High survival of bovine mature oocytes after nylon mesh vitrification, as assessed by intracytoplasmic sperm injection

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Abstract. Intracytoplasmic sperm injection (ICSI) is an alternative technique to in vitro fertilization (IVF) for producing transferable blastocysts, especially in combination with cryopreserved oocytes, when the IVF system does not work sufficiently. The present study was conducted to directly compare the efficacy of producing bovine blastocysts by ICSI and IVF from vitrified-warmed and fresh oocytes. Denuded oocytes with a detectable first polar body were vitrified-warmed using a nylon mesh device. In the non-vitrified control group, blastocyst yields 8 days after IVF and ICSI were 32.0 and 26.8%, respectively. Oocyte vitrification and subsequent IVF resulted in an impaired blastocyst yield (15.0%); however, such a loss of efficacy due to vitrification was not observed in the ICSI group (blastocyst yield, 25.2%). The alignment of cortical granules beneath the oolemma was comparable between the fresh control and vitrified-warmed oocytes. Here, we report the high survival of vitrified-warmed bovine oocytes, as assessed by ICSI.

Key words: Blastocyst, Cortical granule (CG) alignment, Intracytoplasmic sperm injection (ICSI), In vitro fertilization (IVF), Oocyte vitrification

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are essential tools to produce transferable blastocysts in domestic animal breeding and human infertility therapy. In cattle, ICSI can be applied to achieve the best utilization of genetically superior bulls characterized by either low sperm concentration or poor motility or both in their ejaculate [1] and to overcome the low fertilization rate or polyspermic penetration in cryopreserved oocytes after IVF [2]. The low or unstable efficacy of ICSI in producing bovine blastocysts, ranging from 5 to 40%, needs to be improved [3]. Technical difficulties in bovine ICSI are caused by the darkness of the lipid droplet-rich ooplasm, the relatively large size of the sperm heads, and the elasticity and toughness of the oolemma. Furthermore, the inability of injected spermatozoa to induce proper oocyte activation [4] and compromised sperm chromatin remodeling in ICSI oocytes [5, 6] are also responsible for the low efficacy of bovine ICSI. Oocyte centrifugation, piezo-actuated micromanipulation, chemical stimuli to induce oocyte activation (e.g., ethanol, ionomycin, 6-dimethylaminopurine, cycloheximide, and roscovitine, independently or in various combinations), and treatment of spermatozoa or ICSI oocytes with reducing agents (e.g., dithiothreitol [DTT] and glutathione) have contributed to improving the efficacy of bovine ICSI in vitro [2, 3, 7, 8].

Cryopreservation of bovine oocytes can be an integral part of various assisted reproductive technologies, but their insufficient post-warm viability (up to 35% blastocyst yield) leaves this technique at an experimental stage [9, 10]. Even in an advanced cryopreservation protocol for bovine oocytes, such as minimum volume cooling (MVC) vitrification with a Cryotop device, a high incidence of polyspermic penetration into the post-warm oocytes was reported along with a low blastocyst yield [11]. The viability of post-warm bovine oocytes has been considerably improved by chemical application (e.g., L-carnitine, Rho-associated coiled-coil kinase [ROCK] inhibitor, α-tocopherol, resveratrol, and melatonin) [9, 12, 13] and novel MVC devices (e.g., open-pulled straw, Cryotop, nylon mesh, and silk fibroin sheet) [10]; however, blastocyst yield following oocyte vitrification remains inferior when compared with its non-vitrified counterpart in an IVF assay. Premature exocytosis of cortical granules (CGs) and hardening of the zona pellucida induced in bovine oocytes during the vitrification process [14, 15] may be one of the factors responsible for impaired blastocyst yield after IVF.

Little is known about the efficacy of producing bovine blastocysts from mature oocytes after vitrification and subsequent ICSI. In an earlier study [2], bovine blastocysts were harvested at 9.8% and 6.1% of oocytes vitrified on an electron microscope (EM) grid after ICSI and IVF, respectively; both of these rates were lower than harvesting rates obtained with the fresh control oocytes (16.3% and 19.1%, respectively). The present study was conducted to directly compare the efficacy of ICSI and IVF in producing bovine blastocysts from fresh and vitrified-warmed oocytes on a nylon mesh device. The alignment of CGs beneath the oolemma in fresh and vitrified-warmed oocytes was also observed by fluorescent staining with FITC-conjugated Lens culinaris agglutinin (LCA).

We conducted a 2 × 2 factorial design experiment (IVF/ICSI × fresh/vitrified oocytes) using in vitro-matured oocytes from abattoir-derived bovine ovaries and frozen semen from a single Japanese Black bull (Table 1). Oocytes that survived nylon mesh vitrification (> 90%) were randomly assigned to the IVF or ICSI group. Cleavage rates 2 days after IVF and ICSI, ranging from 70.1% to 74.4%, were
comparable, regardless of oocyte vitrification. In the fresh control group, blastocyst yields 8 days after IVF and ICSI were 32.0 and 26.8%, respectively (P ≥ 0.05). Nylon mesh vitrification of mature oocytes and subsequent IVF resulted in an impaired blastocyst yield (15.0%, P < 0.05); however, such a loss of efficacy due to vitrification was not observed in the ICSI group (blastocyst yield, 25.2%). Mavrides and Morroll [16] reported that Cryoloop vitrification of bovine mature oocytes did not affect the cleavage rate after ICSI (16.0% vs. 17.3% in the fresh control) but did affect the cleavage rate after IVF (25.8% vs. 49.5% in the fresh control). Rho et al. [2] reported that EM grid vitrification of bovine mature oocytes affected both the cleavage rate after IVF (40.6% vs. 83.6% in the fresh control) or ICSI (52.2% vs. 70.0% in the fresh control) and the blastocyst yield after IVF (6.1% vs. 19.1% in the fresh control) or ICSI (9.8% vs. 16.3% in the fresh control). The difference between our results and those of previous studies may be attributed to the progress in bovine oocyte vitrification over the last two decades [9, 10, 17]. Very recently, Kagawa et al. [18] reported that bovine ICSI after in vitro maturation and Cryotop vitrification of ovum pick-up (OPU)-retrieved oocytes resulted in a 30.9% cleavage rate and a 16.5% blastocyst yield, with the successful production of two living calves. No data were available for bovine ICSI of fresh OPU oocytes. However, the proportion of ICSI blastocysts re-calculated from cleaved embryos in the vitrification group (55.9%) was comparable to that (55.3%) of IVF blastocysts in the fresh control group but higher than that (23.2%) of IVF blastocysts in the vitrification group [18], which is consistent with our results.

Blastocyst quality is often evaluated using in vitro parameters, such as developmental kinetics (time required to reach blastocysts), total cell number, and inner cell mass (ICM) cell ratio [19]. As a parameter reflecting developmental kinetics, mean proportion of Day 7 blastocysts in total blastocyst harvests ranged from 69.2% to 86.0% (P ≥ 0.05). The developmental stage of blastocysts harvested on Day 8 can be defined as a parameter that reflects the total cell number (Fig. 1). Comparing the proportion of unexpanded blastocysts, quality of the ICSI-derived blastocysts in the fresh control group was higher than that in the vitrification group. In our previous studies, Cryotop vitrification of bovine mature oocytes resulted in the production of IVF-derived Day 8 blastocysts with fewer total cell number (87.9 vs. 118.1 in fresh control [11]; 97.5 vs. 135.7 in fresh control [20]; 107.8 vs. 158.0 in fresh control [21]). In contrast, the quality of the ICSI-derived blastocysts was comparable regardless of oocyte vitrification (Fig. 1). Rho et al. [2] reported that the EM grid vitrification of bovine mature oocytes resulted in the production of ICSI-derived Day 8 blastocysts with fewer total cell numbers (99 vs. 124 in fresh control) and a higher ICM ratio (34 vs. 25% in fresh control). Hatching of Day 8 blastocysts may be assisted by a pin-hole in the zona pellucida drilled by piezo pulses immediately after aspiration of the blastocyst.

### Table 1. In vitro development of vitrified-warmed bovine oocytes after IVF or ICSI

| Oocyte vitrification | No. (%) of oocytes | Developed to blastocysts |
|----------------------|--------------------|-------------------------|
|                      | Inseminated | Cleaved | Day 7 | Day 8 |
| IVF                  | 96         | 72 (74.4 ± 9.5) a| 25 (25.7 ± 5.2) a| 31 (32.0 ± 4.6) a |
| +                    | 87         | 61 (70.1 ± 2.8) a| 9 (10.7 ± 3.0) b| 13 (15.0 ± 3.0) b |
| ICSI                 | 123        | 91 (73.6 ± 9.3) a| 28 (22.7 ± 8.7) a| 33 (26.8 ± 8.4) ab |
| +                    | 99         | 70 (71.2 ± 3.8) a| 22 (21.8 ± 2.4) a| 25 (25.2 ± 1.3) b |

* Different superscripts within the same column indicate significant differences at P < 0.05.
Percentages are expressed as mean ± SEM of four replicates.

Fig. 1. Effect of oocyte vitrification on the developmental stage of bovine blastocysts harvested 8 days after IVF or ICSI (192 hpi). Hd, Hatching/hatched blastocysts; Ex, Expanding/fully expanded blastocysts; Un, Unexpanded/early blastocysts.
before ICSI [22]. Structural flexibility changes in the zona pellucida associated with oocyte vitrification [14, 15] may be responsible for accelerating the hatching process in ICSI-derived Day 8 blastocysts.

The impaired blastocyst yield in IVF, but not ICSI, of vitrified-warmed bovine oocytes (Table 1) may be due to the high incidence of polyspermic penetration, as reported by Hwang et al. [11]. Therefore, the alignment of CGs in fresh versus vitrified-warmed oocytes was assessed by LCA lectin staining (Fig. 2), according to the categories reported by Goud et al. [23]. Nylon mesh vitrification did not induce any adverse effects on CG alignment regardless of recovery culture, as the proportion of oocytes with intact or minor-loss CG alignment was not different between the fresh control and vitrification groups (86.4% and 84.6–87.1%, respectively). Chilling or DMSO exposure of mouse oocytes induces premature exocytosis of CGs, which leads to zona hardening [24, 25]. After conventional straw vitrification, disturbed CG alignment in bovine oocytes was observed using transmission EM [14]. Biochemical changes in the architecture of the zona pellucida, including the transformation of the protein secondary structure from the α-helix to the β-sheet, were observed in bovine oocytes after Cryotop vitrification using non-invasive Raman micro-spectroscopy [15]. Cryoloop vitrification of bovine oocytes also disturbed CG alignment [26]. However, Cryotop vitrification did not affect CG alignment [11, 21], similar to the nylon mesh vitrification in the present study. Yashiro et al. [21] reported a comparable frequency of diploid bovine IVF blastocysts between the fresh control and oocyte vitrification groups (84.6% vs. 81.3%, respectively) based on chromosomal analysis. We speculated that vitrification injuries occurring in the ooplasm would influence the transition from maternal to zygotic genome activation and subsequent blastocyst development in our IVF system. Oocyte activation treatment adapted to ICSI oocytes may help to overcome this inconvenience.

Various MVC vitrification devices have been developed to achieve ultra-rapid cooling rates for bovine oocytes [10]. Among them, a triangular nylon mesh device employed here has been applied to handle up to 50 oocytes; it allows easy removal of excess vitrification solution around the oocytes by its absorption into a paper towel without need for a time-consuming pipetting technique [27]. All oocytes retrieved from a single donor can be cryopreserved by nylon mesh vitrification because the efficiency of oocyte retrieval varies considerably among individual donors [28]. Oikawa et al. [29] reported that donor-dependent low blastocyst yield from bovine IVF oocytes (6.2%) could be improved by applying ICSI (27.1%) in an OPU system. Under ICSI conditions, where a single spermatozoon is injected, the possibility of developmental failure of vitrified oocytes due to polyspermic penetration is negligible. A higher incidence of multiple-aster formation due to dysfunction of the microtubule-organizing center (MTOC) was observed in vitrified-warmed bovine oocytes after IVF, and pronuclear development and migration were delayed in zygotes with multiple-aster formation [30]. Interestingly, bovine ICSI procedures, including sperm pretreatment with DTT and oocyte activation with ionomycin and ethanol, also result in impaired MTOC function [31]. Short-term recovery culture of vitrified-warmed bovine oocytes could reduce the incidence of multiple-aster formation in the presence of the ROCK inhibitor Y-27632 [20] and α-tocopherol [21], suggesting that the post-warming ooplasmic environment is reversibly repaired.

In conclusion, bovine mature oocytes vitrified-warmed on a nylon mesh device can develop into transferable blastocysts after ICSI without losing production efficacy. Whether the resultant blastocysts can participate in calf production would be of great importance in the veterinary field.

Methods

Preparation of mature oocytes

Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine ovaries from a local abattoir were transported to the laboratory in saline solution (23 ± 3°C) within 6 h of slaughter. Oocytes surrounded by at least 2 layers of compact cumulus cells were retrieved from < 8 mm follicles and cultured in maturation medium comprised of HEPES-buffered TCM199 (Earle salts; Gibco™, Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS; Gibco™), 0.2 mM sodium pyruvate, 0.002 AU/ml follicle-stimulating hormone.

Fig. 2. Alignment of CGs in bovine mature oocytes after nylon mesh vitrification with or without 2-h recovery culture. Alignment of the CGs periphery to the oolemma of each oocyte was classified as fully intact (100% stained), minor loss (≥ 90% stained), or major loss (< 90% stained) [23].
(Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 µg/ml estradiol-17β, and 50 µg/ml gentamicin sulfate for 22 h at 38.5°C in 5% CO₂ atmosphere (8–15 oocytes per 100-µl microdrop under mineral oil). Then, the cumulus cells were removed entirely by vortex-mixing for 3 min in HEPES-buffered TC199 supplemented with 3 mg/ml bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 1,000 IU/ml hyaluronidase, and 50 µg/ml gentamicin sulfate. Denuded oocytes with an extruded first polar body were defined as matured, and used for IVF or ICSI with or without nylon mesh vitrification.

**Vitrification and warming**

As described in our previous report, MVC vitrification using a nylon mesh device was performed [27]. Briefly, mature denuded oocytes were rinsed twice in basic solution (HEPES-buffered TC199 supplemented with 20% FBS) and equilibrated with 7.5% ethylene glycol (EG; FUJIFILM Wako, Osaka, Japan) and 7.5% DMSO in basal solution for 3 min at ambient temperature (25 ± 3°C). Twenty to thirty oocytes were placed onto the center triangle of a nylon mesh device (a square opening 37-µm on a side length; Sansyo, Tokyo, Japan) and the device was processed as a developed figure of triangular pyramid, using a capillary pipette. Immediately after the equilibration solution was removed by placing the device on a sterilized paper towel (Kimwipes®, Nippon Paper Crecia, Tokyo, Japan), the oocytes on the device were exposed to vitrification solution comprised of 15% EG, 15% DMSO, and 0.5 M sucrose in the basal solution for 60 sec at ambient temperature using sterilized tweezers. During this 60-sec, the device was placed on new sterilized Kimwipes® paper to minimize the volume of the vitrification solution and then quickly plunged into liquid nitrogen (LN₂).

After storage in LN₂-filled 15-ml conical tubes for at least 1 week, oocytes were warmed by immersing the device in a basal solution containing 1.0 M sucrose prewarmed to 38.5°C for 1 min. Post-warm oocytes were retrieved from the device and transferred to the basal solution at ambient temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose solutions for 3, 5, and 5 min, respectively). The oocytes were then washed twice in HEPES-buffered TC199 supplemented with 5% FBS, 0.2 mM sodium pyruvate, and 50 µg/ml gentamicin sulfate and incubated in 100-µl microdrops of the same medium for 2 h at 38.5°C in 5% CO₂ atmosphere (10–12 oocytes per microdrop under mineral oil).

**IVF**

Commercially available frozen semen from a single Japanese Black bull (bull ID “Sengaku”, Nagano Animal Industry Experiment Station, Nagano, Japan) was thawed in a water bath at 37.0°C for 30 sec. The contents of 0.5-ml straw were layered on top of a Percoll density gradient consisting of 2 ml of 45% Percoll above 2 ml of 90% Percoll in a 15-ml conical tube and centrifuged for 20 min at 700 × g. The sperm pellet was resuspended in 4 ml of modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan) supplemented with 5 mM theophylline, washed twice (5 min at 300 × g each), and then resuspended in the mBO medium supplemented with 10 µg/ml heparin and 5 mg/ml BSA (IVF medium) to obtain a concentration of 4 × 10⁶ sperm cells/ml. Ten to twelve denuded oocytes were placed in an 80-µl microdrop of the IVF medium under mineral oil, and insemminated with 20 µl of the sperm suspension (final sperm concentration, 5 × 10⁶ cells/ml) for 6 h at 38.5°C in 5% CO₂ atmosphere.

**ICSI**

ICSI was performed as described in our previous report [3]. Briefly, motile frozen-thawed bull spermatozoa were selected by 45%/90% Percoll density gradient centrifugation (20 min at 700 × g) as described above. The sperm pellet was treated with 5 mM DTT in a BSA-free mBO medium for 15 min at 38.5°C in 5% CO₂ atmosphere and then washed twice (3 min at 300 × g each) in an mBO medium. Immediately before ICSI, 2 µl of the sperm suspension was mixed with 8 µl of M2 medium [32] containing 10% polyvinylpyrrolidone (PVP). The ICSI was performed using a piezo-driven micromanipulator (PMM-150FU; Prime Tech, Ibaraki, Japan). A single spermatozoon was immobilized by applying several piezo pulses to the midpiece before being aspirated tail first into a blunt-ended injection pipette with an outer diameter of 7–9 µm. A denuded oocyte was held with the holding pipette, as the polar body was either at the 6 or 12 o’clock position. The zona pellucida was drilled by several piezo pulses (speed, 2; intensity, 2). The spermatozoon was repositioned to the tip of the injection pipette, and the injection pipette was mechanically advanced deep into the center of the oocyte, stretching the oolemma extensively. Upon the application of a single piezo pulse, the oolemma was punctured at the pipette tip. The sperm was gently injected into the ooplasm, and the injection pipette was withdrawn. For short-term incubation of oocytes before, during, and after ICSI, HEPES-buffered TC199 supplemented with 5% FBS was used. For supplementary oocyte activation, the ICSI oocytes were first treated with 10 µM monomycin in phosphate-buffered saline (PBS) for 5 min in the dark and then incubated in 100-µl microdrops of HEPES-buffered TC199 + 5% FBS for 4 h at 38.5°C in 5% CO₂ atmosphere. The ICSI oocytes were then treated with 7% ethanol in HEPES-buffered TC199 supplemented with 1 mg/ml PVP for 5 min.

**In vitro culture**

Presumptive zygotes 6 h post-insemination (hpi) were washed three times with modified synthetic oviduct fluid [33] supplemented with 20 µl/ml of essential amino acid solution (50 ×; Gibco™), 10 µl/ml of non-essential amino acid solution (100 ×; Gibco™), and 5% FBS, and cultured in 250-µl microdrops of the same medium at 39.0°C in 5% CO₂, 5% O₂, and 90% N₂ atmosphere (20–30 zygotes per microdrop under mineral oil). Day 0 was defined as the day of the IVF/ICSI. The number of zygotes cleaving was recorded on Day 2 (48 hpi), and the number of zygotes developing to the blastocyst stage was recorded on Day 7 (168 hpi) and Day 8 (192 hpi). Blastocystcs were qualitatively analyzed based on developmental kinetics (% Day 7 blastocysts per total harvest) and developmental stage (unexpanded, expanded, or hatched blastocysts on Day 8).

**CG alignment**

Fresh mature oocytes and vitrified-warmed oocytes with or without 2-h recovery culture were fixed in 4% paraformaldehyde for 30 min at ambient temperature after the zona pellucida was removed in an M2 medium containing 0.75% protease (Pronase; Calbiochem, Darmstadt, Germany). The oocytes were permeabilized overnight in PBS with 0.1% Triton X-100 and 0.3% BSA at 4°C and then incubated for 15 min in PBS with 100 ng/ml FITC-conjugated LCA (Vector Laboratories, Burlingame, CA, USA) in the dark. The oocytes were rinsed three times in PBS with 0.1% PVP and mounted with coverslips in an anti-fade agent (100 mg 1,4-diazabicyclo [2.2.2] octane in 1 ml glycerol). Digital images were collected at 2-µm thickness using a confocal laser-scanning microscope (FV1000-D; Olympus, Tokyo, Japan). Alignment of the CGs periphery to the oolemma of each oocyte was classified as fully intact (100% stained), minor loss (≥90% stained), or major loss (<90% stained) [23].
Statistical analysis
Percentage data in each of the four replicates were arcsine-transformed and compared using a one-way analysis of variance (ANOVA). When the ANOVA was significant, differences among means were analyzed using Fisher’s protected least significant difference test. A statistical value of P < 0.05 was considered to indicate significant difference.

Conflict of interests: The author declares that there is no conflict of interest.

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