Effect of Linoleic Acid-Albumin in the IVM and IVF Media on Survival of Frozen-Thawed Pronuclear Bovine Zygotes

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Abstract: *Bovine oocytes were matured and fertilized in a medium supplemented with or without 0.1% linoleic acid-albumin (LAA). The presumptive pronuclear-stage zygotes were frozen-thawed in the medium containing 1.5 M ethylene glycol with or without 0.1 M sucrose. The post-thaw survival of the zygotes was assessed by blastocyst development in vitro. In non-frozen control groups, 66 vs 64% (p=0.757) of the zygotes that were produced in the presence vs absence of LAA cleaved and 25 vs 27% (p=0.883) developed to blastocysts, respectively. After freezing and thawing, similar proportions (53–65%) of the zygotes appeared to be normal regardless of the LAA treatment in the IVM / IVF media (P≥0.775) and sucrose inclusion in the cryoprotective medium (P≥0.177). However, the post-thaw cleavage rate of LAA-treated zygotes (45–54%) was significantly higher (p<0.008) than that of non LAA-treated zygotes (23–29%). The post-thaw development of LAA-treated zygotes into blastocysts (14%) was also higher (p<0.003) than that of non LAA-treated zygotes (2%). Developmental kinetics and cell numbers of the resultant blastocysts were similar between frozen and non-frozen groups. These results suggest that presumptive pronuclear-stage bovine zygotes matured and fertilized in the presence of LAA are relatively tolerant to the process of freezing and thawing, although the post-thaw survival rate needs to be further improved.*

Key words: *Bovine, Ethylene glycol, Linoleic acid-albumin, Pronuclear eggs, Sucrose.*

The one-cell stage zygote, the biggest single cell, is used as a volumetric indicator of cells in hyperosmotic cryoprotective solutions. Practically, cryopreservation of 1-cell stage zygotes is beneficial in the production of transgenic cattle by pronuclear DNA microinjection. In humans, any possible risks of *in vitro* culture can be avoided if the excess numbers of zygotes produced by a routine IVF program are cryopreserved at the 1-cell stage. However, the post-thaw development of 1-cell stage bovine zygotes derived from IVM and IVF has been reported to be very low [1, 2], while the *in vitro* system that has been recently developed for the bovine species is reliable to evaluate survival of the treated zygotes.

In vitro-produced bovine blastocysts were reported to be more sensitive to cryopreservation than *in vivo*-produced counterparts [3]. The efficacy of cryopreservation of *in vitro*-produced bovine embryos appears to be improved by changes in the IVC system. For example, removal of serum from the medium for culturing presumptive bovine zygotes has been shown to improve the resistance of blastocysts to cryopreservation [4, 5]. It has been recently reported that the supplementation of linoleic acid-albumin (LAA) to the medium for culturing bovine zygotes improved the survival of blastocysts after freezing and thawing [6]. We have confirmed that the supplementation of 0.1% LAA to the IVC medium of bovine zygotes is effective to improve the post-thaw survival of morula-stage embryos [7].

The objective of this study was to examine the effect of LAA supplementation in IVM and IVF media on freezing sensitivity of presumptive pronuclear-stage bovine zygotes.

Materials and Methods

IVM/IVF

Oocytes with compact cumulus cells were recovered by slicing and washing of ovary surfaces of slaughtered cows. Approximately 50 oocytes were placed in 500 μl of 25 mM Hepes-buffered TCM199 supplemented with 5% fetal bovine serum (FBS) under mineral oil. The

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medium was supplemented with LAA at a concentration of 0.1%, according to the method of an experiment conducted with IVF-derived bovine morulae [7]. The oocytes were cultured for 24 h at 39.0°C in humidified 5% CO_2 in air.

A single ejaculate of a Japanese Black bull whose spermatozoa had been frozen in an egg yolk-Tris buffer/glycerol solution was used. Frozen-thawed spermatozoa (0.5 ml) were washed twice with BO medium [8] containing 60 μg/ml heparin by centrifugation at 490 g, and the sperm pellet was mixed with the same volume of BO medium containing 60 μg/ml heparin, 0.5% BSA and 0.2% LAA. Then 200 μl of the sperm suspension (5 to 7 $\times$ 10^6 cells/ml) were placed under mineral oil. Twenty to 30 IVM oocytes with volume of cumulus cells reduced by 30 sec Vortex-mixing were transferred to the drop of sperm suspension after washing twice with the BO/0.25% BSA/0.1% LAA/60 μg/ml heparin medium. Nineteen to 20 hours after insemination, presumptive pronuclear zygotes were stripped of cumulus cells by 5 min Vortex-mixing, and were randomly allocated to 6 groups (4 cryopreservation groups and 2 non-frozen control groups). In our standard procedure (no LAA supplementation), 81% of inseminated oocytes could fertilize and 69% of the fertilized oocytes were at the pronuclear stage [9].

Freezing and thawing

The cryoprotective medium consisted of 1.5 M ethylene glycol with or without 0.1 M sucrose in PB1 medium. Zygotes were transferred into 2 ml of the cryoprotective medium at 25°C and then groups of 15 to 25 zygotes were loaded into 0.25-ml plastic straws. Ten minutes after transferring zygotes into the cryoprotective medium, the straws were placed into the alcohol bath of a programmable freezer at –7°C and were seeded 5 min later at that temperature. After an additional 5 min, the straws were cooled to –35°C at the rate of 0.3°C/min before being plunged into liquid nitrogen [5].

After at least 15 min in liquid nitrogen, the straws were warmed rapidly in a 35°C water bath. The contents of the straws were expelled, and the zygotes were immediately transferred into PB1. The zygotes were washed twice in fresh PB1 for 15 min at 39°C.

Survival assay

Post-thaw zygotes as well as non-frozen control zygotes were washed twice with modified synthetic oviduct fluid (mSOF) [10]. The zygotes were cultured in 50 μl of mSOF under mineral oil at 39.0°C in humidified 5% O_2, 5% CO_2 and 90% N_2 (20 to 30 zygotes per microdrop). Forty-eight hours after insemination (28 hours after culture in mSOF), the cleaving status of zygotes was recorded. At 90 hours after insemination, 5.5 μl of FBS were added to each mSOF drop. The number of blastocysts was recorded on Days 6, 7, 8 and 9 (Day 0 being defined as the day of insemination). The cell numbers of some expanding blastocysts were counted after Giemsa staining, as described previously [9].

Statistics

The percentages of morphologically normal zygotes of frozen-thawed zygotes, cleaved zygotes to ≥2-cell stages of cultured zygotes, cleaved zygotes to ≥4-cell stages of total cleaved zygotes, and blastocysts of total cleaved zygotes in each of 4 replicates were subjected to ANOVA after angular transformation. The day of blastocyst appearance and the total cell number of expanding blastocysts were also analyzed with ANOVA. Means were compared by Fisher's least significant difference test.

Results

The results of cryopreservation of presumptive pronuclear bovine zygotes produced in the presence or absence of LAA are shown in Table 1. More than half (53–65%) of the presumptive zygotes appeared to be morphologically normal after freezing and thawing ($p \geq 0.177$), and were cultured for assessing their subsequent development. The post-thaw cleavage rates of LAA-treated zygotes (45–54%) were higher than those of non LAA-treated zygotes (23–29%; $p<0.008$). Furthermore, proportions of zygotes cleaving ≥4-cell stages in the total cleaved zygotes were similar ($p \geq 0.596$) between LAA-treated, frozen groups (without sucrose 46%, with sucrose 56%) and non-frozen group (59%), while the proportions of the ≥4-cell stage zygotes in non LAA-treated, frozen groups (without sucrose 21%, with sucrose 8%) were significantly lower than that (65%; $p<0.001$) in non-frozen group (data not shown). The post-thaw development of LAA-treated zygotes into blastocysts (frozen with sucrose 14%) was also higher ($p<0.003$) than that of non LAA-treated zygotes (2%). In non-frozen groups, 25 and 27% of zygotes produced in the presence and absence of LAA developed to blastocysts, respectively ($p=0.883$).

The quality of the resultant blastocysts was analyzed by the developmental kinetics and the total cell numbers of the resultant blastocysts, as shown in Table 2. The mean day of initial blastocyst appearance was not different among LAA-treated, frozen-thawed group (Day

Table 1. The post-thaw development into blastocysts of presumptive pronuclear bovine zygotes, produced in the presence or absence of linoleic acid-albumin

	LAA (–)				LAA (+)			
	No. (%) of zygotes				No. (%) of zygotes			
Cryoprotectants	Treated	Cultured[1]	Cleaved[2]	Developed[2]	Treated	Cultured[1]	Cleaved[2]	Developed[2]
EG[3]	154	81 (53)[a]	19 (23)[a]	1 (1)[a]	143	82 (57)[a]	37 (45)[b]	4 (5)[a]
EG plus Suc[3]	141	91 (65)[a]	26 (29)[a]	2 (2)[a]	149	93 (62)[a]	50 (54)[bc]	13 (14)[b]
Non-frozen	—	132	84 (64)[c]	35 (27)[b]	—	132	87 (66)[c]	33 (25)[b]

[a–c] Different superscripts within columns denote significant differences ($p<0.05$). [1] Percentages calculated from the numbers of treated zygotes. [2] Percentages calculated from the numbers of cultured zygotes. [3] EG: ethylene glycol, Suc: sucrose.

Table 2. Developmental kinetics of cryopreserved pronuclear bovine zygotes into blastocysts and cell numbers of the resultant expanding blastocysts

Treatments	LAA	Mean day ± SD of blastocyst appearance (n)	Mean cell number ± SD of expanding blastocysts (n)
Frozen in EG plus Suc[1]	+	7.38 ± 0.77 (13)	114.2 ± 61.6 (5)
Non-frozen	+	7.33 ± 0.78 (33)	124.8 ± 59.0 (20)
Non-frozen	–	7.29 ± 0.86 (35)	96.9 ± 37.9 (22)

[1] EG: ethylene glycol, Suc: sucrose.

7.38), LAA-treated, non-frozen group (Day 7.33) and non LAA-treated, non-frozen group (Day 7.29). In addition, mean total cell number of the expanding blastocysts was not different among LAA-treated, frozen-thawed group (114.2 cells), LAA-treated, non-frozen group (124.8 cells) and non LAA-treated, non-frozen group (96.9 cells).

Discussion

The sensitivity of early cleavage- and morula-stage bovine embryos to low temperatures is higher than that of the later blastocyst-stage embryos [5, 11]. Understanding the mechanisms could be the key to extending the developmental stages of bovine embryos that can be successfully cryopreserved. The content of intracytoplasmic lipid droplets has been reported to be a factor influencing the freezing sensitivity of 2- to 4-cell porcine embryos [12] and 1- to 8-cell bovine embryos [1, 13, 14]. Removal of serum from the medium for culturing presumptive bovine zygotes decreased the content of cytoplasmic lipids in the blastocysts, resulting in their improved post-thaw viability [4, 5, 15]. However, the post-thaw survival of the morula-stage embryos produced in a serum-free co-culture system was improved by a lesser extent [5], suggesting the presence of a mechanism other than that caused by intracytoplasmic lipid content. Nevertheless, delipation of [1], or alpha tocopherol microinjection into [16] pronuclear-stage bovine zygotes improved the development to blastocysts after vitrification (9 to 12% of cryopreserved zygotes), but did not after two-step freezing.

The membrane fatty acid composition that affects the fluidity of the membrane lipid bilayer may be involved in the factors influencing the freezing sensitivity [17]. The positive action of LAA on cryopreservation, obtained in the present study, may be explained as follows. It is that linoleic acid carried to the membrane site by the bound albumin is directly incorporated into the lipid bilayer, and thus increases the membrane fluidity in order to facilitate water loss from the zygotes during cooling. The depletion of membrane cholesterol is also related to the increased membrane fluidity. It has been reported that incubation of adenocarcinoma cells with polyunsaturated fatty acids (linoleic acid, linolenic acid, DHA, EPA etc.) resulted in the modification of membrane fatty acid composition [18, 19] and a decrease in the rates of synthesis and esterification of cholesterol [19]. However, the co-culture of presumptive bovine zygotes with liposomes of cholesterol, phospholipids and sphingomyelin did not influence the freezing sensitivity of the resultant blastocysts [20].

The inclusion of a small amount of sucrose in the ethylene glycol-containing cryoprotective medium seemed to be effective at conserving the morphological integrity and promoting subsequent development of the post-thaw zygotes (Table 1). The extracellular disaccharide may play a role for the partial dehydration of the zygotes before cooling, as reported in the rabbit [21].

Nevertheless, the freezing efficacy of pronuclear-stage bovine zygotes needs to be further improved by increasing the morphological survival after thawing, as well as by optimizing the culture system for post-thaw zygotes.

In conclusion, linoleic acid-albumin supplemented to the IVM and IVF media produces the pronuclear-stage bovine zygotes relatively tolerant to the process of freezing and thawing.

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