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Hollow Fiber Vitrification Provides a Novel Method for Cryopreserving In Vitro Maturation/Fertilization-Derived Porcine Embryos[1]

Miki Maehara,[3] Hitomi Matsunari,[3,4] Kasumi Honda,[3] Kazuaki Nakano,[3] Yasuhiro Takeuchi,[3] Takahiro Kanai,[3] Taisuke Matsuda,[3] Yukina Matsumura,[3] Yui Hagiwara,[5] Norihisa Sasayama,[5] Akio Shirasu,[5] Masashi Takahashi,[6] Masahito Watanabe,[3,4] Kazuhiro Umeyama,[3,4] Yutaka Hanazono,[7] and Hiroshi Nagashima[2,3,4]

[3]*Laboratory of Developmental Engineering, Department of Life Sciences, School of Agriculture, Meiji University, Kawasaki, Japan*
[4]*Meiji University International Institute for Bio-Resource Research (MUIIBR), Kawasaki, Japan*
[5]*Research & Development Institute for Artificial Organs, Research & Development Laboratory, Nipro Corporation, Shiga, Japan*
[6]*Department of Animal and Grassland Research, National Agricultural Research Center for Kyushu Okinawa Region (KONARC), Kumamoto, Japan*
[7]*Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan*

ABSTRACT

In vitro matured (IVM) oocytes have been used to create genetically modified pigs for various biomedical purposes. However, porcine embryos derived from IVM oocytes are very cryosensitive. Developing improved cryopreservation methods would facilitate the production of genetically modified pigs and also accelerate the conservation of genetic resources. We recently developed a novel hollow fiber vitrification (HFV) method; the present study was initiated to determine whether this new method permits the cryopreservation of IVM oocyte-derived porcine embryos. Embryos were created from the in vitro fertilization of IVM oocytes with frozen-thawed sperm derived from a transgenic pig carrying a humanized Kusabira-Orange (*huKO*) gene. Morula-stage embryos were assigned to vitrification and nonvitrification groups to compare their in vitro and in vivo developmental abilities. Vitrified morulae developed to the blastocyst stage at a rate similar to that of nonvitrified embryos (66/85, 77.6% vs. 67/84, 79.8%). Eighty-eight blastocysts that developed from vitrified morulae were transferred into the uteri of three recipient gilts. All three became pregnant and produced a total of 17 piglets (19.3%). This piglet production was slightly lower, albeit not significantly, than that of the nonvitrification group (27/88, 30.7%). Approximately half of the piglets in the vitrification (10/17, 58.8%) and nonvitrification (15/27, 55.6%) groups were transgenic. There was no significant difference in the growth rates among the piglets in the two groups. These results indicate that the HFV method is an extremely effective method for preserving cryosensitive embryos such as porcine in vitro maturation/fertilization-derived morulae.

cryopreservation, hollow fiber vitrification, in vitro produced porcine embryo, IVM/IVF, pig

INTRODUCTION

Pigs are increasingly important in biomedical research, especially in translational research [1–3], and have been used in a wide range of studies, such as for cardiovascular disease, liver and kidney failure, endoscopic surgery, biomaterials, hybrid organs, and wound healing. Furthermore, genetically modified pigs have been created for specialized research purposes and applications, such as cell tracking with fluorescent proteins [3–5], tissue/organ transplantation [6–9], and disease models [10, 11].

Currently, genetically modified pigs are created from in vitro produced embryos by somatic cell nuclear transfer (SCNT) [4, 8, 12] or microfertilization [13, 14] of in vitro matured (IVM) oocytes. Pig production using in vitro produced embryos can be further improved through the cryopreservation of embryos, which enables long-term storage of embryos, thus permitting their transfer into recipients under optimal conditions, including precise estrus-synchronization. Embryo cryopreservation also facilitates the development of a gene bank of genetically modified pigs.

However, the practical cryopreservation of porcine embryos has been hindered by their intrinsic cryosensitivity [15–18]. Furthermore, porcine embryos derived from IVM oocytes are extremely cryosensitive; the cryopreservation of these embryos has only been successful when special measures have been taken [19–23], such as delipation (removal of cytoplasmic lipids) [24, 25]. Recently, Matsunari et al. [26] developed a new vitrification protocol that uses a hollow fiber as the device for holding the embryos; this approach is called the hollow fiber vitrification (HFV) method. Using this method, IVM oocyte-derived parthenogenetic morulae were preserved without delipation and with a high postvitrification survival rate [26].

We therefore conducted this study to examine whether in vitro maturation/fertilization (IVM/IVF)-derived porcine embryos can give rise to normal piglets after vitrification by the

[1]Supported by the Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency, and the Meiji University International Institute for Bio-Resource Research (MUIIBR).
[2]Correspondence: Hiroshi Nagashima, Laboratory of Developmental Engineering, Department of Life Sciences, School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama, Kawasaki 214-8571, Japan.
E-mail: hnagas@isc.meiji.ac.jp

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HFV method. We generated porcine IVM/IVF-derived morulae with frozen sperm from a transgenic boar and demonstrated that the embryos maintained their viability at a high efficiency after vitrification by the HFV method. Furthermore, live piglets could be produced efficiently from the IVM/IVF-derived morulae after vitrification. We found that cryopreservation of porcine IVM/IVF-derived embryos is a practical method for conserving and proliferating valuable transgenic pigs.

MATERIALS AND METHODS

Animal Care

All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC-05-003).

Chemicals

All the chemicals were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise indicated.

In Vitro Maturation of Porcine Oocytes

Pig ovaries were collected at a local abattoir and transported to the laboratory in Dulbecco phosphate-buffered saline (DPBS; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate, 2.5 μg/ml amphotericin B, and 0.1% (w/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes (COCs) were collected by aspiration from ovarian antral follicles with a diameter of 3.0–6.0 mm. The COCs with at least three layers of compacted cumulus cells were selected and cultured in high-performance porcine oocyte maturation medium [27] (POM; Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) supplemented with 10% (v/v) porcine follicular fluid, 10 international units (IU)/ml equine chorionic gonadotropin (eCG; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), 10 IU/ml human chorionic gonadotropin (hCG; ASKA Pharmaceutical), and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP; Research Institute for the Functional Peptides). Follicular fluid added to the maturation medium was collected with COCs, filtered, and stored at −20°C until use.

The COCs were cultured for 20 h with dbcAMP, eCG, and hCG, followed by culture for 24 h without these supplements, in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. In vitro matured oocytes with expanded cumulus cells were subjected to IVF.

In Vitro Fertilization of In Vitro Matured Oocytes

In vitro fertilization of the IVM oocytes was performed with slight modifications of the methods reported by Yoshioka et al. [28] and Funahashi and Nagai [29]. Epididymal sperm from a transgenic boar carrying a single copy of the *huKO* gene in a single autosomal site (H. Matsunari, personal communication) were frozen at a concentration of 1×10^9 cells/ml according to the method of Kikuchi et al. [30] prior to use in IVF. A straw (0.5 ml) containing frozen sperm was thawed by immersion in warm water (37°C) for 30 sec. The sperm were then suspended in 5 ml DPBS supplemented with 0.1% bovine serum albumin (306-1138; Wako Pure Chemical industries, Ltd., Osaka, Japan) and washed three times by centrifugation at $1000 \times g$ for 4 min. After washing, the sperm pellets were resuspended in porcine fertilization medium [31] (PFM; Research Institute for the Functional Peptides) at a concentration of 1×10^7 cells/ml.

For insemination, 20 COCs that had been matured in vitro were placed in a 100-μl drop of PFM containing spermatozoa (1.75×10^6 cells/ml). The oocytes and sperm were incubated for 8 h at 38.5°C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2.

After insemination, the eggs were transferred to Tyrode lactose medium containing 10 mM Hepes and 0.3% (w/v) polyvinylpyrrolidone; cumulus cells and excess sperm were removed by gentle pipetting. Eggs that showed release of polar bodies with normal cytoplasmic morphology were cultured (25–30 eggs/30 μl drop) in porcine zygote medium-5 [31] (PZM-5; Research Institute for the Functional Peptides) for use in later experiments.

Some eggs were fixed in acetic acid:methanol (1:3) after 12 h of culture and then stained with 1% aceto-orcein. Eggs with two pronuclei and two polar bodies were considered to have undergone normal fertilization. Other categories of fertilized eggs were identified as polyspermic: eggs with two pronuclei, two polar bodies, and swollen sperm head(s), and eggs with two polar bodies and three or more pronuclei.

Parthenogenetic Activation of In Vitro Matured Oocytes

The in vitro matured oocytes were washed twice in an activation solution composed of 280 mM mannitol (Nacalai Tesque, Inc., Kyoto, Japan), 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% (w/v) PVA. The oocytes were then positioned between two wire electrodes (1.0 mm apart) in a drop of the activation solution on a fusion chamber slide (CUY500G1, NEPA GENE Co., Ltd., Chiba, Japan). A single direct current pulse of 150 V/mm was applied for 100 μsec using an electrical pulsing machine (LF201; NEPA GENE). Activated oocytes were treated with 5 μg/ml cytochalasin B for 3 h to suppress extrusion of the second polar body.

In Vitro Culture of the Embryos

In vitro culture of the porcine in vitro fertilized and parthenogenetic embryos was performed in 20–35 μl droplets of PZM-5 under paraffin oil (32033-00; Kanto Chemical Co., Inc., Tokyo, Japan) in a plastic Petri dish maintained in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. Embryos were cultured beyond the morula stage by adding 10% (v/v) fetal calf serum to the medium.

Vitrification and Rewarming of the Embryos

The solutions used for the embryo vitrification procedures were prepared as described by Kuwayama et al. [32]. Hepes (20 mM)-buffered tissue culture medium 199 (Nissui Pharmaceutical) supplemented with 20% calf serum was used as the basal solution. The basal solution was also used as the washing solution (WS). The vitrification solution (VS) contained 15% (v/v) dimethyl sulfoxide (DMSO; Nacalai Tesque) and 15% (v/v) ethylene glycol (EG; Nacalai Tesque) as permeable cryoprotectants (CPAs) and 0.5 M sucrose as a nonpermeable CPA.

The morulae obtained in each experiment at 4 days after insemination (Day 4) were randomly assigned to either the vitrification or nonvitrification group. Embryos assigned to the nonvitrification group were cultured in vitro, while the remaining embryos were cryopreserved. Embryos were vitrified following the HFV method reported by Matsunari et al. [26]. First, embryos were placed in 4 ml of equilibration solution (ES) containing 7.5% DMSO and 7.5% EG in a 35-mm plastic dish at room temperature (RT; 24°C–27°C). The embryos were then aspirated in a cellulose triacetate hollow fiber (ca. 25 mm long, inner diameter 185 μm, outer diameter 215 μm; FB-150FH; Nipro Corporation, Osaka, Japan) connected to a hypodermic needle with a square end (Fig. 1A) (length, 5 mm; outer diameter, 0.15 mm; inner diameter, 0.1 mm; Medical Planning Corporation, Miyagi, Japan) using a 1-ml syringe and aspiration tube (Fig. 1B).

The embryos were loaded into the hollow fiber by aspirating 10 to 20 embryos in a 2.0–4.5 mm column of ES flanked by air bubbles (Fig. 1C). The hollow fiber portion was detached from the hypodermic needle with dissecting forceps (Fig. 1D) and was kept in ES for 5–7 min. Following equilibration, the hollow fiber was transferred to VS (4 ml) at RT using forceps (Fig. 1E). The hollow fiber was kept in VS for 1 min, during which it was moved gently in the dish to ensure the dehydration of the embryos inside. After 1 min in VS, the hollow fiber holding the embryos was immersed in liquid nitrogen (LN) while being held vertically with forceps (Fig. 1F). The hollow fiber was kept in LN for a brief period (5–10 min) or housed in a cryotube [26] for longer storage (43 days) in an LN tank.

After preservation in LN, the hollow fiber was rewarmed by quickly immersing it in 4 ml rewarming solution (RS, 38.5°C) containing 1 M sucrose in a dish. In the HFV method, a group of embryos contained in a hollow fiber could be handled as a unit. Therefore, the transfer from RS to the dilution solution (DS, 0.5M sucrose) and from DS to WS was performed by holding the hollow fiber with forceps, just as in the transfer of embryos from ES to VS (Fig. 1E). The hollow fiber was kept in RS for 1 min, in DS for 3 min, and then in the first and second WS for 5 min each. In the second WS, the embryos were expelled from the hollow fiber by gently squeezing the fiber from one end to the other with forceps (Fig. 1G). Both DS and WS were kept at RT.

The appearance of liquid columns in the hollow fibers and the embryos in the columns at the ES, VS, RS, DS, and WS steps are shown in Figure 1C. The length of solution column containing 15 morulae at the ES step (3.6 mm; Fig. 1C) decreased remarkably in the VS (1.8 mm; Fig. 1C); the volumes of these liquid columns were calculated as approximately 0.13 and 0.065 μl, respectively, based on the diameter of the hollow fiber. After rewarming the hollow fiber, the length of the column at the RS step (Fig. 1C) was similar to that at the VS step; the column increased in length as the stage progressed from DS (Fig. 1C) to WS (Fig. 1C).

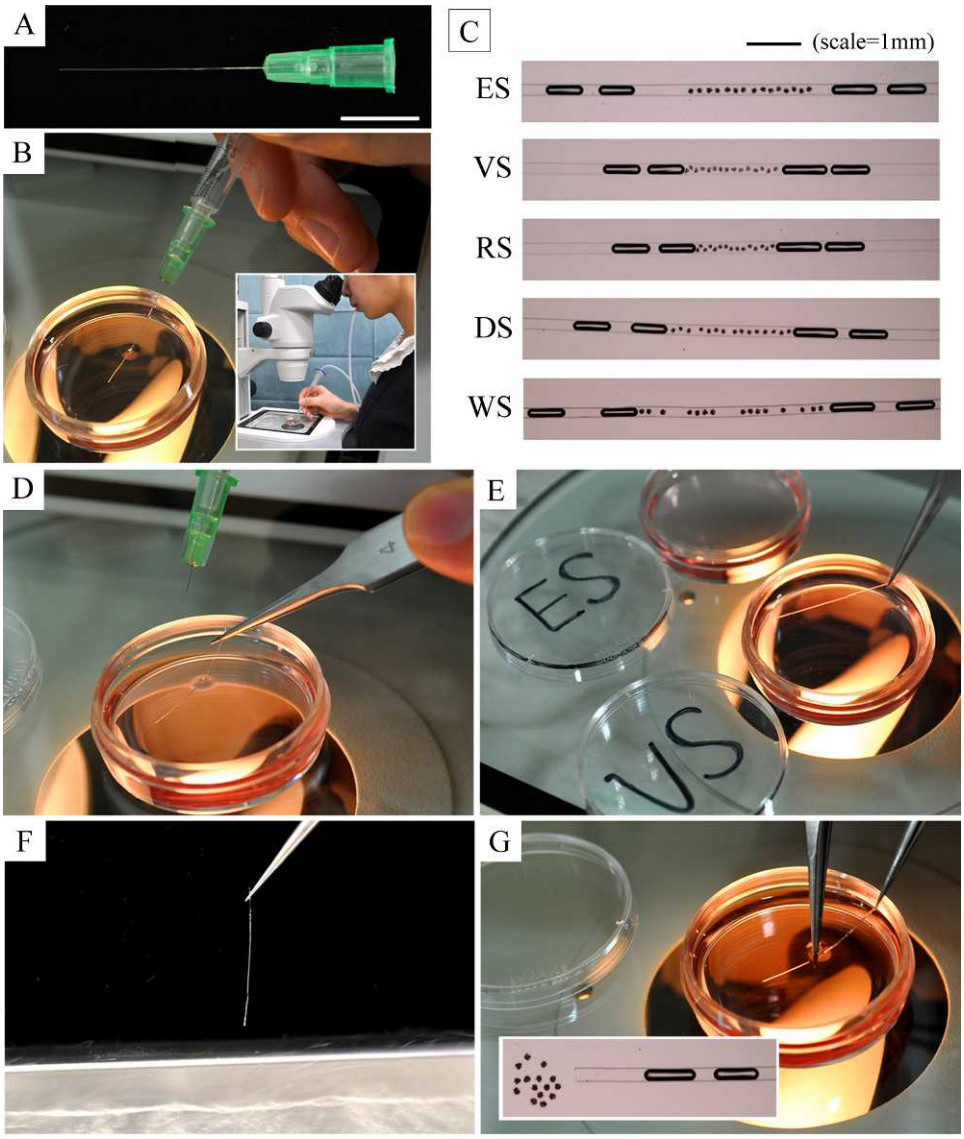

FIG. 1. Procedures for vitrification and rewarming of porcine IVM/IVF-derived morulae by the HFV method. **A**) An HFV device consisting of a 25 mm long hollow fiber segment and a hypodermic needle. Bar = 1 cm. **B**) Aspiration of embryos from the ES into the HFV device attached to a 1-ml syringe and an aspiration tube. **C**) Change of solution column length in the hollow fiber during the embryo vitrification procedures from the ES step to the WS step. **D**) Detachment of the hollow fiber holding the embryos from the hypodermic needle in the ES. **E**) Transfer of the hollow fiber from ES to VS using forceps. **F**) Plunging of the hollow fiber into LN. **G**) Recovery of embryos from the hollow fiber in WS. Inset shows embryos expelled from the hollow fiber.

Survival Assay of the Vitrified Embryos

Survival of the vitrified morulae was assessed by in vitro culture and embryo transfer to the recipients. The in vitro development of vitrified embryos to the blastocyst stage was evaluated against the nonvitrified controls. The cell numbers of the blastocysts obtained were evaluated on Day 6. The blastocysts were mounted onto glass slides with DPBS containing 20% EG and 5 mg/ml Hoechst 33342 and examined under a fluorescence microscope (TE2000-U; Nikon Corporation, Tokyo, Japan) to count cell numbers.

In the embryo transfer experiments, blastocysts obtained from vitrified and nonvitrified embryos were transferred into three recipients for each group. A group of 25 to 32 blastocysts were transferred to each recipient on Day 5 or 6, and the piglet production efficiency was compared between the vitrified and nonvitrified groups. In addition, some of the vitrified morulae preserved in LN for 43 days were used in the transfer experiment. The survival of the vitrified embryos was evaluated by development to Day 30 fetuses.

Embryo Transfer

Crossbred (Large White/Landrace × Duroc) prepubertal gilts, weighing between 100 and 105 kg, were the recipients of the vitrified and nonvitrified embryos. The gilts were given a single intramuscular (i.m.) injection of 1000 IU eCG (ASKA Pharmaceutical) to induce estrus. Ovulation was induced by an i.m. injection of 1500 IU hCG (Kyoritsu Seiyaku Corporation, Tokyo, Japan), which was given 66 h after the injection of eCG. Blastocysts developed from the vitrified and nonvitrified embryos were surgically transferred into the oviducts of recipients approximately 147 h after hCG injection. Pregnant recipients were allowed to farrow naturally.

Identification of Transgenic Pigs

Genomic DNA was extracted from tail biopsies of newborn piglets using DNeasy Blood and Tissue Kit (QIAGEN, Inc., Hilden, Germany). For identification of transgenic pigs, DNA samples were analyzed using PCR with specific primers for amplifying *huKO* in the transgene. The primer sequences were as follows: 5′-TACTTCATGGACGGCAGCGTGAAC-3′ and 5′-AGGTGGTCTTGAACTGGCACTTGTG-3′. The cycle conditions were 95°C for 1 min, followed by 30 cycles of 95°C for 30 sec, 68°C for 20 sec, and 72°C for 30 sec.

Permeability Test of the Hollow Fiber

Parthenogenetic morulae were obtained by culturing electrically activated oocytes for 4 days and used to test the permeability properties of the hollow

fiber. Between 5 and 10 morulae were placed in a dish containing 4 ml of isotonic solution (WS, 283 mOsm) and aspirated into the HFV device. The hollow fiber portion was detached from the device and transferred to 4 ml of ES (3080 mOsm). After 7 min of equilibration, the hollow fibers were transferred to VS (6310 mOsm) for 1 min. Then, the hollow fiber was sequentially transferred to the solutions (4 ml) of the rewarming/rehydration process: RS (2110 mOsm) for 1 min, DS (897 mOsm) for 3 min, and WS (283 mOsm) for 5 min. Volume changes in the embryos in the hollow fibers during the sequential exposure to the solutions with different osmolarities were analyzed. Images of the embryos were captured using a DS-2Mv digital camera (Nikon Corporation) attached to a TE2000-U inverted microscope (Nikon Corporation) while they were in the isotonic solution (WS), at 1, 4, and 7 min in ES, at 1 min in VS, at 1 min in RS, at 1 and 3 min in DS, and at 1 and 5 min in WS. The major and minor axes of each morula were measured in the captured images, and the average length was used as an indicator of embryo size. Similarly, the diameters of embryos that were not held in hollow fibers were measured during sequential exposure to the same solutions.

Statistics

Statistical analyses were performed with SPSS 16.0 software (SPSS Inc., Chicago, IL). The differences in proportional data between the two groups were analyzed with the chi square-test. The differences in blastocyst cell number and embryo diameters between groups were analyzed with Student *t*-test. The level of significance was set at $P < 0.05$.

RESULTS

Survival of IVM/IVF-Derived Porcine Morulae after Vitrification by the HFV Method

A total of 484 IVM oocytes were subjected to IVF in three repeated experiments. In each experiment, 10–15 oocytes were randomly selected and used to estimate the rate of successful fertilization. This analysis indicated that 74.4% (32/43) of the oocytes were fertilized, of which 46.9% (15/32) were judged to be monospermic.

A total of 393 putative fertilized eggs derived from the IVM/IVF experiments were cultured further. Of these, 169 eggs (43.0%) developed to form morphologically normal morulae on Day 4, and eight (2.0%) developed into early blastocysts. Morulae with eight or more equally sized blastomeres were considered normal. Each morula was assigned to either the vitrification or nonvitrification treatment group, and the ability of the morulae in these groups to subsequently develop to the blastocyst stage during in vitro culture were compared (Table 1). This analysis showed that there was no significant difference between the two groups: 66/85 (77.6%) vitrified morulae developed into blastocysts compared to 67/84 (79.8%) nonvitrified morulae. Similarly, there were no significant differences between the two groups in the rate of embryos proceeding to the hatched blastocyst stage (9/85, 10.6% vs. 14/84, 16.7%) or the average number of cells in the blastocysts (82.2 vs. 84.2) (Table 1).

Pig Production Efficiency from IVM/IVF-Derived Morulae after Vitrification by the HFV Method

A total of 88 blastocysts (Day 5 or 6) derived from vitrified morulae (Fig. 2) were transferred into three recipients (25–32 blastocysts each); all three gilts became pregnant (Table 2). As a control, the same numbers of blastocysts derived from nonvitrified morulae (Fig. 2) was transferred into three recipients, all of which became pregnant. The efficiency of piglet production (piglets produced/embryos transplanted) ranged from 12.5% (4/32) to 25.8% (8/31) for vitrified embryos and 28.1% (9/32) to 36.0% (9/25) for nonvitrified embryos (Fig. 2). Overall, the efficiency of piglet production with vitrified embryos (17/88, 19.3%) was slightly lower, albeit nonsignificantly different, than with nonvitrified embryos (27/88, 30.7%) (Table 2).

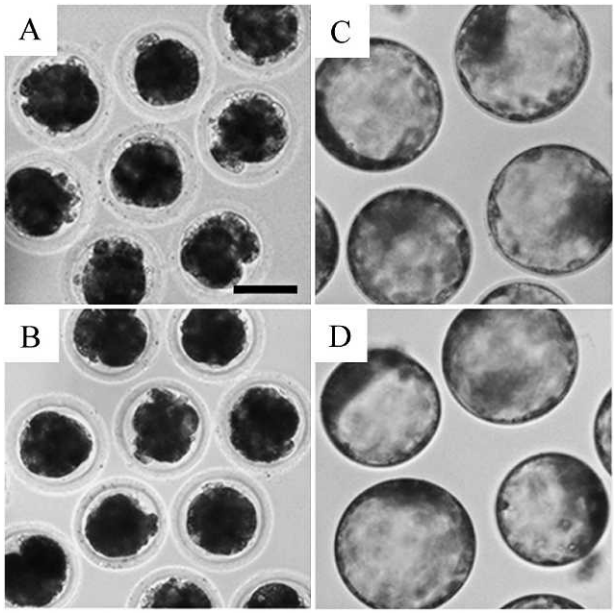

FIG. 2. Morphological appearance of the IVM/IVF-derived morulae after vitrification by the HFV method. IVM/IVF-derived morulae recovered after vitrification (**A**) and nonvitrified controls (**B**). Blastocysts developed from vitrified (**C**) and nonvitrified (**D**) morulae. Bar = 100 μm.

The proportion of normally fertilized embryos was 43.0% (40/93) in the three transfer experiments. From this observation, 38 of the 88 transferred embryos in both the vitrified and nonvitrified groups were estimated to be normal. Thus, the production efficiency of piglets in the two groups, with respect to the transfer of the putative normal blastocysts, was 44.7% (17/38) in the vitrification group and 71.1% (27/38) in the nonvitrification group (Table 2).

In the additional experiment in which 32 blastocysts were transferred to one recipient after a longer preservation period (43 days), seven (21.9%) fetuses, including three transgenics, were obtained.

Transgene Transmission and Growth of Piglets Obtained from Vitrified Embryos

The proportion of transgenic piglets was consistent with the expected Mendelian ratio in both the vitrification (10/17, 58.8%) and nonvitrification groups (15/27, 55.6%) (Table 2).

TABLE 1. In vitro development of porcine IVM/IVF-derived morulae after vitrification by the HFV method.

Embryos	No. of morulae cultured	Embryos developed to blastocysts (%)	Embryos developed to hatched blastocysts (%)	Cell number of blastocysts (mean ± SEM)
Vitrified	85	66 (77.6)*	9 (10.6)*	82.2 ± 5.5*
Nonvitrified	84	67 (79.8)*	14 (16.7)*	84.2 ± 6.0*

* No significant difference.

TABLE 2. Production efficiency of piglets from IVM/IVF-derived morulae vitrified by the HFV method.

Recipients	Embryos	No. of embryos transferred	Pregnancy (%)	No. of piglets (%)
1-V	Vitrified	32[a]	+	4[e] (12.5)
1-C	Nonvitrified	32[a]	+	9[e] (28.1)
2-V	Vitrified	31[b]	+	8 (25.8)
2-C	Nonvitrified	31[b]	+	9[e] (29.0)
3-V	Vitrified	25[c]	+	5 (20.0)
3-C	Nonvitrified	25[c]	+	9[e] (36.0)
Total	Vitrified	88[d]	3 (100)	17[f] (19.3)*
	Nonvitrified	88[d]	3 (100)	27[g] (30.7)*

[a-d] Estimated numbers of normally fertilized embryos were 14 (a), 13 (b), 11 (c), and 38 (d), respectively.
[e] Includes 1 stillborn piglet in each litter.
[f] Includes 10 (58.8%) transgenic piglets.
[g] Includes 15 (55.6%) transgenic piglets.
* No significant difference.

Fourteen piglets from the vitrification group and 22 piglets from the nonvitrification group were monitored until they reached 4 wk of age; there were no significant differences in growth between the two groups of piglets (Fig. 3).

Permeability Properties of the Hollow Fiber

Five hollow fibers, each holding 5–10 parthenogenetic morulae, were used to analyze cytoplasmic volume changes in ES, VS, RS, DS, and WS (Fig. 4). In each hollow fiber, the diameters of three embryos that could be viewed simultaneously in the viewfinder of the digital camera were measured. The average diameter of the parthenogenetic morulae in isotonic solution in the hollow fibers was 105.2 ± 0.9 μm (n = 15). The embryos shrank immediately after transfer of the hollow fibers to ES, and their average diameter was 87.5 ± 1.0 μm 1 min after transfer. The diameters gradually increased with time in ES: 94.2 ± 0.8 μm at 4 min and 97.3 ± 0.9 μm at 7 min. After transfer of the hollow fibers to VS, the average diameter was significantly reduced to 83.5 ± 1.3 μm. When the hollow fiber was moved from VS to RS, the size of the embryos slightly increased (95.5 ± 1.0 μm), and then they gradually shrank again in DS to a diameter of 86.1 ± 1.1 μm at 3 min. The embryos inside the hollow fiber recovered their original sizes (104.5 ± 1.0 μm) during 5 min in WS. We found that the shrinkage and recovery patterns of embryos not held in hollow fibers were not significantly different from embryos in the hollow fibers (Fig. 4). The average diameter of 15 morulae was measured in five repeat experiments with the following results: isotonic solution, 106.9 ± 1.1 μm; 1 min in ES, 89.0 ± 1.0 μm; 4 min in ES, 95.2 ± 1.3 μm; 7 min in ES, 99.5 ± 0.9 μm; 1 min in VS, 86.2 ± 1.5 μm; 1min in RS, 95.2 ± 1.3 μm; 3 min in DS, 87.3 ± 1.2 μm; and 5 min in WS, 103.4 ± 1.7 μm.

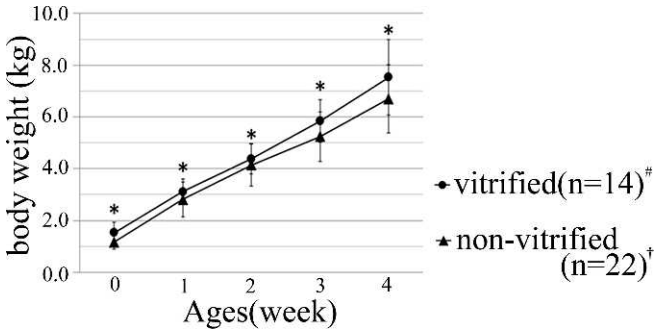

FIG. 3. Growth of piglets developed from vitrified and nonvitrified embryos. *No significant difference. #Piglets that developed from vitrified embryos included nine females and five males, of which five females and two males were transgenic. †Piglets that developed from nonvitrified embryos included 10 females and 12 males, of which four females and eight males were transgenic.

DISCUSSION

In this study, we demonstrated that a new vitrification method using flexible cellulose triacetate hollow fibers (pore size, 7.5 nm; membrane thickness, 15 μm) to house embryos is effective for the cryopreservation of porcine IVM/IVF-derived morulae. The HFV method is a simple yet reliable method in which embryos are aspirated into a piece of hollow fiber (approx. 20 mm long). The fiber plus embryos can then be handled as a single unit, enabling the vitrification of a large number of embryos simultaneously. Most of the established methods for cryopreserving embryos involve handling individual embryos using a glass capillary. This requirement is avoided in the HFV method because the fiber plus embryos can be handled using dissecting forceps throughout the processes of equilibration, vitrification, rewarming, dilution, and washing.

The transparent cellulose triacetate membrane chosen for use as the hollow fiber allows for the observation of the embryos housed inside using a microscope. We exploited this property in the experiment to examine changes in the cytoplasmic volume of embryos housed in the hollow fiber following transfer from an isotonic solution to the hypertonic ES. The embryos could clearly be seen to shrink in an initial reaction to the increase in osmolarity of the solution outside the hollow fiber before they regained volume during equilibration (7 min). The fact that these reactions were similar in embryos in a culture dish and in the hollow fiber indicates that the hollow fiber membrane allows the smooth passage of water and CPA molecules. In the present study, embryos were aspirated from ES into the hollow fibers and were subsequently equilibrated and vitrified. We placed embryos in ES prior to loading into hollow fibers to simplify the experimental procedure. In a preliminary experiment, we confirmed that the postvitrification viability was similar when the embryos were aspirated from the isotonic solution into the hollow fibers and then equilibrated in ES. This preliminary experiment also confirmed the permeability of the hollow fiber to CPAs.

When a hollow fiber with ES inside was placed in VS, the volume of solution inside the fiber was reduced to approximately one-half within 1 min. We interpret this phenomenon to indicate that the difference in osmolarity inside and outside the fiber induced loss of water from ES. The concentrations of CPAs, such as DMSO and EG, may also have equilibrated rapidly with the external VS by passage through the permeable hollow fiber membrane; however, sucrose would only have

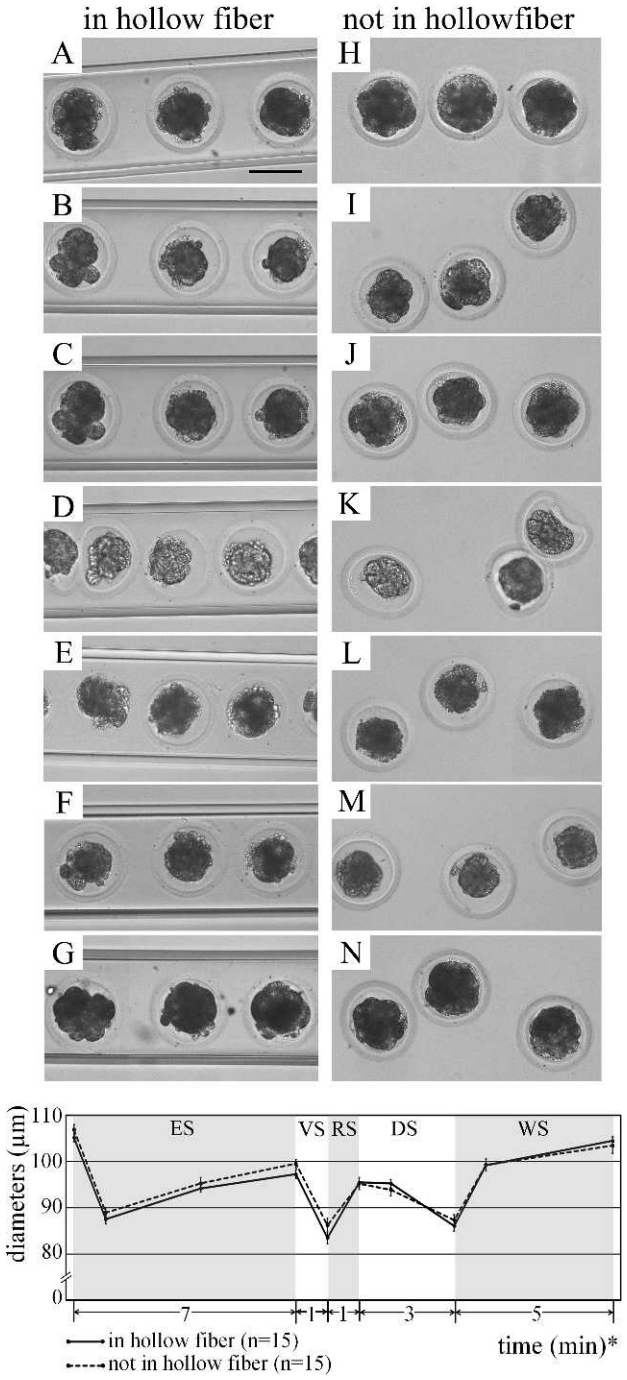

FIG. 4. Cytoplasmic volume changes of porcine morulae, indicating the permeability property of the hollow fiber. The volume changes of the morulae housed in a hollow fiber during sequential exposure to isotonic solution (**A**), hypertonic ES (**B** and **C**), and hypertonic VS (**D**) were similar to embryos not housed in a hollow fiber (**H**–**K**). The behavior of embryos during the recovery processes from VS was also similar in the both groups (**E**–**G** and **L**–**N**). **A** and **H**) Original cytoplasmic sizes of the parthenogenetic morulae in isotonic solution (WS; 283 mOsmol): 105.2 ± 0.9 μm versus 106.9 ± 1.1 μm. **B** and **I**) At 1 min in hypertonic ES (3080 mOsmol): 87.5 ± 1.0 μm versus 89.0 ± 1.0 μm. **C** and **J**) At 7 min in ES: 97.3 ± 0.9 μm versus 99.5 ± 0.9 μm. **D** and **K**) At 1 min in hypertonic VS (6310 mOsmol): 83.5 ± 1.3 μm versus 86.2 ± 1.5 μm. **E** and **L**) At 1 min in RS (2110 mOsmol): 95.5 ± 1.0 μm versus 95.2 ± 1.3 μm. **F** and **M**) At 3 min in DS (897 mOsmol): 86.1 ± 1.1 μm versus 87.3 ± 1.2 μm. **G** and **N**) At 5 min in WS (283 mOsmol): 104.5 ± 1.0 μm versus 103.4 ± 1.7 μm. The average cytoplasmic diameters of the embryos in both groups were not different significantly in each step. The sequential volume changes of the embryos shown in the graph indicate that the shrinkage and recovery patterns of the embryos during the equilibration (**A**–**C**, **H**–**J**), vitrification (**D** and **K**), rewarming (**E** and **L**), dilution (**F** and **M**), and washing (**G** and **N**) processes were similar between the HFV and conventional methods. Each plot from the left side of the graph represents the mean ± SEM size of the embryo at 0 1, 4, and 7 min in ES, at 1 min in VS, at 1 min in RS, at 1 and 3 min in DS, and at 1 and 5 min in WS, respectively. *Numbers on the x axis represent duration in each solution. Bar = 100 μm.

entered the hollow fiber membrane very slowly. The volume of solution within the hollow fiber was reduced for at least 10 min. The hollow fiber has a limit on the mass of permeating molecules of 50 000 g/mol, which is much larger than the molecular weight of sucrose. However, under nonpressurized conditions, sucrose molecules did not seem to enter the hollow fiber membrane rapidly. Therefore, we conclude that with the HFV method, embryos were vitrified inside the hollow fiber in a solution containing the concentration of permeable CPAs necessary for successful vitrification, whereas the concentration of sucrose was very low or not present. Further analyses will be needed to determine whether loading embryos into a hollow fiber containing nonpermeable CPAs, such as sucrose, would improve the viability of the vitrified embryos.

The permeability property of the hollow fiber is interpreted to be beneficial not only for the equilibration and vitrification steps but also for the processes of rewarming, rehydrating, and diluting the CPAs. Embryo behavior in the hollow fiber during each step of RS, DS, and WS indicates that the hollow fiber membrane does not impede the rehydration and CPA removal from the embryos. In fact, the stepwise recovery of the embryo volume in RS, DS, and WS was accompanied by a stepwise increase in the solution column length in the hollow fibers, indicating a smooth transfer of the solution and CPA molecules through the hollow fiber membrane.

In this study, we demonstrate for the first time that superior embryonic viability can be attained from porcine IVM/IVF-derived morulae that have been vitrified without delipation [33]. Delipation of embryos [24] is an effective approach for species with cryosensitive embryos, such as pigs [20, 24, 34]. In fact, the successful cryopreservation of in vitro produced porcine embryos reported so far involved delipation procedures [19–23, 34]. Delipation is believed to be effective in minimizing cryoinjuries that are ascribed to the high cytoplasmic lipid content. It has been reported that porcine embryos exposed to low temperatures show the coalescence of intracellular lipid droplets, and this effect is associated with irreversible changes to the intracellular structure [25]. The removal of cytoplasmic lipid droplets may eliminate potential cytoplasmic elements that cause cellular damage. Porcine embryo cryosensitivity is also influenced by other factors, such as the lipid composition of the cell membrane. It has been suggested that delipation might result in changes to the lipid composition of the cytoplasmic and/or intracellular membranes, which may indirectly improve the cryotolerance of embryos.

Although considered essential for cryopreserving porcine IVM/IVF-derived embryos [20, 34], delipation increases the complexity of the cryopreservation protocol. Thus, we developed a method that could provide efficient vitrification of IVM/IVF-derived embryos without the need for special pretreatment to improve the practicality of embryo cryopreservation technologies in pigs.

Nakano et al. [19] previously reported that IVM oocyte-derived SCNT embryos at the morula stage could be vitrified after performing delipation in a noninvasive manner. They reported that the efficiency of producing piglets from the vitrified cloned embryos was equivalent to that attained with

nonvitrified SCNT embryos. The HFV method is anticipated to eventually permit the cryopreservation of SCNT embryos without delipation.

Embryo vitrification methods [35, 36] have improved significantly in recent years. Highly effective approaches, such as the open pulled straw method [37], the Cryotop method [38], the cryoloop vitrification [39], the solid-surface vitrification [40], and microdroplet method [41], have been developed around the minimum volume cooling (MVC) concept [36, 42]. These methods allow ultrarapid temperature drops during vitrification and ultrarapid temperature increases during rewarming by minimizing the volume of the vitrification solution, which prevents the formation of ice crystals that can critically injure embryos during cryopreservation [43, 44]. However, cryopreservation of cryosensitive IVM/IVF-derived porcine morulae has been unattainable by conventional vitrification methods, including those with the MVC concept. In a preliminary experiment, we compared the HFV method and the Cryotop method in vitrifying IVM/IVF-derived porcine morulae. Because the Cryotop method is suitable for vitrifying a small number of ova, we conducted the comparison using three embryos per device; HFV gave improved results (data not shown).

The critical difference between the HFV and conventional methods cannot be explained by a single factor. The complex factors described below are thought to be involved in the effectiveness of the HFV method. The HFV method may cause less physical stress on embryos than conventional methods when vitrifying embryos in a very small amount of solution. Using a vitrification method developed for handling small numbers of embryos, such as the Cryotop method, to vitrify many embryos requires crowding the embryos into a very small volume of VS, which inevitably places physical stress on the embryos, that is, the embryos are somewhat squashed in the microdrop of the VS. Any increased stress on the embryos is likely to result in reduced viability. The HFV method is less likely to create a situation in which detrimental stress is imposed on the embryos. When the hollow fiber that contains the embryos is transferred from the ES to the VS, the volume of the solution in the fiber is reduced by half due to the higher osmolarity of the surrounding VS. For example, to vitrify 20 porcine embryos in a single device, the volume of vitrification solution in the hollow fiber is estimated to be approximately 0.07 μl, which is smaller than the threshold volume of 0.1 μl below which the effects of the MVC concept become obvious [32]. However, the embryos inside the hollow fiber appeared to be less squashed than those handled in a hemispherical microdrop of VS in the Cryotop method. The hemispheric microdrop may impose significant surface tension on the embryos when a large number of embryos are crowded into a single microdrop. Conversely, with the HFV method, embryos are held in a solution column, which has only a limited surface area within a hollow fiber. Less surface tension from the VS may be imposed directly onto the embryos with the HFV method as long as no more than 20 porcine embryos are held in the approximately 2 mm-long VS column (0.07 μl). Thus, our HFV method is unique because, although it follows the MVC concept, it allows the vitrification of a large number of embryos in a single device.

The HFV method may also avoid overexposing embryos to toxic CPAs. Unlike conventional methods, in which individual embryos are manipulated using a glass capillary, the HFV method handles the embryos indirectly by using a hollow fiber. Capillary-mediated manipulation of multiple embryos in highly viscous VS is technically difficult. Handling embryos in VS for an extended period of time could overexpose the embryos to high concentrations of CPA, resulting in cytotoxicity. By contrast, the HFV method allows the embryos in the hollow fiber to be rapidly moved from one solution to another, reducing the risk of exposure to CPA toxicity, which may be one of the reasons why embryos cryopreserved with the HFV method consistently exhibit high rates of viability.

There were no significant differences in the in vitro development of vitrified and nonvitrified embryos in this study. Conversely, an apparently lower rate of piglet production was obtained using vitrified embryos, although this difference was not statistically significant compared to nonvitrified embryos. However, the viability of vitrified embryos could be further improved. The cryotolerance of porcine embryos is higher at the blastocyst stage than at earlier cleavage stages [45, 46]. The morula stage embryos were vitrified in this study to demonstrate the effectiveness of the HFV method using embryos that were at a known cryosensitive stage [16, 46, 47]. The vitrification of blastocysts should also be studied to maximize the viability of embryos after cryopreservation.

In this study, IVM/IVF-derived morulae obtained from the same batch of oocytes in each experiment were equally assigned to the vitrification and nonvitrification groups to enable a strict comparison between the two groups. Therefore, the in vitro and in vivo survival of the vitrified embryos had to be assessed after a brief preservation in LN. In separate experiments, however, we confirmed that the vitrified hollow fiber could be stored in a cryotube, and long-term preservation in an LN tank was also performed without a decrease in embryo survival [26]. Therefore, the HFV method is a practical embryo cryopreservation method.

The use of animal models, such as genetically modified pigs, will undoubtedly advance our understanding of intractable human diseases, such as diabetes and cystic fibrosis, and will also be vital to preclinical research on xenotransplantation. The principal problem in using a large model animal, such as the pig, is that rearing and breeding genetically modified animals requires substantial effort and cost. For this reason, we have frozen the sperm of genetically modified pigs from several lines, including gene knockout pigs, and have used the sperm to produce pigs for research by IVF (data not shown). Additionally, the ability to cryopreserve in vitro fertilized embryos created with the sperm of genetically modified pigs will not only improve the efficiency of production of genetically modified pigs but will also bring major changes to the preservation and distribution (transport) of genetic resources. In this study, no differences in growth were found between piglets derived from vitrified embryos and those from nonvitrified embryos, indicating that the piglets produced after vitrification were normal.

In conclusion, we show that the HFV method is an extremely effective method for cryopreserving cryosensitive embryos, such as porcine IVM/IVF-derived morulae. IVM/IVF using frozen sperm and embryo cryopreservation by the HFV method will expand the range of reliable technologies for producing genetically modified pigs, which are expected to play an important role in future biomedical research and in preserving precious genetic resources.

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