

## Hollow Fiber Vitrification: A Novel Method for Vitrifying Multiple Embryos in a Single Device

Hitomi MATSUNARI<sup>1,2,3</sup>#, Miki MAEHARA<sup>1</sup>, Kazuaki NAKANO<sup>1</sup>, Yuka IKEZAWA<sup>1</sup>, Yui HAGIWARA<sup>4</sup>, Norihisa SASAYAMA<sup>4</sup>, Akio SHIRASU<sup>4</sup>, Hisayoshi OHTA<sup>5</sup>, Masashi TAKAHASHI<sup>6</sup> and Hiroshi NAGASHIMA<sup>1,3</sup>

<sup>1</sup>Laboratory of Developmental Engineering, Department of Life Sciences, School of Agriculture, Meiji University, Kawasaki 214-8571, Japan

<sup>2</sup>Research Fellow of the Japan Society for the Promotion of Science, Japan

<sup>3</sup>Meiji University International Institute for Bio-Resource Research, Kawasaki 214-8571, Japan

<sup>4</sup>Research & Development Institute for Artificial Organs, Research & Development Laboratory, Nipro Corporation, Shiga 525-0055, Japan

<sup>5</sup>Tokyo Development Foundation for Agriculture, Forestry and Fisheries, Tokyo 198-0024, Japan

<sup>6</sup>Department of Animal and Grassland Research, National Agricultural Research Center for Kyushu Okinawa Region (KONARC), Kumamoto 861-1192, Japan

#Present: Japan Science and Technology Agency, ERATO, Nakauchi Stem Cell and Organ Regeneration Project, Tokyo 102-0075, Japan

**Abstract.** Current embryo vitrification methods with proven efficacy are based on the minimum volume cooling (MVC) concept by which embryos are vitrified and rewarmed ultrarapidly in a very small amount of cryopreserving solution to ensure the high viability of the embryos. However, these methods are not suitable for simultaneously vitrifying a large number of embryos. Here, we describe a novel vitrification method based on use of a hollow fiber device, which can easily hold as many as 40 mouse or 20 porcine embryos in less than 0.1  $\mu$ l of solution. Survival rates of up to 100% were obtained for mouse embryos vitrified in the presence of 15% DMSO, 15% ethylene glycol and 0.5 M sucrose using the hollow fiber vitrification (HFV) method, regardless of the developmental stage of the embryos (1-cell, 2-cell, morula or blastocyst; n = 50/group). The HFV method was also proven to be effective for vitrifying porcine *in vitro*- and *in vivo*-derived embryos that are known to be highly cryosensitive. For porcine embryos, the blastocyst formation rate of *in vitro* maturation (IVM)-derived parthenogenetic morulae after vitrification (48/65, 73.8%) did not decrease significantly compared with non-vitrified embryos (59/65, 90.8%). Transfer of 72 *in vivo*-derived embryos vitrified at the morula/early blastocyst stages to 3 recipients gave rise to 29 (40.3%) piglets. These data demonstrate that the HFV method enables simultaneous vitrification of multiple embryos while still adhering to the MVC concept, and this new method is very effective for cryopreserving embryos of mice and pigs.

**Key words:** Cryopreservation, Hollow fiber, Mouse embryos, Pig embryos, Vitrification

(J. Reprod. Dev. 58: 599–608, 2012)

Embryo cryopreservation is widely and routinely used both for research in reproductive biology and for the development of practical applications in animal industries and human reproductive medicine. The cryopreservation of oocytes and embryos is essential for the long-term preservation of valuable genetic resources in experimental and livestock animals [1–5], and the cryopreservation of embryos improves pregnancy rates in assisted reproductive technology applications [6–8]. Various protocols have been developed for the cryopreservation of animal embryos, and many are used in research and clinical applications [1, 5, 9].

In recent years, embryo vitrification has been applied to an increasing number of animal species, and high post-cryopreservation

embryo viability has been achieved [10, 11]. The basic concept of vitrification is as follows: a solution containing a high concentration of a cryoprotective agent (CPA) is rapidly cooled, causing it to transform from the liquid phase to the solid phase without forming ice crystals. As a result, cells suspended in the solution are preserved in an ultra-low temperature glassy (amorphous) material [10, 12]. To create this glassy state, a solution containing a high concentration of CPA (normally 4–6 M) needs to be cooled ultrarapidly. However, use of a CPA at high concentrations has the drawback of increasing the cytotoxicity of the solution.

It has been reported that the concentration of the CPA necessary for creating the glassy state can be reduced by ultrarapid cooling of very small quantities of a cell suspension [11, 13]. Vitrification in the presence of a relatively low concentration of CPA is suitable for the cryopreservation of oocytes and early stage embryos, which are sensitive to the cytotoxicity of CPAs. Methods that involve a reduction in the volume of vitrification solution are termed “minimum volume cooling (MVC) methods” [14]. Various devices and strategies have

Received: December 31, 2011

Accepted: May 29, 2012

Published online in J-STAGE: July 4, 2012

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Correspondence: H Nagashima (e-mail: hnagas@isc.meiji.ac.jp)

been developed to put MVC protocols into practical use such as open pulled straw (OPS) [15], Cryotop [13], nylon loop [16], solid surface [17] and microdroplet vitrification [18]. MVC-based vitrification is the current state-of-the-art approach for the cryopreservation of mammalian oocytes and embryos, including those of humans [13, 19]. However, to ensure high rates of viability, the MVC concept assumes the cryopreservation of either one or a very small number of oocytes/embryos. This limitation is a drawback for the cryopreservation of oocytes and embryos of polytocous animals. For example, cryopreservation of the embryos of mice, rats or pigs requires a method that allows for the simultaneous preservation of several dozen embryos (i.e., the number necessary for embryo transfer) as a single unit. However, to vitrify this number of oocytes or embryos in one unit, the volume of the vitrification solution would have to be increased, making it difficult to adhere to the MVC concept. The present study was initiated to develop a vitrification protocol that allows for easy, simultaneous cryopreservation of large numbers of oocytes or embryos from polytocous animals such as mice and pigs while adhering to the MVC concept.

Recently, Takahashi *et al.* [20] reported that high viability was attained after the vitrification of bovine embryos in a hollow fiber. We applied this method to mouse embryos and achieved very high viability after cryopreservation. We also found that the hollow fiber vitrification (HFV) method was effective in preserving the early embryos of pigs, which are known to be highly cryosensitive [21–23]. Based on these successful applications of the HVF technique, we provide here a detailed description of the hollow fiber MVC method.

## Materials and Methods

### Animal care

All of the animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC-11-008). The mice were housed under controlled conditions ( $24 \pm 2$  C, humidity 70%, 12 h light/12 h darkness photoperiod) with food (CE-2, CLEA Japan, Tokyo, Japan) and water available *ad libitum*.

### Chemicals

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

### Collection of mouse embryos

B6D2F1 (BDF1) female mice (7–10 weeks old; CLEA Japan) were induced to superovulate by intraperitoneal (i.p.) injection of 7.5 IU of equine chorionic gonadotropin (eCG, ASKA Pharmaceutical, Tokyo, Japan) and 8.25 IU of human chorionic gonadotropin (hCG, Kyoritsu Seiyaku, Tokyo, Japan) given 44–46 h apart. Females were paired with males of the same strain immediately after the injection of hCG and checked for mating the next morning; the day of the discovery of a vaginal plug was designated as day 1.

The collection of embryos at the pronuclear (1-cell), 2-cell, morula and blastocyst stages was carried out at 20, 44, 72 and 87 h, respectively, after the hCG injection. The pronuclear stage embryos were collected from the ampulla of the oviducts in a drop of embryo culture medium (CZB medium) [24] and treated with hyaluronidase

(0.4 mg/ml, H-4272) to remove the surrounding cumulus cells. Two-cell stage embryos were recovered by oviductal flushing. Morulae and blastocysts were collected from the uterine horns. Embryo culture medium was used for both oviductal and uterine flushing.

### Collection of pig embryos

Pregnant gilts (Tokyo X breed, 11 months old) were aborted by an intramuscular (i.m.) injection of 1 mg of the prostaglandin F $_{2\alpha}$  analog cloprostenol (Estrumate, Intervet, Osaka, Japan) between 31 and 47 days after mating, followed by a second injection of 0.5 mg cloprostenol 24 h later. At the same time as the second cloprostenol injection, 1000–1500 IU eCG (ASKA Pharmaceutical) was administered by an i.m. injection. Ovulation was induced by an i.m. injection of 750–1500 IU hCG (Kawasaki Pharmaceutical, Kanagawa, Japan) 66 h after the eCG. Embryo donors were mated with a fertile boar of the same breed on the next day and the day after. The embryo donors were sacrificed approximately 120 h after hCG, and embryos were collected by downward flushing of the oviducts and the upper half of the uterine horns using Dulbecco's phosphate buffered saline (DPBS, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 1% fetal bovine serum (FBS). Embryos at the 4–8-cell stage to the morula stage were selected and used in the experiments described below.

### Preparation of pig parthenogenetic embryos

Pig ovaries were collected at a local abattoir and transported to the laboratory in DPBS containing 75  $\mu$ g/ml potassium penicillin G, 50  $\mu$ g/ml streptomycin sulfate, 2.5  $\mu$ g/ml amphotericin B and 0.1% (w/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes (COCs) were collected by aspiration from ovarian antral follicles that had a diameter of 3.0–6.0 mm. COCs with at least three layers of compacted cumulus cells were selected and cultured in NCSU23 medium [25] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor, 10% (v/v) porcine follicular fluid, 75  $\mu$ g/ml potassium penicillin G, 50  $\mu$ g/ml streptomycin sulfate, 10 IU/ml eCG (ASKA Pharmaceutical) and 10 IU/ml hCG (ASKA Pharmaceutical). The COCs were cultured for 22 h with eCG and hCG in a humidified atmosphere of 5% CO $_2$  and 95% air at 38.5 C. The COCs were then cultured for 22 h without eCG and hCG in an atmosphere of 5% CO $_2$ , 5% O $_2$  and 90% N $_2$  at the same temperature [26]. *In vitro* matured oocytes with expanded cumulus cells were treated with 1 mg/ml hyaluronidase dissolved in Tyrode's lactose medium containing 10 mM HEPES and 0.3% (w/v) polyvinylpyrrolidone (Hepes-TL-PVP) and were separated from the cumulus cells by gentle pipetting. Oocytes with an evenly granulated ooplasm and an extruded first polar body were selected for subsequent experiments.

The oocytes were washed twice in an activation solution composed of 280 mM mannitol (Nacalai Tesque, Kyoto, Japan), 0.05 mM CaCl $_2$ , 0.1 mM MgSO $_4$  and 0.01% (w/v) PVA. They were then aligned between two wire electrodes (1.0 mm apart) in a drop of the activation solution on a fusion chamber slide (CUY500G1, Nepa Gene, Chiba, Japan). A single direct current pulse of 150 V/mm was applied for 100  $\mu$ sec using an electrical pulsing machine (LF201; Nepa Gene). Activated oocytes were treated with 5  $\mu$ g/ml cytochalasin B for 3 h to suppress extrusion of the second polar body. Parthenogenetic morulae obtained on day 4 were used for the vitrification experiments.

### Embryo culture

Mouse embryos were cultured at 37.5 C in a humidified atmosphere of 5% CO<sub>2</sub> in air in 20 µl drops of CZB medium supplemented with 5 mg/ml BSA, 0.146 mg/ml glutamine and 0.029 mg/ml Na pyruvate under mineral oil (light white, MP Biomedicals, Illkirch, France) in a plastic 35 mm dish (Iwaki 1000-035, Asahi Techno Glass, Tokyo, Japan).

*In vitro* culture of the porcine activated embryos and *in vivo*-derived embryos was performed in 20 µl droplets of porcine zygote medium-5 (PZM-5, Research Institute for the Functional Peptides, Yamagata, Japan) under paraffin oil (32033-00, Kanto Chemical, Tokyo, Japan) in a plastic 35 mm dish maintained in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> at 38.5 C. Embryos were cultured beyond the morula stage by adding 10% (v/v) FBS to the medium. The resulting blastocysts were mounted onto glass slides with DPBS containing 20% ethylene glycol (EG) and 5 mg/ml Hoechst 33342 and examined under a fluorescence microscope (TE 2000, Nikon, Tokyo, Japan) to count cell numbers.

### Procedure for the HFV method

The vitrification solution described by Kuwayama *et al.* [19] was used in the present study to cryopreserve mouse and porcine embryos. A solution of 20 mM Hepes buffered Tissue Culture Medium 199 (Nissui Pharmaceutical) supplemented with 20% calf serum (12133C, SAFC Biosciences, Kansas City, KS, USA) was used as the basal solution. Dimethyl sulfoxide (DMSO), EG and sucrose were used as CPAs. The equilibration solution contained 7.5% DMSO and 7.5% EG and the vitrification solution contained 15% DMSO, 15% EG and 0.5 M sucrose. The rewarming solution contained 1 M sucrose, and the dilution solution contained 0.5 M sucrose. The basal solution was used as the washing solution.

The cryopreservation of embryos using the HFV method was performed as reported by Takahashi *et al.* [20] and Nakano *et al.* [27] with modifications (Fig. 1). An HFV device was constructed using a hollow fiber (Fig. 1-A-a; inner diameter 185 µm, outer diameter 215 µm, FB-150FH, Nipro, Osaka, Japan) cut into approximately 25 mm lengths. Each piece of fiber was connected to a hypodermic needle with a square end (Fig. 1-A-b; outer diameter 0.15 mm, inner diameter 0.1 mm, length 5 mm, Medical Planning, Miyagi, Japan). The HFV device (Fig. 1-A-c) was attached to a 1 ml syringe connected to an aspiration tube (Fig. 1-B). Embryos were first placed in the equilibration solution (4 ml; room temperature) in a 35 mm dish and were then aspirated into the HFV device (Fig. 1-C). As shown in Fig. 1-B, the column of solution containing the embryos was sandwiched by air bubbles in the hollow fiber.

The hollow fiber was detached from the hypodermic needle using dissecting forceps (Fig. 1-D, 1-E-1) and placed in the equilibration solution for varying times depending on the source of the embryos: mouse embryos, 5–7 min; porcine embryos, 5–6 min. Equilibration was considered complete when embryos had shrunk and then regained their volumes in the equilibration solution. Following equilibration, the hollow fiber was transferred to the vitrification solution (4 ml) using dissecting forceps (Fig. 1-E-2). For mouse embryos, the vitrification solution was cooled in an ice bath. For porcine embryos, room temperature vitrification solution was used. The hollow fiber was gently moved in the dish to ensure the dehydration of embryos

inside the hollow fiber (Fig. 1-E-2). After 1–2 min for mouse embryos or 1 min for pig embryos, the hollow fiber was immersed into liquid nitrogen (LN) while being held by forceps to maintain its vertical orientation (Fig. 1-E-3).

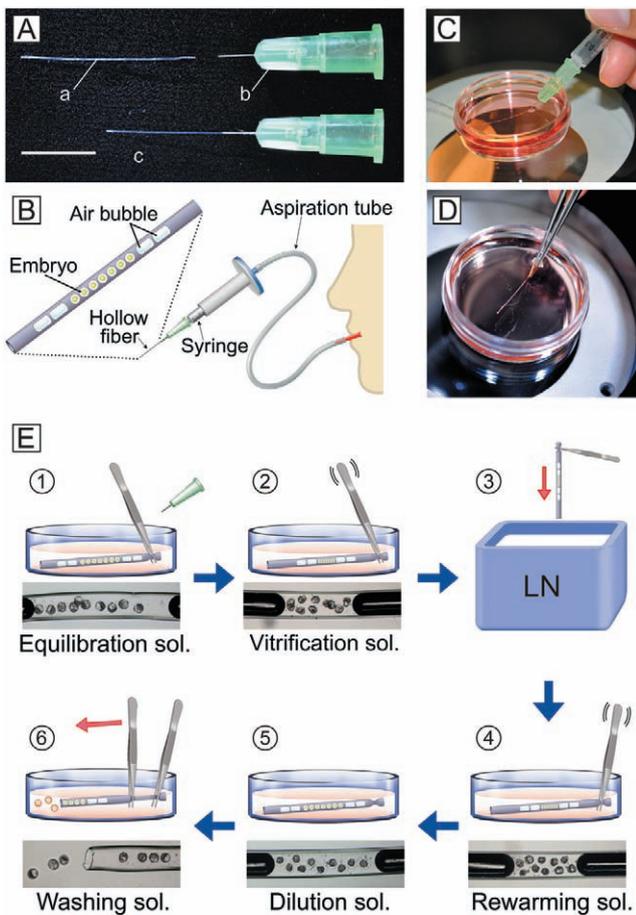
After being kept in LN for at least 1 min, the hollow fiber was moved from LN to the rewarming solution (4 ml; 37.5 C for mouse embryos and 38.5 C for porcine embryos) in a dish (Fig. 1-E-4). The hollow fiber was kept in the rewarming solution for 1 min, during which the fiber was gently moved (Fig. 1-E-4). Next, the hollow fiber was transferred using forceps to a dilution solution for 3 min (Fig. 1-E-5) and then to the washing solution (Fig. 1-E-6). In the washing solution, the embryos were expelled from the hollow fiber by gently squeezing the fiber from one end to the other with forceps as shown in Fig. 1-E-6. The recovered embryos were washed twice using a glass capillary pipette and then cultured.

A modified version of the HFV device (Fig. 2-A) was used in the experiments in which embryos were stored for a long period of time (Exp. 2 and 4). This modified device was constructed by connecting a 25 mm hollow fiber (Fig. 2-A-a) to the tip of an ethylene-tetrafluoroethylene peripheral cannula of an indwelling needle (Fig. 2-A-b; SR-OT1852C Surflo<sup>®</sup> I.V. catheter 18G×2, Terumo, Tokyo, Japan) using epoxy adhesive (Fig. 2-A-c; 16051, Konishi, Osaka, Japan). The device incorporates a polypropylene extendable protective sheath (Fig. 2-A-d; Telescopic Straw, Nippon Straw, Tokyo, Japan), which serves to prevent damage to the hollow fiber (Fig. 2-A-e, f). A cryotube (Fig. 2-A-g; CryoTube<sup>®</sup> 337516, 4.5 ml, Thermo Fisher Scientific, MA, USA) was used to store the device in an LN tank for long-term preservation. The 1 ml syringe with aspiration tube from the original device was used to handle the embryos with the modified device (Fig. 2-B).

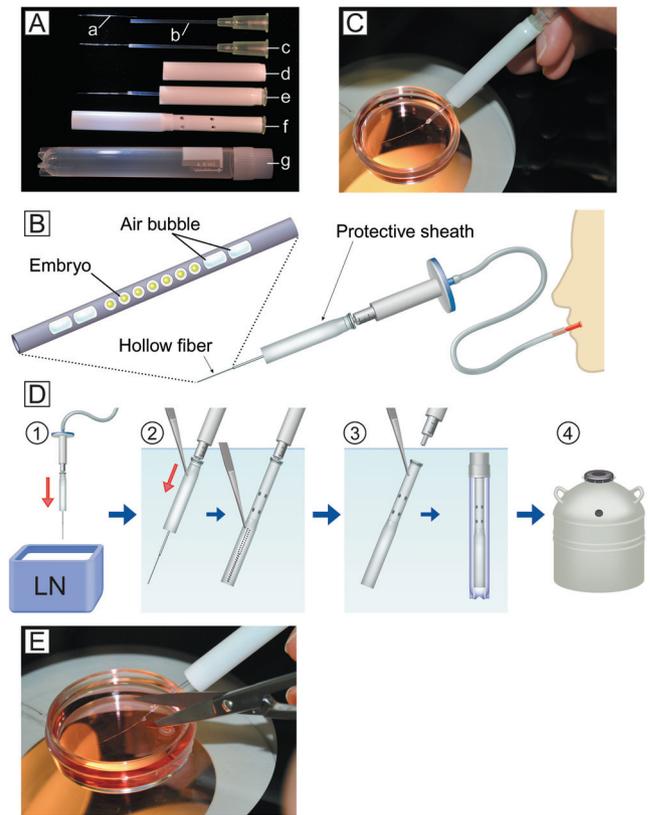
To vitrify embryos using the modified device, the embryos were first aspirated into the hollow fiber in equilibration solution while keeping the protective sheath at the upper position in the same manner as for the original device (Fig. 2-B, C). The hollow fiber portion of the device that held embryos was kept immersed in the equilibration solution during the equilibration period by holding the device by hand as shown in Fig. 2-C. Using the modified device, the hollow fiber portion was kept attached to the cannula that was connected to the syringe during the processes of equilibration and vitrification.

After the equilibration period, the device holding the embryos was moved to vitrification solution, and after a predetermined holding time, the device was immersed into LN by holding it vertically (Fig. 2-D-1). After immersing the device in LN, the protective sheath was extended by sliding the outer portion down using forceps (Fig. 2-D-2). The device was detached from the syringe and placed in a cryotube (Fig. 2-D-3) in LN and stored in an LN tank (Fig. 2-D-4).

For rewarming and recovery of the embryos, the device was removed from the cryotube using forceps and reattached to a 1 ml syringe; the protective sheath was then slid up to its original upper position. All of these processes were carried out in LN. The device was quickly moved from LN, and the hollow fiber portion was immersed in the rewarming solution. The hollow fiber was cut from the catheter using scissors while in the rewarming solution (Fig. 2-E) and kept there for 1 min. Then, the isolated hollow fiber was processed, using forceps, as described above for the original device to dilute the CPAs and recover the embryos.



**Fig. 1.** The HFV device and schematic representation of the embryo vitrification procedures using the HFV method. **A:** A 25-mm-long hollow fiber segment (a) is connected to a hypodermic needle (b) to produce an HFV device (c). Scale bar = 1 cm. **B:** Embryos are aspirated into the HFV device, which is attached to a 1 ml syringe and an aspiration tube. Embryos are loaded into the hollow fiber with a column of equilibration solution sandwiched between air bubbles. **C:** Aspiration of embryos into the HFV device from the equilibration dish. **D:** Handling of a hollow fiber containing embryos using dissecting forceps. **E:** (1) The hollow fiber containing the embryos is detached from the hypodermic needle and kept in the equilibration solution for a predetermined period: 5–7 min for mouse or 5–6 min for pig embryos (upper). Ten to 20 porcine or 10 to 40 mouse embryos are held in a 2–3-mm-long column of equilibration solution in the hollow fiber (lower). (2) After the equilibration period, the hollow fiber is transferred to the vitrification solution using forceps (upper). In the vitrification solution, the volume of solution inside the fiber noticeably reduces due to the hyperosmolarity of the vitrification solution (lower). (3) The hollow fiber is immersed into liquid nitrogen while being held by forceps to maintain its vertical orientation. (4) The hollow fiber is rewarmed by immersion in a pre-warmed 1 M sucrose solution and then transferred stepwise to a dilution solution containing 0.5 M sucrose (5) and a washing solution (6) using forceps. Note that the column of solution containing the embryos regains its volume as the hollow fiber is transferred from the rewarming solution to the dilution solution (4 and 5, lower) (6) In the washing solution, the embryos are recovered by gently squeezing the hollow fiber from one end to the other with forceps. Abbreviations: sol: solution, LN: liquid nitrogen.



**Fig. 2.** The modified HFV device for long-term storage and a schematic representation of embryo vitrification procedures. **A:** Assembling the modified HFV device. (a) Hollow fiber (approximately 25 mm long), (b) ethylene-tetrafluoroethylene peripheral cannula of indwelling needle, (c) hollow fiber and peripheral cannula connected using epoxy adhesive, (d) polypropylene extendable protective sheath, (e, f) assembled device with the extendable protective sheath at the upper (e) and extended position (f), (g) cryotube for long-term storage of the device. **B:** Embryos are aspirated into the HFV device with the protective sheath in the upper position as for the original device shown in Fig. 1. **C:** Handling of the modified HFV device for aspiration of embryos from the equilibration solution. **D:** (1) For vitrifying embryos, the device is immersed into LN with a vertical orientation. (2) In LN, the protective sheath is extended by sliding down the outer portion using forceps to prevent damage to the hollow fiber part. (3, 4) The device is detached from the syringe in LN, placed in a cryotube (3) and stored in an LN tank (4). **E:** In the rewarming solution, the hollow fiber is cut from the catheter using scissors. The processes for dilution of cryoprotectants and washing and recovery of embryos from the hollow fiber are carried out in the same manner as with the original device in Fig. 1.

### Embryo transfer

Vitrified and control non-vitrified embryos were transferred either at the 2-cell or blastocyst stage. ICR female mice (10–12 weeks old, CLEA Japan) were used as recipients after being mated with vasectomized males of the same strain. The day on which the presence of a vaginal plug was noted was considered to be day 1 of pseudopregnancy. Females with estrus induced by i.p. injections of 4 IU eCG and hCG and those with natural estrus were used for transfer of the 2-cell and blastocyst stage embryos, respectively.

Two-cell stage embryos were transferred to the oviducts of pseudopregnant recipients on day 1 under anesthesia using avertin. Ten embryos were transferred into each oviduct. On the other hand, 5 or 6 blastocysts were transferred into each uterine horn of the recipients on day 4. All females showing signs of pregnancy were sacrificed on day 18 of gestation, and the number of normal fetuses in each uterine horn was counted.

Crossbred (Large White/Landrace × Duroc) prepubertal gilts, weighing from 100 to 105 kg, were used as recipients of the vitrified porcine embryos. The gilts were administered a single i.m. injection of 1000 IU of eCG (ASKA Pharmaceutical) to induce estrus. Ovulation was induced by an i.m. injection of 1500 IU of hCG (Kyoritsu Seiyaku) given 66 h after the injection of eCG. Blastocysts developed from vitrified embryos were surgically transferred into the uterine horns of recipients 125–147 h after hCG injection. Pregnant recipients were allowed to farrow naturally.

### Statistics

Statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Differences in proportional data between the two groups were analyzed using the  $\chi^2$  test. Differences in blastocyst cell number between groups were analyzed using the Student's *t*-test. The level of significance was set at  $P < 0.05$ .

### Experimental design

Vitrification of mouse embryos at various developmental stages: One-cell, 2-cell, morula and blastocyst stage embryos were collected and randomly assigned to vitrification and non-vitrification groups. Embryos in the vitrification group were immediately vitrified, and those in the non-vitrification group were placed in culture. In the vitrification group, 10 embryos were placed into an HFV device and vitrified. The vitrified embryos were held in LN for approximately one minute and then rewarmed. Embryos that developed from their original embryonic stage in the post-rewarming culture were judged viable. For embryos that were at the morula stage or earlier, the rate of development to blastocysts was recorded. For blastocysts, the rate of development into expanded blastocysts was evaluated. In both the vitrified and non-vitrified embryo groups, day 5 blastocysts were stained with Hoechst 33258, and their cell numbers were counted.

For *in vivo* assessment of embryo viability, embryos vitrified at the 1-cell stage were cultured for 24 h and transferred to recipients at the 2-cell stage. Embryos vitrified at the 2-cell stage were also transferred to recipients after being developed to the blastocyst stage in culture for 40–45 h. Non-vitrified embryos at the same developmental stages were transferred as controls.

Long-term cryopreservation of mouse embryos by the HFV method: The modified HFV device with a protective sheath was used in this experiment (Fig. 2). Twenty to 40 embryos at the 2-cell stage were placed in the device and vitrified. The embryos were stored for 6 or 12 months to compare the effects of the storage period on embryo viability. The embryos were rewarmed and cultured for 72 h, and their rate of development into blastocysts (day 5) was recorded.

Vitrification of IVM-derived porcine parthenogenetic morulae using the HFV method: To determine whether the HFV method was effective for the cryopreservation of IVM-derived porcine parthenogenetic morulae, which are highly cryosensitive, embryos were randomly

assigned to either a vitrification or non-vitrification group. Ten to 20 parthenogenetic morulae (day 4) were vitrified in each HFV device. As a non-vitrification control group, parthenogenetic morulae were placed into culture. Developmental rates of the embryos to the blastocyst stage (day 7) and the number of cells in blastocysts were compared between the vitrified/rewarmed embryos and non-vitrified embryos (control).

Vitrification of *in vivo*-derived porcine embryos by the HFV method: The aims of this experiment were to assess the efficacy of the HFV method for vitrifying porcine *in vivo*-derived embryos and to determine whether production of live piglets from the vitrified embryos was possible.

Embryos at the 4–8-cell or morula stage were collected from the superovulated female pigs. The 4–8-cell stage embryos were developed by *in vitro* cultured for 16–30 h to the morula or early blastocyst stages; the embryos were then vitrified. The morula stage embryos were vitrified within 2 h after collection. Embryos were cryopreserved using the modified HFV device, and the vitrified embryos were stored in LN for 52 to 313 days until rewarming and transfer. After rewarming, the recovered embryos were cultured for 20–34 h, and those that developed to blastocysts were transferred. Twenty-one to 26 embryos were transferred to each recipient gilt. In some recipients, 10 blastocysts (day 5) developed from vitrified parthenogenetic morulae (see Exp. 3) were cotransferred with the aim of ensuring the maintenance of pregnancy. Recipient gilts were allowed to farrow naturally.

## Results

### *Survival of mouse embryos vitrified at various developmental stages*

One-cell, 2-cell, morula and blastocyst stage mouse embryos were cryopreserved using the HFV method, and their viabilities were evaluated according to their *in vitro* development (Table 1). All vitrified embryos at all the developmental stages tested (50 embryos examined in each of the four experimental groups) and all control non-vitrified embryos (43–55 embryos) were found to be viable. The proportions of vitrified embryos developing to the blastocyst stage were similar to those for non-vitrified embryos regardless of the embryonic stage at which vitrification was carried out. Blastocyst formation rates in vitrified embryos ranged from 46/50 (92.0%) to 50/50 (100.0%) compared with 51/55 (92.7%) to 50/50 (100.0%) for the non-vitrified embryos. The morphological characteristics of the developing embryos were similar in vitrified and non-vitrified embryos (Fig. 3). There was also no difference in the number of cells in the blastocysts obtained from the two groups (Table 1).

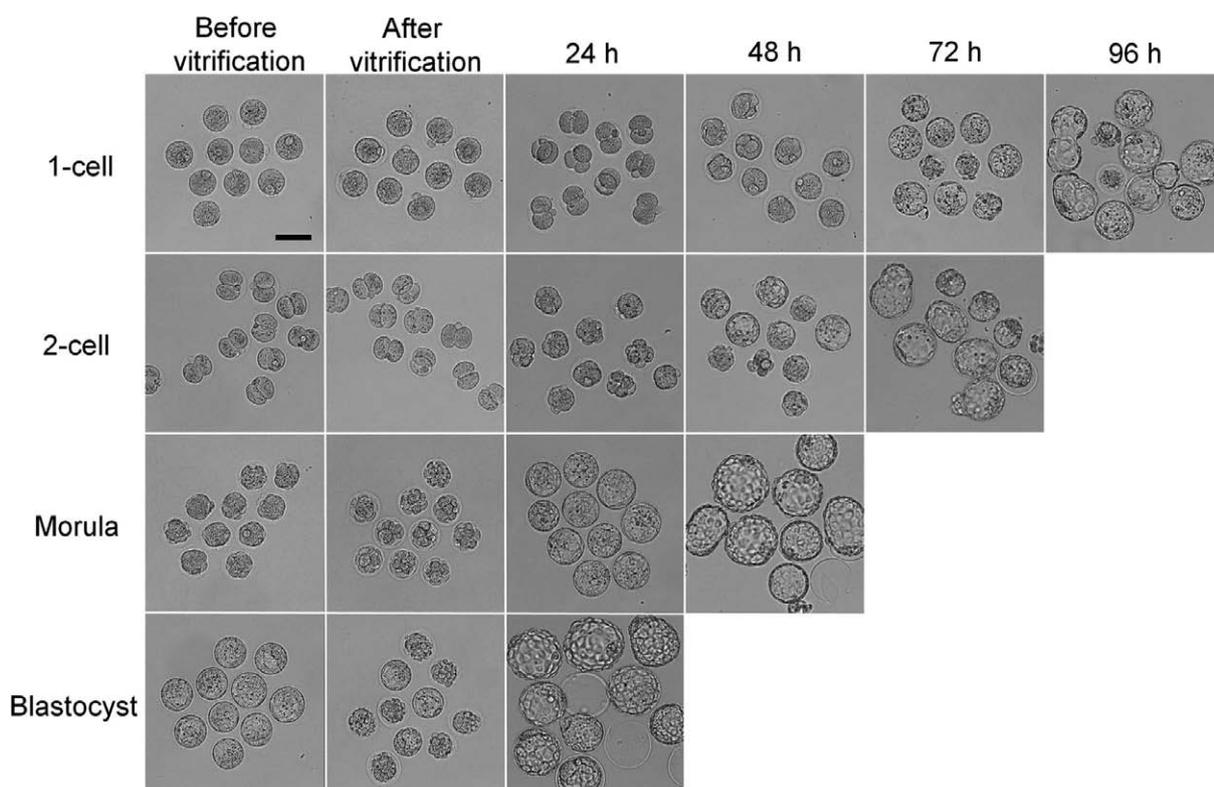
### *Development of the vitrified embryos after transfer*

Two hundred and fourteen embryos in 9 HFV devices were vitrified at the 1-cell stage. After rewarming and culture, 209 (97.7%) embryos developed to the 2-cell stage and were used for transfer into the oviducts of 8 estrus-synchronized recipient mice (20 embryos / recipient). Six (75.0%) of the recipients became pregnant, and a total of 40 fetuses were recovered by autopsy at 18 days after transfer. The efficiency of fetus production (number of fetuses / number of embryos transferred) of the six pregnant mice was 33.3% (40/120).

**Table 1.** Vitrification of mouse embryos at various developmental stages

Embryonic stage	Vitrification	No. of embryos:			Cell numbers in the blastocysts (mean $\pm$ SEM)
		Vitrified or cultured as control	Survived (%)	Developed to blastocysts on day 5 (%)	
1-cell	+	50	50 (100.0)	46 (92.0)	68.4 $\pm$ 2.9
	-	55	55 (100.0)	51 (92.7)	70.9 $\pm$ 2.3
2-cell	+	50	50 (100.0)	47 (94.0)	78.7 $\pm$ 2.1
	-	47	47 (100.0)	45 (95.7)	84.0 $\pm$ 2.8
Morula	+	50	50 (100.0)	50 (100.0)	98.1 $\pm$ 2.5
	-	43	43 (100.0)	43 (100.0)	90.0 $\pm$ 3.0
Blastocyst	+	50	50 (100.0)	50 (100.0)	100.7 $\pm$ 2.9
	-	50	50 (100.0)	50 (100.0)	115.6 $\pm$ 3.6

For each embryonic stage (5 replicates), vitrified and non-vitrified group values did not differ significantly.



**Fig. 3.** *In vitro* development of mouse embryos vitrified using the HFV method at various developmental stages. Mouse embryos at the 1-cell, 2-cell, morula and blastocyst stages were vitrified. After rewarming, the recovered embryos were cultured *in vitro* for up to 4 days to assess their survival. Scale bar = 100  $\mu$ m.

In the control group, in which non-vitrified embryos were transferred at the 2-cell stage, the pregnancy rate was 66.7% (6/9), and the fetus production efficiency was 29.2% (35/120).

A total of 32 blastocysts developed from vitrified 2-cell embryos were transferred to 3 recipients (10–12 embryos / recipient). All of the recipients became pregnant, and a total of 21 (65.6%) fetuses were recovered at autopsy. In the control group, 22 (64.7%) fetuses were obtained after transfer of 34 blastocysts in 3 recipients (all pregnant). Thus, in the two types of transfer experiments conducted, vitrified and non-vitrified embryos gave similar rates of fetus production.

#### *Survival of mouse embryos after long-term cryopreservation using the modified HFV device*

A total of 138 mouse embryos at the 2-cell stage were vitrified in 4 modified HFV devices and stored for 6 (n=2) or 12 (n=2) months in LN. The rates of development to the blastocyst stage for embryos stored for 6 and 12 months were 96.3% (78/81) and 94.7% (54/57), respectively.

**Table 2.** Vitrification of IVM-derived porcine parthenogenetic morulae using the hollow fiber vitrification method

Treatments	No. of embryos:			Cell numbers in the blastocysts (mean $\pm$ SEM)
	Vitrified or cultured as control	Developed to blastocysts (%)	Developed to hatched blastocysts (%)	
Vitrified*	65	48 (73.8) <sup>a</sup>	21 (32.3) <sup>a</sup>	86.8 $\pm$ 6.5 <sup>a</sup>
Non-vitrified*	65	59 (90.8) <sup>a</sup>	30 (46.2) <sup>a</sup>	77.0 $\pm$ 4.8 <sup>a</sup>

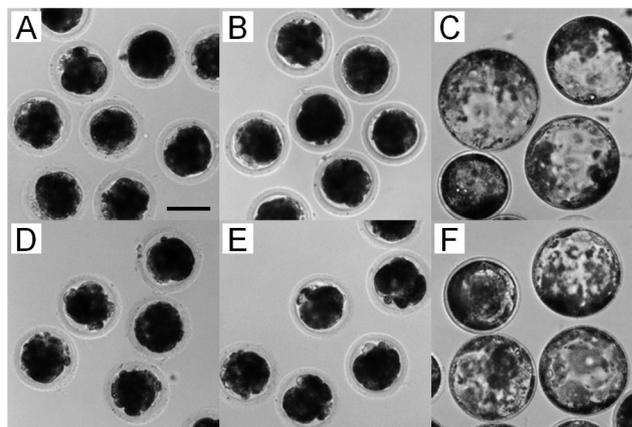
\* 6 replicates. <sup>a</sup> Nonsignificant difference.

#### Survival of IVM-derived porcine parthenogenetic morulae cryopreserved using the HFV method

Table 2 shows the viability of porcine parthenogenetic morulae that were vitrified using the HFV method. Figure 4 represents the morphological appearance of the vitrified and non-vitrified embryos. There were no significant differences in the proportions of morulae developing to the blastocyst stage between the vitrified and non-vitrified groups, although the proportion in the vitrification group (48/65, 73.8%) was slightly lower than that in the non-vitrification group (59/65, 90.8%). The viability of the vitrified embryos was also high, with 32.3% (21/65) reaching the hatched blastocyst stage compared with 46.2% (30/65) for the control non-vitrified group (not significantly different). There was no difference between the numbers of cells in blastocysts from the two groups: 86.8  $\pm$  6.5 for the vitrification group versus 77.0  $\pm$  4.8 for the non-vitrification group.

#### Production of piglets from *in vivo*-derived porcine embryos cryopreserved using the HFV method

A total of 77 embryos were collected from four donors: eight morulae were vitrified immediately after collection (Table 3); the remaining 69 embryos were cultured, and 65 (94.2%) developed to the morula or early blastocyst stages (Fig. 5-A). Of 73 morulae/early blastocysts vitrified using the modified HFV device (3 to 14 embryos/device, Fig. 5-B), 72 embryos (98.6%) developed to blastocysts after rewarming (Fig. 5-C). These blastocysts were transferred to three recipients, each receiving 21–26 embryos. All the recipients became pregnant, with each giving birth to 9 or 10 piglets (Fig. 5-D). The production efficiency (piglets/transferred embryos) ranged from 36.0% (9/25) to 47.6% (10/21), with an overall rate of 40.3% (29/72). Of the 29 piglets born (16 females and 13 males), only one was a stillbirth. The piglets grew normally till weaning, except for



**Fig. 4.** Survival of IVM-derived porcine parthenogenetic morulae vitrified using the HFV method. A–C: IVM-derived porcine parthenogenetic morulae before (A) and after (B) vitrification and day 6 blastocysts developed from the vitrified embryos (C). D–F: Non-vitrified control embryos at the same time points as the vitrified embryos. Scale bar = 100  $\mu$ m.

three that died accidentally. After weaning, 17 pigs were fattened for six to eight months and showed similar growth characteristics to standard Tokyo X pigs (data not shown). The males subjected to fattening were castrated.

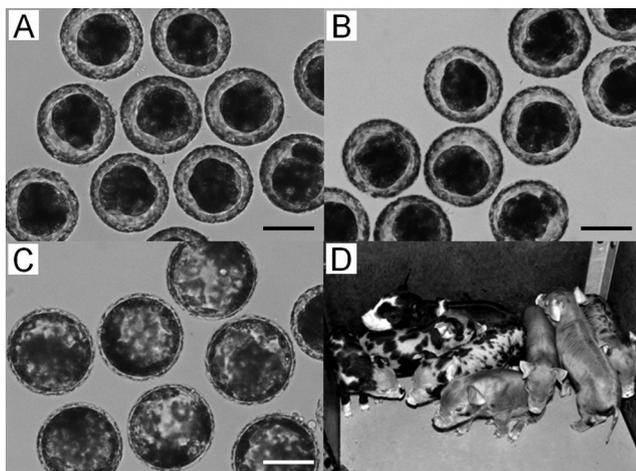
## Discussion

A number of hollow fibers made from different types of material such as polysulfone, polypropylene and ethylene vinyl alcohol were

**Table 3.** Production efficiency of piglets from *in vivo*-derived embryos vitrified by the HFV method

Embryo donors	No. of embryos collected	Culture prior to vitrification	No. of embryos vitrified	Culture prior to transfer	No. of embryos transferred (%)	No. of piglets farrowed (%) <sup>7</sup>	Timing of transfer to recipients (after hCG)
1	25	17 h	25 <sup>2</sup>	20 h <sup>5</sup>	25 (100) <sup>6</sup>	9 (36.0)	125 h
2	21	16–23 h	21 <sup>3</sup>	24 h <sup>5</sup>	21 (100) <sup>6</sup>	10 (47.6)	144 h
3, 4	31 <sup>1</sup>	20–30 h	27 <sup>4</sup>	34 h	26 (96.3)	10 (38.5)	151 h
Total	77	16–30 h	73	20–34 h	72 (98.6)	29 (40.3)	125–151 h

<sup>1</sup> Embryos were collected from 2 donors. <sup>2</sup> Eight morulae were vitrified 2 h after collection. Seven compact morulae and 10 early blastocysts were vitrified after *in vitro* culture for 17 h. <sup>3</sup> Seventeen compact morulae and 4 early blastocysts developed *in vitro* were vitrified. <sup>4</sup> Twenty-two compact morulae and 5 early blastocysts developed *in vitro* were vitrified. Four embryos with retarded development were not used for vitrification. <sup>5</sup> IVM-derived parthenogenetic morulae were also cultured after vitrification and rewarming, and those developed to blastocysts were cotransferred to the recipients. <sup>6</sup> Ten parthenogenetic blastocysts were cotransferred. <sup>7</sup> No. of piglets/No. of embryos transferred  $\times$  100.



**Fig. 5.** Morphological appearance of *in vivo*-derived porcine embryos before and after vitrification and piglets developed from the vitrified embryos. **A:** *In vivo*-derived porcine embryos at the morula stage; before vitrification. **B:** Embryos at the morula stage after vitrification and rewarming. **C:** Vitrified embryos that developed *in vitro* to the blastocyst stage prior to transfer. **D:** A litter of 8 live piglets obtained by transfer of 25 vitrified embryos. Scale bars = 100  $\mu\text{m}$ .

considered for use as a vitrification device. Eventually, however, we chose a cellulose triacetate hollow fiber of the type used in dialysis. This fiber has high permeability with an inner diameter of 185  $\mu\text{m}$  and a film thickness of 15  $\mu\text{m}$  (pore size 15 nm, permeating molecule size MW 50,000). The inner diameter (185  $\mu\text{m}$ ) of this hollow fiber is sufficiently large to accommodate early cleavage stage mouse embryos (90  $\mu\text{m}$ ) and blastocysts (100–120  $\mu\text{m}$ ) as well as cleavage stage porcine embryos (approximately 150  $\mu\text{m}$ ). The hollow fiber is made of a transparent film material, meaning that the embryos inside the fiber are visible from the outside and that morphological changes in the embryos can be observed during the experimental procedure.

One of the features of the HFV method is that it allows many embryos to be handled as a unit by placing them inside a single hollow fiber. In conventional vitrification methods, exposure of embryos to equilibration and vitrification solutions is performed by manipulating individual embryos using a fine glass pipette. In contrast, the HFV method uses a hollow fiber that carries a group of embryos. This fiber is easily handled using forceps and can be placed in the equilibration and vitrification solutions, where the embryos can absorb CPAs or dehydrate. As CPAs and water pass easily through the pores of the hollow fiber, equilibration can be performed while retaining embryos in the device. In the present study, the embryos were already in equilibration solution before being aspirated into the hollow fiber. However, embryo viability is not affected if the embryos are first placed in normal medium and then equilibrated in the hollow fiber (data not shown).

With most vitrification methods, embryos are retained in the vitrification solution for a maximum of 2 min before immersion into LN [28–30]. This process avoids exposing embryos to the toxicity of high concentrations of CPAs contained in the vitrification solution. However, because the vitrification solution has a high osmolarity

and a high viscosity, handling the embryos in this solution during this short period of time requires considerable skill on the part of the operator. Inter-operator differences in the viability of vitrified embryos, which often occur, are believed to be caused by the difficulty of handling embryos in the vitrification solution. However, with the HFV method, the hollow fiber that contains the embryos is easily manipulated using forceps, reducing the possibility of differences in inter-operator skill having a significant effect on embryo viability.

To date, all of the vitrification methods based on the MVC concept, such as OPS [15], Cryotop [13], nylon loop [16], solid surface [17] and microdroplet vitrification [18], require ultrarapid cooling/rewarming to ensure the high viability of the embryos. The thin (15  $\mu\text{m}$ ) film thickness of the hollow fiber is thought to aid the rapid temperature drop of the vitrification solution contained inside. It is also assumed that the LN permeates the hollow fiber film and comes into direct contact with the vitrification solution surrounding the embryos. The direct contact between the LN and the vitrification solution produces the ultrarapid cooling that is critical to attaining high viability in vitrified embryos [10, 31]. In addition, the high permeability and thermal conductivity of the hollow fiber are thought to allow a rapid temperature increase during rewarming. Thus, our HFV method is unique in that it meets requirements of an MVC concept-based protocol while allowing for the simultaneous vitrification of many embryos in one device.

In the current MVC methods, the volume of the vitrification solution surrounding the embryos is believed to have a significant impact on embryo viability [13]. The Cryotop method requires a maximum microdroplet volume of 0.1  $\mu\text{l}$  of vitrification solution [13]. The Cryotop method is very effective for cryopreserving embryos and has been used in various mammalian species, including humans [19, 32], cattle [28], mice [33], pigs [34, 35] and rabbits [36]. Thus, it is appropriate to consider the 0.1  $\mu\text{l}$  maximum volume as standard for MVC methods. We calculated that the volume of vitrification solution in an HFV device carrying 10 porcine embryos was approximately 0.03  $\mu\text{l}$ . This estimate was based on the diameter of the hollow fiber and the fact that the column length of the vitrification solution containing 10 embryos in the fiber was approximately 0.9 mm.

If 10 porcine embryos are aspirated into a 2 mm column of equilibration solution in the hollow fiber, the volume of solution containing embryos is calculated to be 0.07  $\mu\text{l}$ . When the fiber is transferred from the equilibration solution to the vitrification solution, the volume of the solution inside the fiber reduces noticeably due to the hyperosmolarity of the vitrification solution. As a result, embryos are plunged into LN with an extremely small (0.03  $\mu\text{l}$ ) volume of vitrification solution despite the device containing 10 porcine embryos. With regard to mouse embryos, which are smaller than porcine embryos, the same volume of solution can contain approximately 20 embryos. Assuming that 0.1  $\mu\text{l}$  of vitrification solution is the standard for the MVC approach, the HFV device could carry even more embryos in this volume of liquid. Indeed, in this study, 100% embryo viability was attained when 40 mouse embryos were placed in a single HFV device. The maximum number of embryos that one HFV device can carry while still maintaining high embryo viability will vary depending on the embryonic stages and species.

In this study, the viability of vitrified mouse embryos was equal to that of non-vitrified embryos regardless of embryonic stage. However,

the number of cells in the blastocysts that developed from embryos vitrified at an early developmental stage (e.g., 1-cell, 2-cell) was smaller than in those vitrified at a later developmental stage. We interpret this difference to be an effect of the culture period prior to cell counting at the blastocyst stage.

In the embryo transfer experiment, the proportion of vitrified mouse embryos that developed to fetuses was similar to that of non-vitrified embryos. This result proves the effectiveness of the HFV method. The developmental rate of non-vitrified embryos into fetuses was relatively low in the oviductal transfer experiment, although this result may have been due to technical problems related to embryo transfer and to use of gonadotropin-treated recipients.

Overall, we have confirmed that the HFV method is a highly effective approach for the cryopreservation of mouse embryos. However, it is known that the embryos of BDF1 mice, the strain used in this study, are highly tolerant of cryopreservation. Therefore, we examined the effectiveness of the HFV method for cryosensitive embryos. In the present study, we evaluated the effectiveness of the HFV method using IVM-derived porcine morulae. Porcine embryos are known to be highly cryosensitive [37, 38], and IVM-derived porcine morulae are even more cryosensitive than *in vivo* derived embryos. To date, successful application of cryopreservation with IVM-derived porcine morulae has required treating the embryos to increase their cryotolerance by removing cytoplasmic lipid droplets, i.e., delipitation [30, 39]. By contrast, in this study, we were able to attain very high viability in the vitrification of IVM-derived porcine morulae without delipitation. Thus, we conclude that application of the HFV method is not limited to species/strains with cryotolerant embryos. In fact, our research confirmed that live offspring could be produced very efficiently by transferring porcine embryos vitrified by the HFV method.

In two of the three transfers of vitrified embryos, vitrified parthenogenetic blastocysts were cotransferred to ensure the occurrence of pregnancy. However, pigs became pregnant with or without cotransfer and the efficiency of producing piglets was the same in the cotransfer and non-cotransfer groups. Indeed, litter sizes of 9 or 10 piglets were obtained from each of the 3 recipients. As we used gilts, a litter size of about 10 piglets is the maximum number that can be conceived by recipients. Furthermore, the *in vitro* development of vitrified embryos after rewarming was nearly 100%. Therefore, it is likely that similar numbers of piglets could be obtained by transferring fewer embryos than in this study; in other words, a higher production efficiency of piglets could be achieved.

Production of pigs from vitrified *in vivo*-derived embryos has been achieved using blastocysts [40–44]. Pig blastocysts at later stages are known to be more cryotolerant than early cleavage stage embryos [45–48]. In this study, however, morulae and early blastocysts were vitrified, and the viability of the embryos was similar to or higher than in previous studies [49, 50], indicating the effectiveness of the HFV method. We suggest that the HFV method will not only be useful for conservation of high value-added breeds such as Tokyo X but also for common breeds and strains.

To compare our new protocol with conventional methods, we performed a preliminary experiment in which we vitrified IVM-derived porcine morulae using the Cryotop method [13]. It was technically difficult to load 10 porcine embryos into a Cryotop device (Kitazato

BioPharma, Shizuoka, Japan) within the predetermined time (1 min), and the proportion of viable embryos after vitrification was significantly lower compared with the HFV method (data not shown). Inter-operator differences in the skill level necessary for the Cryotop method were apparent when attempting to minimize the volume of vitrification solution during the loading of multiple eggs into the device, leading to inconsistent results. By contrast, using the HFV method, the vitrification process was very stable, and the method allowed for the handling of multiple embryos as a single unit. We suggest that this stability is the reason for the higher viability attained using the HFV method. We also compared the HFV method with a conventional method suitable for vitrifying multiple embryos (20  $\mu$ l of 20% DMSO/20% EG/0.5 M sucrose in a 0.25 ml straw) in a preliminary study. Survival of the IVM-derived porcine morulae vitrified by the conventional method was extremely low (data not shown). The HFV method may be a rare method that allows for cryopreservation of IVM-derived porcine embryos at the morula stage.

For the HFV method to be truly practical, its operability and stability during long-term storage are important issues to verify. By using the original HFV method, long-term storage of a hollow fiber containing vitrified embryos in a cryotube is possible without a protective sheath. However, cellulose acetate hollow fibers are very fragile in LN, and careful handling is required when transferring the hollow fiber from LN to a cryotube, as the fiber is easily broken if it hits the wall of the tube. To prevent such accidents, we developed a modified device that has a sheath covering the hollow fiber portion and examined the utility of this modified device in a long-term storage experiment. Embryos vitrified using this device were safely stored and were unaffected by external events, such as the relocation of the LN storage tank and several earthquakes. In fact, we demonstrated that transfer of porcine embryos vitrified and stored using the modified HFV device enabled efficient live-pig production. In addition, we confirmed that the modified HFV device could be stored in a dry shipper without any decrease in embryo survival (data not shown). A drawback of the modified device is its complex structure, e.g., the sheath, which makes constructing the devices more difficult. The modified HFV device used in the present study is a prototype, and further modifications are in development.

In this study, we showed that the HFV method is extremely effective for the cryopreservation of early embryos in mice and pigs. Our novel protocol allows for the simultaneous vitrification of multiple embryos while adhering to the MVC concept and hence is suitable for cryopreservation of embryos of polytocous species. We conclude that the HFV method will be widely applicable not only as a research tool in reproductive biology but also as a practical technology in a variety of areas including animal industries, experimental animal breeding, conservation biology and reproductive medicine.

### Acknowledgments

This work was supported by the Japan Science and Technology Agency, ERATO, Nakauchi Stem Cell and Organ Regeneration Project and the Meiji University International Institute for Bio-Resource Research (MUIBR).

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