High incidence of multiple aster formation in vitrified-warmed bovine oocytes after in vitro fertilization

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Abstract

In vitro-matured bovine oocytes do not tolerate vitrification as well as mature murine or human oocytes. Delayed first cleavage in vitrified and in vitro-fertilized bovine oocytes may be responsible for the decreased yield of blastocysts in vitro. Since formation of sperm-aster and the subsequent assembly of microtubule network play an important role for migration and fusion of both pronuclei, aster formation in vitrified-warmed oocytes was analyzed by confocal laser-scanning microscopy. At 10 h post-insemination (hpi), proportions of oocytes fertilized normally were comparable between the vitrified and fresh control groups (67 and 70%, respectively). Proportions of oocytes that exhibited microtubule assembly were similar between the two groups (95% each), but the proportion of oocytes with multiple asters was higher in the vitrified group when compared with the fresh control group (68 vs 29%, $P < 0.05$). Both migration and development of two pronuclei were adversely affected by multiple aster formation. In the next experiment, multiple asters observed in 5.5 versus 8 hpi pronuclear zygotes were located near the male pronucleus, suggesting that those multiple asters were not the cytoplasmic asters of maternal origin. In conclusion, multiple aster formation frequently observed in vitrified-warmed bovine oocytes may be related to loss of ooplasmic function responsible for normal microtubule assembly from the sperm-aster.

Keywords: Bovine oocytes; Cryotop vitrification; MTOC; Multiple asters; Single sperm-aster

1. Introduction

Although successful pregnancies from frozen-thawed oocytes have been reported in several mammals, including mice [1], rabbits [2], cattle [3], and humans [4], developmental rate needs to be improved. Proposed reasons for high sensitivity of oocytes to cryopreservation include the large cell size and low permeability of water and cryoprotectants (CPA) [5]. Depolymerization of microtubules induced by CPA treatment and cryopreservation resulted in meiotic spindle disassembly and chromosome misalignment [6]. Treatment with CPA induced a transient rise of intracellular free calcium level, premature exocytosis of cortical granules, and hardening of zonae pellucidae [7,8]. Application of vitrification improved the efficacy of oocyte cryopreservation, especially in mice [9,10] and humans [11,12]. However, vitrification of oocytes from large domestic species enriched with cytoplasmic lipid droplets still requires substantial improvement [13-17].

A centrosome is composed of a pair of centrioles surrounded by the pericentriolar materials such as γ-tubulin, centrin and pericentrin, and acts as the microtubule-organizing center (MTOC). In cattle, a sperm brings a centrosome into an oocyte during fertilization [18] and a single sperm aster is formed by polymerization of microtubules (α- and β-tubulin). The microtubule network plays a key role in the migration of male and female pronuclei to the center of a zygote and the subsequent fusion and mitotic cleavage [19,20]. Conversely, rodent sperm lose their centrioles during spermiogenesis [21]. Hence, the oocytes use their own MTOC dispersed in the cytoplasm for aster formation; that is called cytoplasmic aster [22,23]. Timing of first cleavage in IVF-derived bovine oocytes is important for yield and quality of blastocysts,

as oocytes cleaving earlier are more likely to become blastocysts [24,25], and the resulting blastocysts have higher cryosurvival potential [26] and higher pregnancy rates [24] than those cleaving later. Thus, developmental kinetics can be used as a proxy of embryo quality.

In the present study, profiles of cleavage and blastocyst development were first examined for vitrified bovine oocytes, and then function of MTOC / aster(s) in the vitrified oocytes after IVF was analyzed.

2. Materials and methods

2.1. In vitro maturation

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Abattoir-derived bovine ovaries were transported to the laboratory in saline (maintained at 10 to 12 ºC) within 24 h after slaughter. The contents of 2-8 mm follicles were aspirated with an 18-G needle connected to a 10-mL syringe. Oocytes surrounded with at least two layers of compact cumulus cells were matured in Hepes-buffered Tissue Culture Medium (TCM)-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/mL FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 µg/mL 17ß-estradiol, and 50 µg/mL gentamycin sulfate for 22 h at 38.5 ºC under 5% CO_2 in air. Then, cumulus cells were removed by a brief vortex-mixing in the Hepes-buffered TCM-199 supplemented with 3 mg/mL bovine serum albumin (BSA), 0.2 mM sodium

pyruvate, 1000 IU/mL hyaluronidase, and 50 µg/mL gentamycin sulfate. Oocytes with an extruded first polar body were defined as matured and were used for experiments.

2.2. Vitrification and warming

Matured oocytes were subjected to vitrification according to the method described previously by Tsujioka et al. [27], with minor modifications. Briefly, oocytes were equilibrated 7.5% ethylene glycol (EG; Wako Pure Chemical Industries Co., Osaka, Japan) and 7.5% dimethylsulfoxide (DMSO; Wako) in Hepes-buffered TCM-199/20% FBS base medium for 3 min at room temperature, and then transferred into a vitrification solution consisting of 15% EG, 15% DMSO and 0.5 M sucrose in the base medium for approximately 60 s at room temperature. Within this 60 s, up to eight oocytes were loaded onto the top of the polypropylene strip of a Cryotop (Kitazato BioPharma Co., Shizuoka, Japan) with a minimal amount of the vitrification solution, and then quickly immersed into liquid nitrogen (LN_2).

After storage for 1 to 10 wk in LN_2 tank, oocytes were warmed by immersing the polypropylene strip of a Cryotop into 3 mL of the base medium containing 1 M sucrose at 38.5 ºC, and kept for 1 min. The oocytes were transferred to the base medium at room temperature in a stepwise manner (0.5, 0.25, and 0 M sucrose for 3, 5, and 5 min, respectively). They were cultured in Hepes-buffered TCM-199 supplemented with 5% FBS, 0.2 mM sodium pyruvate and 50 µg/mL gentamycin sulfate (TCM-199/5% FBS) for 1 to 2 h at 38.5 ºC under 5% CO_2 in air before subjecting to IVF.

2.3. In vitro fertilization and culture

Commercially available frozen semen from a Japanese Black bull was used. After thawing in a water bath at 37 ºC for 30 s, the contents of a 0.5-mL straw was layered on the top of 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 $700 \times g$. The pellet was re-suspended 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 $300 \times g$ each) and then re-suspended in the mBO medium supplemented with 5 mg/mL BSA and 10 µg/mL heparin (IVF medium) to yield a concentration of 4×10^7 sperm cells/mL. In Experiments 1 and 2, 10 to 12 matured oocytes in the IVF medium were co-incubated with the above sperm suspension at a final concentration of 8×10^6 sperm cells/mL for 6 h in 100-µL microdrops under mineral oil at 38.5 ºC under 5% CO_2 in air. In Experiment 3, a 100-µL sperm suspension at a final concentration of 8×10^6 sperm cells/mL in IVF medium was preincubated for 2 h before introduction of the 10 to 12 vitrified oocytes. The period for gamete co-incubation was shortened to 2 h for restriction of the fertilization window.

In Experiment 1, up to 30 presumptive zygotes (6 hpi) were cultured in a 250-µL microdrop of modified synthetic oviduct fluid (mSOF) [28], supplemented with 30 µL/mL essential amino acids solution ($\times$ 50, Gibco-11130), 10 µL/mL non-essential amino acids solution ($\times$ 100, Gibco-11140) and 5% FBS at 39.0 ºC under 5% CO_2, 5% O_2 and 90% N_2 for 8 d.

2.4. Immunostaining of pronuclear zygotes

In Experiment 2, fresh or vitrified oocytes after IVF (6 hpi) were cultured for an

additional 4 h (10 hpi) in TCM-199/5% FBS at 38.5 ºC under 5% CO_2 in air, and then immunostained. In Experiment 3, vitrified oocytes after IVF (2 hpi) were cultured for an additional 3.5 and 6 h (5.5 and 8 hpi, respectively), and then immunostained. According to the method described previously [29], the oocytes were extracted for 15 min by buffer M (25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA and 50 mM imidazole hydrochloride, pH 6.8) containing 5% (v/v) methanol and 1% (v/v) Triton X-100, after zonae pellucidae had been removed with 0.75% protease in M2 medium. The oocytes were then fixed with cold methanol for 10 min and permeabilized overnight in PBS containing 0.1% (v/v) Triton X-100. Microtubules were labeled with a monoclonal antibody against α-tubulin (T5168; diluted 1:1000). The primary antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (F1010; diluted 1:200). Nuclear DNA was visualized by counterstaining with 2.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI). Preparations were mounted with coverslips in antifade agent, and digital images were collected at 2 µm distance using a confocal laser scanning microscope (FV1000-D; Olympus, Tokyo, Japan). The digital images were stacked and assessed with Image-J software (National Institutes of Health, Bethesda, ML, USA; accessed on-line). Zygotes with 2PN were defined as those fertilized normally, and the larger pronucleus was defined as male pronucleus.

2.5. Experimental design

In Experiment 1, cumulus-free bovine matured oocytes were vitrified-warmed, and then fertilized in vitro. The presumptive zygotes were placed in microdrops and cultured to allow development into blastocysts for up to 8 d. Cleavage was checked first at 27

hpi and again at 48 hpi, and the blastocyst yields from earlier-cleaving and delayed-cleaving embryos were separately recorded. Non-vitrified matured oocytes served as fresh controls. In Experiment 2, pronuclear-stage zygotes were produced by IVF of vitrified-warmed and fresh control oocytes (gamete co-incubation for 6 h and additional culture for 4 h). The microtubules of the 10 hpi zygotes were immunostained with a monoclonal antibody against α-tubulin and nuclear DNA were counterstained with DAPI. Under a confocal laser-scanning microscopy, aster formation in the normally fertilized zygotes with two pronuclei (2PN) was analyzed. The number of asters, the distance between male and female pronuclei, and the size of both pronuclei were recorded. In Experiment 3, it was investigated whether the observed multiple asters were the cytoplasmic asters (maternal origin) or the fragmentation of sperm-aster (paternal origin). The positioning of multiple asters in the vitrified and IVF oocytes at 5.5 and 8 hpi was compared after the fertilization window was narrowed to 2 h.

2.6. Statistical analysis

In Experiment 1, cleavage rates at 27 and 48 hpi and blastocyst yields were arcsin-transformed and compared between vitrified and fresh control groups by Student's *t*-test. Arcsin-transformed blastocyst yields were compared between subgroups of oocytes cleaved earlier (0-27 hpi) and later (27-48 hpi) by Student's *t*-test. In Experiment 2, rates of oocytes for normal fertilization, polyspermic fertilization, overall aster formation, single aster formation and multiple aster formation were arcsin-transformed and compared between vitrified and fresh control groups by Student's *t*-test. Mean aster number per zygote exhibiting multiple asters was also

analyzed by Student's *t*-test. The distance between male and female pronuclei and the pronuclear size were compared by one-way ANOVA. When the ANOVA was significant, differences among means were analyzed by a Tukey's test. In Experiment 3, mean distance from aster to male pronucleus was analyzed between 5.5 and 8 hpi groups by Student's *t*-test. Mean aster number per zygote was also analyzed by Student's *t*-test. A value of $P < 0.05$ was defined as a significant difference.

3. Results

3.1. Early cleavage and blastocyst yield (Experiment 1)

All 635 post-warm oocytes appeared to be intact (morphological cryosurvival, 100%). After IVF and in vitro culture of the presumptive zygotes, the cleavage rate 48 hpi was lower than that of fresh control counterparts (63 vs 75%, $P < 0.05$; Table 1). The adverse effects of vitrification were more prominent in the developmental rate to blastocyst stage (16 and 42% in vitrified and fresh control groups, respectively; $P < 0.05$). The proportion of oocytes cleaving earlier in the fresh control group (63%) was more than twice that in the vitrified group (24%). The earlier-cleaving oocytes developed to blastocysts at a higher rate ($P < 0.05$) when compared to the later-cleaving ones (41%, 16/39 vs 15%, 10/65 and 60%, 61/102 vs 35%, 7/20 in vitrified and fresh control groups, respectively).

3.2. Analyses of aster formation (Experiment 2)

Presumptive zygotes 10 hpi with or without vitrification at their metaphase-II stage were assessed for the presence of aster(s), as shown (Table 2). Counterstaining with DAPI indicated the comparable incidence of normal fertilization between vitrified and fresh control groups (67 and 70%, respectively). Polyspermic penetration occurred at a similar rate between the two groups (20 ± 4 and $11 \pm 3\%$, respectively). Immunostaining for α-tubulin indicated that proportions of zygotes exhibiting aster formation were also comparable between vitrified and fresh control groups (95% of 2PN zygotes each). However interestingly, relative ratio of zygotes with a single aster (Fig. 1A) versus multiple asters (Fig. 1B) was significantly different between the two groups. Incidence of multiple aster formation in zygotes derived from vitrified oocytes (68%) was more than double that in zygotes derived from fresh control oocytes (29%, $P < 0.05$). Mean aster number per zygote exhibiting multiple asters was 5.4 ± 0.5 and 4.9 ± 0.5 in vitrified and fresh control groups, respectively ($P > 0.05$).

With regards to pronuclear migration and development, zygotes with multiple asters were retrospectively compared with those with a single aster, as shown (Table 3). Distances between male and female pronuclei in the zygotes exhibiting multiple asters (42 and 45 μm in vitrified and fresh control groups, respectively) were significantly longer than those in the zygotes exhibiting a single aster (30 and 27 μm in vitrified and fresh control groups, respectively; $P < 0.05$) regardless of vitrification. In addition, areas of both pronuclei in the zygotes exhibiting multiple asters were significantly smaller than those in the zygotes exhibiting a single aster ($P < 0.05$). There were no significant differences in the pronuclear size between vitrified and fresh control groups.

3.3. Origin of multiple asters (Experiment 3)

Vitrified oocytes were inseminated with a different IVF regimen and multiple asters observed in the presumptive zygotes 5.5 hpi (n=14) and 8 hpi (n=24) were analyzed. Mean number of asters per zygote with multiple asters increased from 3.2 ± 0.3 at 5.5 hpi to 5.3 ± 0.6 at 8 hpi. When distances between the center of each aster and the center of male pronucleus were measured, the mean value among the zygotes increased significantly in a time-dependent manner (9.1 ± 1.1 at 5.5 hpi vs 21.7 ± 2.9 at 8 hpi, $P < 0.05$). Since the 5.5-hpi asters were located near the male pronucleus (Fig. 1C), it was speculated that the multiple asters have not been derived from the cytoplasmic asters of maternal origin.

4. Discussion

In Experiment 1, cleavage rate of vitrified-warmed bovine oocytes 27 hpi was much lower than that of fresh control oocytes, whereas the difference in cleavage rate at 48 hpi was smaller than that at 27 hpi (Table 1). Incidence of delayed cleavage and overall lower cleavage rate in vitrified-warmed oocytes after IVF were also reported in sheep [16]. Higher potential of oocytes cleaving earlier, compared to those cleaving later, to reach blastocysts [24,25], to survive cryopreservation [26] and to achieve pregnancy [24] have been reported in cattle. Although genetic factor [30], bull individuals for sperm [25], chromosomal normality [31], embryonic sex [24,31,32], and culture conditions [33] are involved in the developmental kinetics of embryos, the principle factor that controls the timing of first cleavage remains unclear. Developmental arrest was observed more frequently in vitrified group than fresh control group (Table 1), suggesting that oocyte activation by sperm penetration was suboptimal in the

vitrified-warmed oocytes. Vitrification induced the reduction of maturation promoting factor (MPF) in ovine oocytes before fertilization [34], the damage of mitochondria in bovine oocytes [35], and of endoplasmic reticulum in mouse oocytes [36]. Post-warm oocytes may have been recovered by 2-h culture prior to IVF in the present study, but the harmful effect of remaining intracellular CPA (EG and/or DMSO) could not be estimated.

In Experiment 2, the influence of oocyte vitrification on aster formation which may be deeply involved in the first cleavage [19,20] was investigated. Since the normal fertilization rate was comparable between vitrified and fresh control groups (Table 2), significant difference in the overall cleavage rate (Table 1) was not due to failure of fertilization [37] or polyspermic (abnormal) fertilization [38]. A high incidence of oocytes with multiple asters was notable in the vitrified group (Table 2, Fig, 1B). Except for rodents in which multiple cytoplasmic asters function as MTOC [22,23], each paternal centrosome organizes only a single aster and functions as an MTOC in many mammalian species. Conversely, parthenogenesis [39] and Taxol treatment to stabilize microtubules [19,40] can induce formation of cytoplasmic asters. Navara et al. [41] reported the incidence of multiple asters in bovine oocytes after IVF as done in the present study, but no further analysis of these multiple asters was performed. In the bovine zygotes with multiple asters, pronuclear migration and development were disturbed when compared to those with a single aster (Table 3), suggesting that multiple asters were not equally functional as a single sperm aster. In addition to the vitrification process, prolonged transportation period (approximately 1 d after slaughter) and storage condition (10-12 ºC in saline) of bovine ovaries were potential causes of zygotes with multiple asters.

Experiment 3 was conducted to investigate whether the observed multiple asters were the cytoplasmic asters (maternal origin) or the fragmentation of sperm-aster (paternal origin). Based on the comparison of aster positioning between 5.5 and 8 hpi samples, the multiple asters frequently observed in the vitrified oocytes were considered not to be typical cytoplasmic asters of maternal origin reported in rodents oocytes [22,23], parthenogenetically activated horse oocytes [39], and Taxol-treated porcine [19] and human [40] oocytes. Because sperm cells lose most of the pericentriolar materials during spermiogenesis, sperm-derived centrosome can function as MTOC after recruiting centrosomal proteins dispersed in oocytes [18,21]. Vitrification procedures, including exposure to highly concentrated CPA and ultra-rapid cooling in the cryodevice, may adversely affect recruitment of the centrosomal proteins by the sperm centrosome. If participation of oocyte-derived γ-tubulin into MTOC was not completely organized within a limited period after fertilization, multiple asters may be induced near the male pronucleus and be dispersed gradually. A low glutathione concentration reported in vitrified porcine oocytes [15] may also be responsible for multiple aster formation, since a sperm centrosome needs a reducing agent to function [18]. Otherwise, low-quality oocytes may be simply unable to maintain the single sperm aster. Mean number of asters per zygote with multiple asters increased with culture period, suggesting either the time-dependent increase or the difficult counting of overlapping asters.

In conclusion, the present study was apparently the first to document that vitrification of bovine matured oocytes increased formation of multiple sperm asters after IVF, and that the multiple asters contributed to the migration and development of pronuclei to a lesser extent. Thus, formation of multiple asters may be involved in the

delayed first cleavage of vitrified-warmed bovine oocytes after IVF and impaired

development into blastocysts.

Acknowledgments

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Table 1. Development into blastocysts of in vitro-matured bovine oocytes after vitrification and in vitro fertilization.

| Groups | No. (%) of oocytes | | | | |
| | Inseminated | Cleaved | | Developed to blastocysts | |
		Earlier: 27 hpi	Total: 48 hpi	Earlier: 27 hpi	Total: 48 hpi
Fresh control	163	102 (63 ± 3) [a]	122 (75 ± 2) [a]	61 (38 ± 3) [a]	68 (42 ± 3) [a]
Vitrified	165	39 (24 ± 2) [b]	104 (63 ± 2) [b]	16 (10 ± 3) [b]	26 (16 ± 3) [b]

hpi: h post-insemination.

Percentages were expressed as mean ± SEM of four replicates in each group.

[a,b] Different superscripts denote significant difference between vitrified or fresh control groups ($P < 0.05$).

Table 2. Formation of single or multiple asters in pronuclear-stage bovine zygotes (6 hpi).

Group	No. (%) of oocytes		No. (%) of 2PN zygotes		
	Inseminated	Fertilized :2PN	Formed aster(s)	With single aster	With multiple asters
Fresh control	97	68 (70 ± 4)	64 (95 ± 3)	45 (67 ± 5)[a]	19 (29 ± 4)[a]
Vitrified	86	56 (67 ± 4)	53 (95 ± 2)	16 (28 ± 5)[b]	37 (68 ± 5)[b]

hpi: h post-insemination.

Percentages were expressed as mean ± SEM of seven replicates in each group.

[a,b]Within a column, means without a common superscript differed (P < 0.05).

Table 3. Migration and development of pronuclei in zygotes with a single or multiple asters.

Groups	Aster formation	Distance between pronuclei (μm)	Pronuclear size (μm^2)	
			Male	Female
Fresh control	Single	27 ± 3^a	300 ± 18^a	148 ± 7^a
	Multiple	45 ± 5^b	177 ± 27^b	78 ± 6^b
Vitrified	Single	30 ± 4^a	315 ± 45^a	129 ± 20^a
	Multiple	42 ± 3^b	199 ± 22^b	79 ± 7^b

Number of 2PN zygotes analyzed corresponds to Table 2.

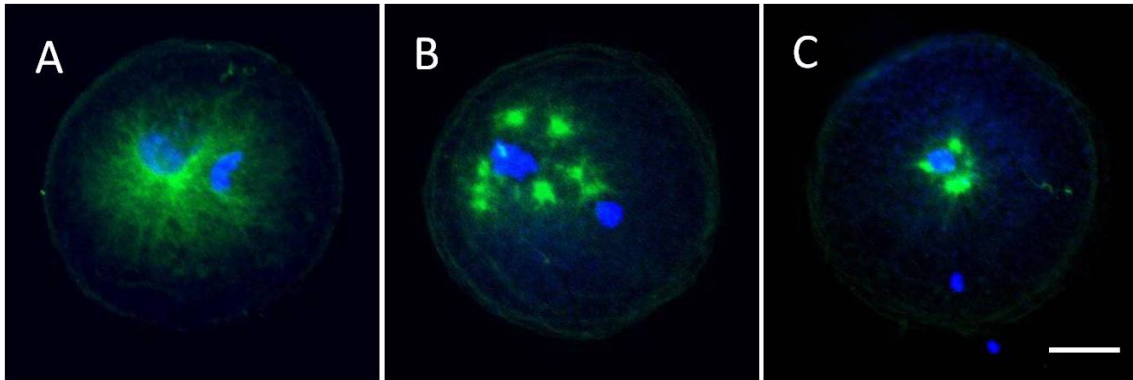[a,b]Within a column, means without a common superscript differed (P < 0.05).

Fig. 1. (A) A bovine oocyte forming a single sperm aster 10 h post-insemination (hpi).
(B) An oocyte forming multiple asters 10 hpi. Note that pronuclear development and
migration are not comparable with those in the panel-A. (C) An oocyte with multiple
asters 5.5 hpi. These asters were located near the male pronucleus. Scale bar = 30 μm.