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Application of hollow fiber vitrification for cryopreservation of bovine early cleavage stage embryos and porcine morula-blastomeres

Ayuko UCHIKURA¹⁾, Hitomi MATSUNARI^{1, 2)}, Kazuaki NAKANO¹⁾, Shota HATAE¹⁾ and Hiroshi NAGASHIMA^{1, 2)}

¹⁾Department of Life Sciences, School of Agriculture, Meiji University, Kawasaki 214-8571, Japan

²⁾Meiji University International Institute for Bio-Resource Research (MUIBR), Kawasaki 214-8571, Japan

Abstract. A novel hollow fiber vitrification (HFV) method was applied to materials that have previously been difficult to cryopreserve, thereby expanding the potential application of this method. The results showed that zona-free porcine morulae and their isolated blastomeres remained viable even after vitrification. The rate of development to blastocysts after vitrification was similar for zona-free and zona-intact morulae (21/23, 91.3% for both). Vitrified blastomeres had a developmental potential equal to that of non-vitrified blastomeres (blastocyst formation rate after reaggregation: 16/17, 94.1% for both). The HFV method was also effective for the cryopreservation of *in vitro* matured/fertilized bovine embryos at the 2- to 4-cell, 8- to 16-cell and morula stages. The blastocyst formation rates of vitrified embryos (66.1–82.5%) were similar to those of non-vitrified embryos (74.5–82.5%). These results indicate that this novel HFV method is an effective tool for embryo cryopreservation that can enhance current practices in reproductive biology.

Key words: Bovine embryos, Cryopreservation, Hollow fiber vitrification (HFV), Porcine morula-blastomeres, Zona-free embryos

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Embryo vitrification methods, such as the open pulled straw [1], cryoloop [2], solid surface [3] and Cryotop [4] techniques, exhibit high efficacy and are based on the concept of minimum volume cooling [5, 6], whereby embryos are vitrified in a very small volume of solution. Maximal embryo survival is achieved by stabilizing the vitrified state and inhibiting ice crystal formation during the cooling and rewarming processes by minimizing the solution volume [7, 8]. We recently developed a novel hollow fiber vitrification (HFV) method in which embryos are held inside a small piece of hollow fiber (cellulose triacetate) with a small amount of vitrification solution containing cryoprotective agents. The fiber allows the simultaneous vitrification of up to 30 embryos or more [9, 10]; the cryopreservation of numerous embryos using a single device is necessary for embryo transfer in multiparous animals such as pigs [10]. This HFV method has been used to vitrify highly cryosensitive porcine morulae and has shown high efficiency in producing offspring [9]. Additionally, the HFV method is effective for cryopreserving *in vitro* matured/fertilized (IVM/IVF) porcine embryos [10].

Pigs have been widely used as experimental animals in a variety of biomedical studies, including organ regeneration [11], xenotransplantation [12–16], disease models [17–19] and stem cell therapy [20]. At the same time, techniques for manipulating porcine early embryos

have become increasingly important in various experimental settings. For example, pluripotency of induced pluripotent stem (iPS) cells has been verified by aggregation chimera techniques using blastomeres of porcine early stage embryos [20]. Furthermore, in basic research for organ regeneration from pluripotent stem cells, a new technique of chimeric pig production has been developed using early stage embryos [11]. Producing chimeras from large animals such as pigs involves diverse constraints that do not exist in rodent research. One such problem is synchronization of the developmental stages of the large numbers of component embryos used in constructing chimeric embryos. It is also difficult to prepare the large numbers of embryos in different developmental stages required for a single experiment. These kinds of constraints are resolved, however, with cryopreservation of embryos or their blastomeres. The same can be said for research using early embryos from cows, another large domestic animal that is similar in agricultural importance to pigs. Development of a practical method for efficiently cryopreserving multiple bovine early cleavage stage embryos would accelerate research, including genetic modification, embryo cloning, production of chimeras and analyses of early development mechanisms. However, early cleavage stage bovine embryos are more cryosensitive than embryos in the post-morula stages [21, 22], and reports on the cryopreservation of early stage bovine embryos have been limited [23–25].

Based on the effectiveness of the HFV method for cryopreserving porcine embryos, in the present study, we examined its efficacy for materials that are typically considered difficult to cryopreserve. Here we show that the HFV method can be used to cryopreserve *in vitro* derived porcine zona pellucida-free morulae and blastomeres isolated from them, as well as bovine IVM/IVF embryos at early cleavage stages.

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

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Experiments were performed using parthenogenetic morulae (day 4) produced via electrical activation of porcine IVM oocytes. Enzymatic removal of the zona pellucida had no effect on the *in vitro* development rate of the parthenogenetic porcine morulae into blastocysts (Table 1, Fig. 1); the rate was comparable between the zona-free and zona-intact embryos (22/22, 100% vs. 20/22, 90.9%). Of 23 zona-free morulae that were vitrified, 21 (91.3%) developed into blastocysts after rewarming and culture. This rate was equal to that of the zona-intact morulae (21/23, 91.3%; Table 1). Day 7 blastocysts obtained by culturing the zona-free morulae were morphologically normal, and the embryos were of the same quality as the hatched blastocysts derived from the zona-intact embryos (Fig. 1). Furthermore, the blastocysts that developed from the zona-free morulae after vitrification were of an equal quality to non-vitrified embryos (Fig. 1).

Blastomeres obtained from each of 17 parthenogenetic morulae (10–29 blastomeres/embryo) were loaded into hollow fibers and vitrified (Fig. 2). The recovery rates of blastomeres from each hollow fiber after vitrification and rewarming ranged from 91.7% (22/24) to 100% (29/29). All blastomeres were successfully recovered after vitrification in 13 (76.5%) of 17 cases. The recovery rates of blastomeres in the remaining 4 cases were 91.7–94.4%. After reaggregation of the vitrified blastomeres in microwells and subsequent culture of them, blastocysts were obtained from 16/17 blastomere aggregates (94.1%; Table 1), which was equal to the rate of formation

from non-vitrified blastomeres (16/17, 94.1%; Table 1, Fig. 2). These results demonstrate the availability of zona-free porcine morulae and isolated blastomeres in the form of cryopreserved materials. This achievement is a major technical innovation for biomedical research.

Bovine IVM/IVF embryos were vitrified at the 2- to 4-cell (day 1), 8- to 16-cell (day 3) and morula (day 5) stages, and their post-rewarming viability was evaluated based on their *in vitro* development into blastocysts (Fig. 3). The post-vitrification survival rate was equal to that of the non-vitrified controls (82.5–94.6%, 40–60 embryos/group) regardless of the embryo stage at the time of vitrification (Table 2). The rates of embryo development into blastocysts after vitrification (37/56, 66.1%, for the 2- to 4-cell stage; 40/60, 66.7%, for the 8- to 16-cell stage; and 33/40, 82.5%, for the morula stage) were also equal to those of the non-vitrified controls (41/55, 74.5%; 47/59, 79.7%; and 33/40, 82.5%, respectively; Table 2).

These results demonstrated that the HFV method was effective for the cryopreservation of bovine IVM/IVF embryos at the early cleavage stages. Considering that the cryosensitivity of bovine embryos is not as extreme as that of porcine embryos, vitrification of isolated bovine blastomeres using the HFV method is assumed to be possible. It is known that post-cryopreservation survival rates of bovine embryos are generally lower for pre-morula stages than for the morula stage and thereafter [21, 22]. In this study, however, post-vitrification survival rates of the 2- to 4-cell and 8- to 16-cell stage embryos were not significantly inferior to those of morulae,

Table 1. Survival of porcine zona-free morulae and morula-blastomeres vitrified by the HFV method

Specimens	Vitrification	No. of embryos *		Cell numbers in blastocysts (mean ± SEM)
		Vitrified or cultured as control	Developed to blastocysts (%)	
Zona-free morulae	–	22	22 (100) ^a	133.4 ± 12.0 ^a
	+	23	21 (91.3) ^a	96.2 ± 15.4 ^a
Zona-intact morulae	–	22	20 (90.9) ^a	111.1 ± 8.1 ^a
	+	23	21 (91.3) ^a	92.7 ± 11.7 ^a
Morula-blastomeres	–	17 [#]	16 (94.1) ^a	67.5 ± 9.8 ^a
	+	17 [†]	16 (94.1) ^a	60.5 ± 7.1 ^a

* Four replicate experiments were conducted. [#] From 17 morulae used for blastomere isolation, 10 to 25 blastomeres per embryo were obtained. [†] From 17 morulae used for blastomere isolation, 10 to 29 blastomeres per embryo were obtained. ^a Values with the same superscript are not different significantly within the same experimental groups.

Table 2. Blastocyst formation rates of bovine IVM/IVF embryos vitrified at the early cleavage stage by the HFV method

Embryonic stages	Vitrification	No. of embryos *			Cell number in the blastocysts (mean ± SEM)
		Vitrified or cultured as control	Survived (%) **	Developed to blastocysts by day 8 (%)	
2–4 cell	+	56	53 (94.6) ^a	37 (66.1) ^a	136.3 ± 9.5 ^a
	–	55	55 (100) ^a	41 (74.5) ^a	145.3 ± 10.2 ^a
8–16 cell	+	60	56 (93.3) ^a	40 (66.7) ^a	155.8 ± 12.7 ^a
	–	59	59 (100) ^a	47 (79.7) ^a	155.4 ± 10.5 ^a
Morula	+	40	33 (82.5) ^a	33 (82.5) ^a	133.3 ± 5.3 ^a
	–	40	33 (82.5) ^a	33 (82.5) ^a	157.7 ± 10.5 ^a

* At least 3 experiments were performed for each group. ** Cleaved in culture after vitrification/rewarming. ^a Values with the same superscript are not different significantly within the same experimental groups.

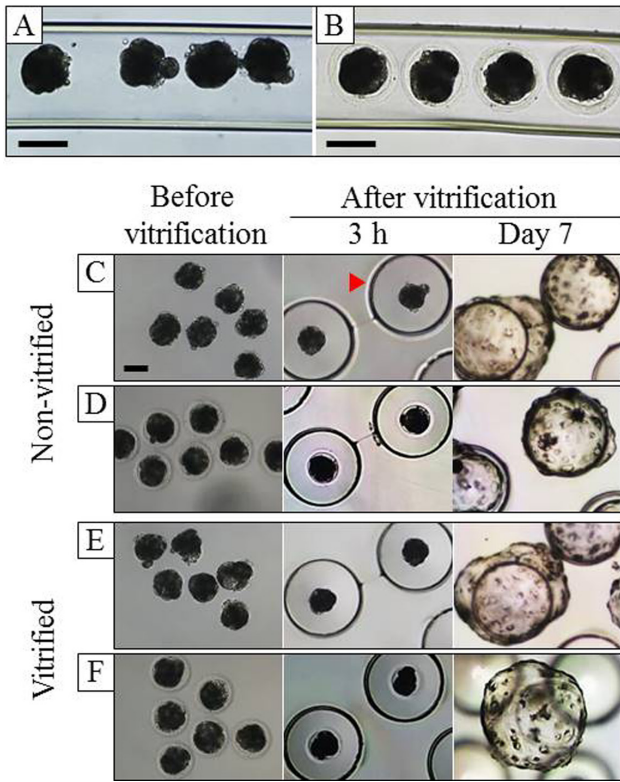


Fig. 1. Development of porcine zona-free morulae after cryopreservation by the HFV method. A, B: Zona-free (A) and zona-intact morulae (B) loaded in the hollow fiber. C–F: Zona-free (C, E) and zona-intact morulae (D, F) were cultured in microwells (arrowhead) after being vitrified or as the non-vitrified controls. Scale bar = 100 μm.

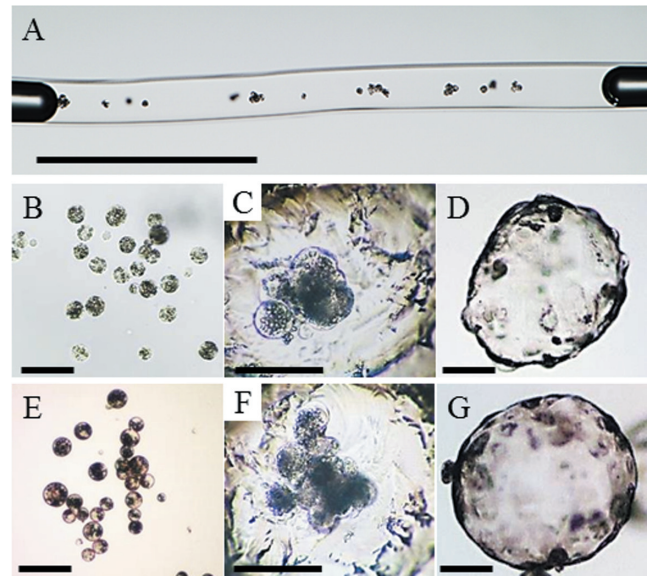


Fig. 2. Cryopreservation of isolated morula-blastomeres by the HFV method and post-rewarming development. A: Isolated morula-blastomeres loaded in a hollow fiber. B–D: Non-vitrified group. The isolated morula-blastomeres (B), blastomere aggregate in a microwell (C) and blastocyst developed from the control aggregate (D). E–G: Vitrified group. The isolated morula-blastomeres before vitrification (E) and after aggregation following vitrification/rewarming (F) and the blastocyst developed from the aggregate (G). Scale bar = 1 mm for A and 100 μm for B–G.

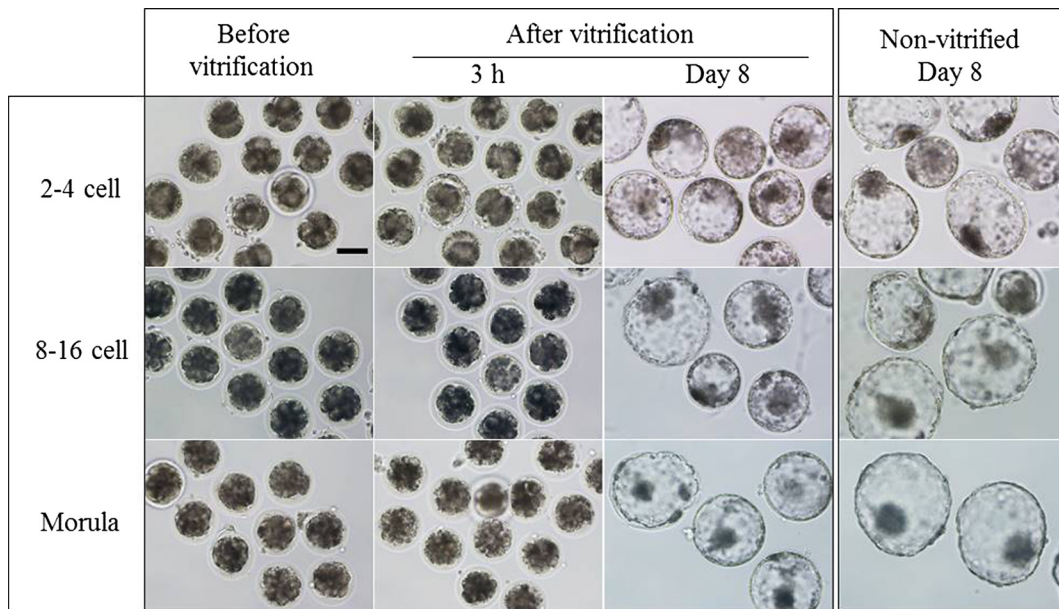


Fig. 3. *In vitro* development of IVM/IVF bovine embryos vitrified using the HFV method at early developmental stages. Bovine IVM/IVF embryos at the 2- to 4-cell, 8- to 16-cell and morula stages were vitrified/rewarmed, and their subsequent development was compared with that of non-vitrified controls. Scale bar = 100 μm.

suggesting the effectiveness of the HFV method for cryosensitive embryos.

In conclusion, the HFV method was shown to be a highly effective method for embryo cryopreservation that could be used as a tool in basic research of reproductive biology.

Methods

Chemicals

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

Preparation of porcine parthenogenetic embryos

Preparation of IVM porcine oocytes was performed as previously described [10]. Cumulus-oocyte complexes (COCs) were aspirated from the follicles of porcine ovaries collected at a local abattoir. COCs with at least three layers of compacted cumulus cells were cultured in NCSU23 medium [26] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor, 10% (v/v) porcine follicular fluid, 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulfate, 10 IU/ml equine chorionic gonadotropin (eCG; ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (hCG; ASKA Pharmaceutical) for 22 h in a humidified atmosphere of 5% CO₂ and 95% air at 38.5°C. COCs were then cultured for 22 h without eCG and hCG in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at the same temperature [27]. IVM 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 and were isolated from cumulus cells by gentle pipetting. Oocytes with an evenly granulated ooplasm and an extruded first polar body were selected for subsequent experiments.

Oocytes were washed twice in an activation solution composed of 280 mM mannitol (Nacalai Tesque, Kyoto, Japan), 0.05 mM CaCl₂, 0.1 mM MgSO₄ and 0.01% (w/v) polyvinyl alcohol (PVA). Oocytes were then aligned between two wire electrodes (separated by 1.0 mm) in a drop of 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 µsec using an electrical pulsing instrument (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 porcine parthenogenetic embryos was performed in 20–35 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 Petri dish (Iwaki 1000-035, Asahi Techno Glass, Tokyo, Japan) in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5°C.

IVM/IVF bovine embryos

IVM/IVF bovine embryos (Holstein oocytes inseminated with Japanese Black semen) were purchased from the Animal Biotechnology Center, Livestock Improvement Association of Japan (Tokyo, Japan). Putative fertilized eggs were transported in IVD101 medium (Research Institute of Functional Peptide) at 38.5°C.

Embryo and blastomere vitrification and rewarming

The vitrification solution (VS) and rewarming solution (RS) used in this study were prepared using HEPES (20 mM)-buffered tissue culture medium 199 (Medium 199, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20% calf serum as the basal solution, which was also used as the washing solution (WS). Basal solution containing 7.5% (v/v) dimethyl sulfoxide (DMSO; Nacalai Tesque) and 7.5% (v/v) ethylene glycol (EG; Nacalai Tesque) as membrane-permeant cryoprotectants was used as the equilibration solution (ES). The ES also contained 0.25 M trehalose as a nonpermeant cryoprotectant. The VS was basal solution containing 15% DMSO, 15% EG and 0.5 M trehalose. Basal solution containing 1 M sucrose was used as the RS at 38.5°C. Basal solution containing 0.5 M sucrose was used as the dilution solution (DS). ES, VS, RS, DS and WS (4 ml each) were used in 35 mm plastic dishes. Each solution except for the RS was used at room temperature (RT; 24–27°C).

For vitrification and rewarming, the embryos were placed in ES and then aspirated into a hollow fiber (~25 mm long with inner and outer diameters of 185 and 215 µm, respectively) (FB-150FH, Nipro, Osaka, Japan) connected to a hypodermic needle with a square end (5 mm long with inner and outer diameters of 0.1 and 0.15 mm, respectively; Medical Planning, Miyagi, Japan) using a 1 ml syringe and aspiration tube. The embryos were loaded into the hollow fiber by aspiration in a 2.0 to 4.5 mm column of ES flanked with air bubbles (Figs. 1 and 2). The fiber was detached from the hypodermic needle using dissecting forceps and incubated in ES for 7 min. Following equilibration, the fiber was transferred to VS using forceps for 1 min, during which time it was moved gently in the dish to ensure dehydration of the embryos inside. The fiber holding the embryos was then immersed in liquid nitrogen (LN) while being held vertically with forceps.

After cryopreservation in LN for at least 1 min, the fiber was rewarmed by rapid immersion 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, embryos or blastomeres were expelled from the hollow fiber by gently squeezing the fiber from one end to the other with forceps. Isolated blastomeres were vitrified in the same manner as the embryos, except that they were placed in WS before being loaded into the hollow fiber.

Preparation and post-vitrification evaluation of zona-free porcine morulae and morula-blastomeres

The zona pellucida of parthenogenetic porcine morulae (day 4) was digested with 0.25% (w/v) pronase in Dulbecco's phosphate-buffered saline (DPBS, Nissui Pharmaceutical). Four to six parthenogenetic or zona-intact morulae (controls) were held in a single hollow fiber and vitrified as described above (Fig. 1). After rewarming, the embryos were removed from the fiber and cultured in PZM-5 containing 10% fetal bovine serum (FBS) in a 35 mm plastic dish (Link KID, DNP, Tokyo, Japan) with microwells (diameter, 280 µm; depth, 180 µm; Fig. 1) on the bottom surface. The development into blastocysts until day 7 of zona-free and zona-intact morulae was compared.

In order to isolate blastomeres, parthenogenetic morulae (day 4) were decompacted by incubation in Ca²⁺- and Mg²⁺-free DPBS containing 0.1 mM EDTA-2Na and 0.01% (w/v) PVA for 15–20 min, followed by removal of the zona pellucidae, as described above. Blastomeres were isolated from zona-free embryos by gentle

pipetting with a finely drawn glass capillary. The blastomeres isolated from a single morula were held in a single hollow fiber and vitrified. After rewarming, the blastomeres recovered from the hollow fiber were loaded into a microwell (diameter, 400 μm ; depth, 300 μm) formed using an aggregation needle (BLS, Budapest, Hungary) on the bottom surface of a 35 mm dish; the aggregates were cultured until day 7 in PZM-5 supplemented with 10% FBS.

Evaluation of early cleavage stage IVM/IVF bovine embryos after vitrification

The insemination date was considered to be day 0; seven to 14 embryos from day 1 (2- to 4-cell stage), day 3 (8- to 16-cell stage) and day 5 (morula stage) were used for vitrification as described above. Vitrified/rewarmed and non-vitrified control embryos were cultured in a 30 μl drop of IVD101 medium in a humidified atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 at 38.5°C until day 8, at which point they were compared in terms of blastocyst formation rates. IVD101 medium supplemented with 10% FBS was used to culture the post-morula stage embryos.

Counting of blastocyst cell number

Blastocysts obtained in the experiments described above (porcine, day 7; bovine, day 8) were mounted onto glass slides with DPBS containing 20% EG and 5 mg/ml Hoechst 33342 and examined under a fluorescence microscope (TE 2000, Nikon, Tokyo, Japan) to count cell numbers.

Statistical analysis

Statistical analyses were performed using the SPSS v.16.0 software (SPSS, Chicago, IL, USA). Differences in the proportional data between two groups were analyzed with the χ^2 test or Fisher's exact test. For comparisons among three groups or more, the data were subjected to arcsine transformation and evaluated by one-way analysis of variance (ANOVA) followed by multiple comparisons by Tukey's test. Differences in the blastocyst cell number between groups were analyzed with Student's *t*-test or ANOVA followed by multiple comparisons by Tukey's test. The level of significance was set at $P < 0.05$.

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