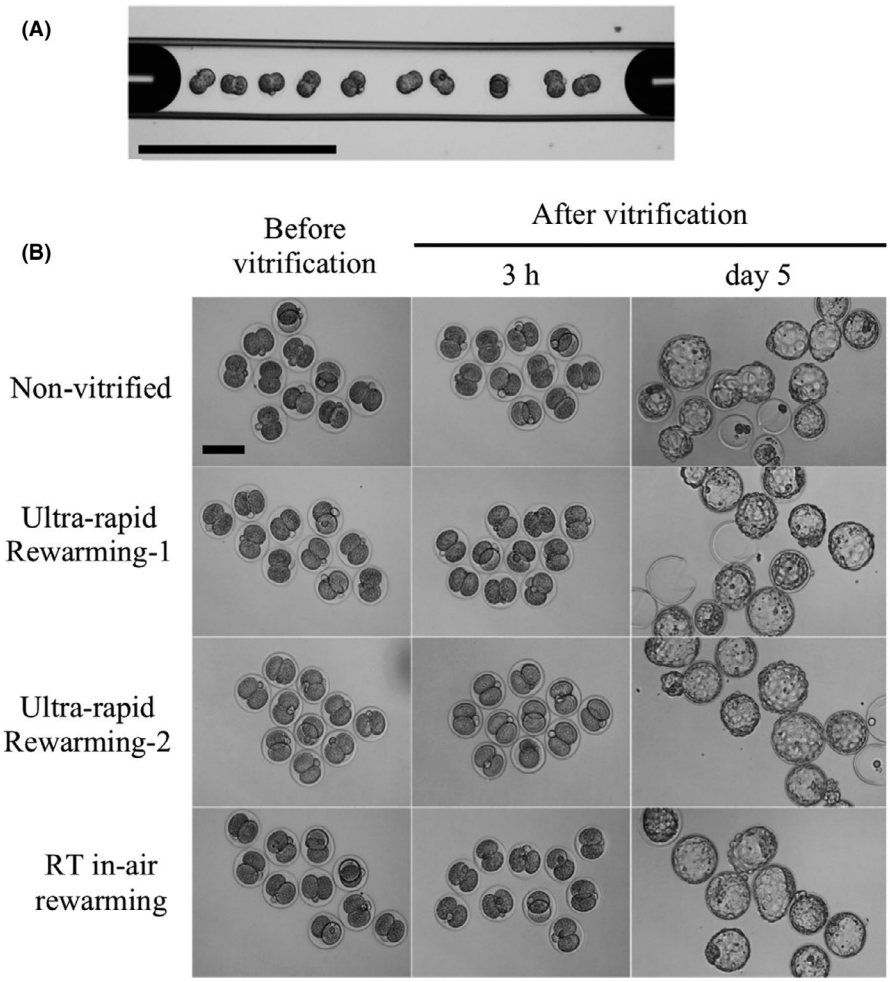

FIGURE 4 In vitro development of mouse 2-cell stage embryos after cryopreservation by the HFV method. (A) Mouse 2-cell stage embryos loaded in a hollow fiber. (B) Morphological features of the vitrified embryos rewarmed under suboptimal conditions were equal to those of the embryos rewarmed using the standard protocol and of the non-vitrified controls. Scale bar = 1 mm (A) and 100 μm (B)

TABLE 5 Production efficiency of fetuses from mouse 2-cell stage embryos vitrified by the HFV method

Treatments	No. of embryos transferred[†]	Pregnancy (%) [††]	Developmental efficiency to fetuses (%)[‡,††]
Non-vitrified	80	4/4 (100)[a]	66/80 (82.5)[a]
Ultra-rapid rewarming-1	80	4/4 (100)[a]	55/80 (68.8)[a]
Ultra-rapid rewarming-2[§]	80	4/4 (100)[a]	55/80 (68.8)[a]
RT in-air rewarming[¶]	80	4/4 (100)[a]	56/80 (70.0)[a]

[†]Twenty embryos/devices were vitrified. Twenty vitrified-warmed 2-cell stage embryos were transferred to each recipient.

[‡]E18 fetuses were recovered.

[††a]No significant difference ($P < .05$).

[§]The hollow fibers removed from LN were maintained on the surface of a 37.5°C warming plate for 3 s.

[¶]The hollow fibers removed from LN were held for 5 s in RT air and then immersed in RS at RT for 1 min.

method, osmotic stress may be reduced since embryos are exposed to solutions with large osmotic pressure differences via the hollow fiber membrane.

Another trait of the HFV method is that embryos are not handled with a glass capillary during the pre-vitrification process, that is, equilibration with cryoprotectants as well as the post-vitrification process, that is, rewarming, dilution, and washing. Therefore, exposure time to the solution in each step can be precisely controlled in the HFV method. In addition, minimizing the volume of VS loaded with an embryo is essential with the CT method, which can be a destabilizing factor for the CT method. Indeed, one reason that the HFV method produces favorable results is due to its reduced reliance on the operator's technical skills.

In Experiment 3, a wide range of rewarming conditions were tested for the cryopreservation of embryos by the HFV method. The HFV method was designed on the basis of the MVC concept,[52,53] wherein minimized amounts of VS holding embryos allow for an ultra-rapid temperature increase during rewarming.[52,53] RT in-air rewarming tested in this study reproduced conditions of spontaneous sample melting at room temperature due to mishandling. The ability to ensure embryo viability even in cases of accidental spontaneous melting, which is a contraindication of embryo vitrification, has great practical significance in embryo cryopreservation technology. Conversely, in a preliminary experiment, when vitrified porcine embryos were rewarmed, following the slow rewarming protocol in the HFV method, the viability of the embryos declined as compared to that when the standard rewarming method was used. This result strongly indicates the importance of the rewarming method for vitrified embryos.

Cryopreserved embryos are widely utilized for production of experimental and domestic animals. Embryos distributed from organizations such as embryo banks often end up in the hands of unspecified users, leading to rewarming in an inappropriate manner and an overall lack of quality control. It is common to rewarm (thaw) vitrified embryos by directly immersing the cryopreservation device from the LN to a warm water bath at 37-40°C, since ultra-rapid temperature raise is critical for embryos viability.[54] Conversely, it is believed that slow warming is damaging to cells, since the increased time allows for small intracellular ice crystals formed during cooling to grow to damaging size during warming.[55]

The results presented here demonstrate that the HFV method is effective for the cryopreservation of embryos with compromised cryotolerance. Our findings also show that cryopreservation methods and devices can be more rigorously evaluated using embryos with compromised cryotolerance, such as IVP porcine embryos. We hope this study will provide a basis for the improvement of current cryopreservation technologies.

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DISCLOSURES

Conflict of interest: Ayuko Uchikura, Hitomi Matsunari, Miki Maehara, Shiori Yonamine, Sayaka Wakayama, and Teruhiko Wakayama declare that they have no conflict of interest. Hiroshi Nagashima is a founder and shareholder of PorMedTec Co., Ltd. *Human rights statements and informed consent*: This article does not contain any studies with human patients performed by any of the authors. *Animal studies*: All of the animal experiments performed in this study were approved by the Institutional Animal Care, Use and Ethics Committee of Meiji University (IACUC16-0009). All animal care and experimental procedures were performed in accordance with the Japan Act on Welfare and Management of Animals and all applicable regulations. *Approval by ethics committee:* Not applicable to human Ethics committee.

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