Effects of Centrifugation and Lipid Removal on the Cryopreservation of *in Vitro* Produced Bovine Embryos at the Eight-Cell Stage

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The effects of intracellular lipid polarization and lipid removal treatments on the postthawed *in vitro* development of frozen bovine embryos at the 8-cell stage were studied. As the first step, bovine presumptive zygotes were centrifuged at 16,000g for 20 min for the cytoplasmic lipid polarization and their lipid layers were removed by micromanipulation in order to examine the influence of these treatments on the developmental capacity of bovine zygotes. As the second step, bovine embryos developed to the 8-cell stage following centrifugation treatment at various forces (8000, 12,000, and 16,000g) or lipid removal treatment at the 1-cell stage were frozen in 1.8 M ethylene glycol + 0.05 M trehalose supplemented with 5% polyvinylpyrrolidone in a one-step procedure. There were no significant differences among the control (nontreatment), lipid-polarized, and lipid-removed groups with respect to the developmental capacity of fresh nonfrozen zygotes (experiment 1). The rates of survival and development to the blastocyst of frozen-thawed 8-cell embryos increased slightly with increasing force of centrifugation (experiment 2). The rate of development into blastocysts of the frozen-thawed 8-cell embryos was significantly higher in the groups that underwent centrifugation (at 16,000g for 20 min; $P <$ 0.05) or lipid removal ($P < 0.01$) treatments than the control (intact) group. However, there were no significant differences among the groups with respect to the rate of development to the expanded/hatched blastocyst stage. In addition, the mean cell numbers of embryos developed into blastocysts (day 8) derived from frozen-thawed 8-cell embryos tended to be low in the centrifugation and lipid removal groups compared to the controls (experiment 3). These results suggest that although the centrifugation with or without lipid removal treatments has no detrimental effects on the developmental capacity of bovine zygotes, the freezing tolerance of bovine 8-cell embryos was not improved by these treatments.   © 1998 Academic Press

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The effective cryopreservation of *in vitro* produced (IVP) bovine embryos at the early cleavage stage would not only provide a greater availability of materials for basic research and an improved manageability of their *in vitro* culture and subsequent embryo transfer, but would also broaden the range of practical applications of genetic engineering, such as the preservation of donor blastomeres for the purpose of cloning by nuclei transfer (16). It would also allow the preservation of embryos for production of chimeric calves by aggregation of the 8-cell stage embryos (1). Moreover, in regard to the currently available cryopreservation methods, freezing of the embryos might be much simpler in the cleavage stage rather than in earlier stages

such as immature and mature oocytes, which have shown relatively low survival rates after freezing and thawing (11, 12, 14). However, it has been suggested that IVP bovine embryos have a higher sensitivity to low temperature compared with *in vivo* derived embryos (7). Bovine embryos have been shown to contain a large number of lipid droplets at the early cleavage stages, and the decline of lipid droplets at the blastocyst stage is coincident with a loss in sensitivity to cooling (8, 15). Leibo *et al.* (7) suggested that intracellular lipids were at least partially responsible for the increased chilling and freezing sensitivity exhibited by IVP bovine embryos at the compact morula stage.

It has also been demonstrated that the tolerance of porcine embryos at the early cleavage stage to cryopreservation can be highly increased after decreasing their relatively high

content and/or high composition of cytoplasmic lipid droplets by aspirating the lipids with a micropipette; the embryos continued to develop to blastocysts *in vitro* and to live offspring *in vivo* (10). However, this procedure harbors some operational problems for routine application, such as its complexity, laboriousness, and requirement of micromanipulation skills. Otoi *et al.* (12) recently suggested that polarization of cytoplasmic lipid droplets by centrifugation may improve the freezing tolerance of the spindles or organelles in mature bovine oocytes. We therefore suspected that, by centrifuging the bovine zygotes at higher speed and for longer times compared with other conventional levels (4, 9, 10, 16), the redistribution throughout the cytoplasm of the polarized lipid layer might be delayed and consequently the freezing tolerance of bovine embryos at the early cleavage stage could be enhanced by this treatment.

To test this hypothesis, the intracellular lipids of bovine zygotes were polarized by centrifugation at various speeds, and the sensitivity of the embryos at the 8-cell stage to freezing was then assessed. In addition, the effect of a lipid removal treatment on the cryopreservation of bovine 8-cell embryos was investigated.

MATERIALS AND METHODS

Zygote Preparation

The methods used for the *in vitro* maturation, *in vitro* fertilization (IVF), and subsequent culture in the experiments were modifications of the procedure described by Boediono *et al.* (2).

Ovaries were collected from cows at a local abattoir and were brought to the laboratory in physiological saline [0.9% (w/v) NaCl] at 25 to 30°C within 3 h. The cumulus–oocyte complexes (COCs) in follicular fluid (5 to 10 per ovary) were aspirated from follicles (2–7 mm in diameter) with a 5-ml syringe fitted with an 18-gauge needle. After being washed twice with modified phosphate-buffered saline (Embryoteck, Nihonzenyaku, Fukushima, Japan), the COCs were cultured for 21 to 23 h in maturation medium [25 mM Hepes TCM-199 with Eagle's salts (Gibco, Grand Island, NY) supplemented

with 5% superovulated cow serum (SCS), 0.01 mg/ml follicle-stimulating hormone (Denka Pharmaceutical Co., Kawasaki, Japan), 20 mM taurine (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 50 μg/ml gentamicin (Sigma Chemicals, St. Louis, MO)] at 38.5°C under 5% CO_2 in air.

Frozen semen was thawed in a water bath (37°C) and washed with 5 mM caffeine (Sigma) in Brackett and Oliphant (BO) medium (3) by centrifugation at 500g for 5 min. The resultant sperm pellet was resuspended in BO medium supplemented with 2.5 mM caffeine, 3 mg/ml bovine serum albumin (BSA, fraction V; Sigma), 20 mM taurine, and 20 mg/ml heparin (Shimizu Pharmaceutical Co., Ltd., Shimizu, Japan) to a final concentration of 5×10^6/ml. A 100-μl aliquot of the sperm suspension was covered with mineral oil (Sigma). COCs that had been cultured for maturation *in vitro* were transferred into sperm microdrops (10–15 oocytes per microdrop) for insemination. After incubation for 5 h, the COCs were transferred and washed several times in culture medium [TCM-199 supplemented with 5% SCS, 5 μg/ml insulin (Wako), 20 mM taurine, and 50 μg/ml gentamicin] and then cultured for an additional 13 h.

Lipid Removal from Zygotes

After IVF and culture, the zygotes were incubated with 300 IU/ml hyaluronidase (Sigma) in culture medium for 20 min, and their adherent cumulus cells were then removed by pipetting. After being rinsed in fresh medium, the denuded zygotes were then transferred into 1.5-ml test tubes (Sarstedt, Germany) containing 500 μl of the culture medium with 7.5 μg/ml cytochalasin D (Sigma). After being cultured for 10 min, the tubes were centrifuged at 16,000g for 20 min at 38.5°C by a high-speed refrigerated centrifuge (MR-150; Tomy Seiko, Tokyo, Japan) in order to polarize the intracellular lipid droplets. In some zygotes, an estimated 90% of the resultant lipid layer was then aspirated using a beveled suction pipette (30 μm in diameter) attached to a micromanipulator (Leitz, Germany) in Dulbecco's PBS without

Ca^{2+} or Mg^{2+} [DPBS(−); Gibco] supplemented with 5% SCS, 5 μg/ml insulin, 20 mM taurine, and 50 μg/ml gentamicin.

Freezing and Thawing

The solution applied as a carrier for the cryoprotectant was DPBS (Gibco) supplemented with 3 mg/ml BSA. After 2 days of IVF (onset of IVF, day 0), embryos at the 8-cell stage were selected and then immersed directly into 1.8 M ethylene glycol (EG; Wako) containing 0.05 M trehalose (T; Wako) and 5% polyvinylpyrrolidone (PVP; Denka) by a one-step method for 5 min at room temperature (25°C). Following this exposure, approximately 0.1 ml of the embryo-cryoprotectant solution was loaded into a 0.25-ml plastic straw (FHK, Tokyo, Japan). The straws (10 to 30 embryos per straw) were placed horizontally in the ethanol bath of a programmable freezer (ET-1; FHK) kept at 0°C and cooled to −7°C at a rate of 1°C/min. They were seeded at −7°C, held for 10 min, cooled at a rate of 0.3°C/min to −30°C, and then plunged into liquid nitrogen (LN$_2$). After being maintained at −196°C for 1 to 2 weeks, the cryopreserved straws were placed in air for 5 s and then plunged into a 37°C water bath for 10 s. The contents were subsequently transferred directly to 2.5 ml of the culture medium and washed three times to dilute the cryoprotectant.

Survival Evaluation

Frozen-thawed embryos were evaluated morphologically following incubation of the embryos on cumulus layer in the culture medium for 24 h (on day 3). The number of embryos that had normal-shaped blastomeres and the number that had cleaved to more advanced stages were recorded as the criteria of survival. The embryos that had irregularly shaped and shrunken blastomeres with dark yellow cytoplasm were considered to be degenerated. These embryos were cultured for an additional 6 days (until day 9) to examine their ability to develop into blastocysts.

Experiment 1

As the first step, the influence of centrifugation treatment and subsequent lipid removal treatment on the developmental capacity of bovine zygotes was examined. The zygotes were centrifuged at 16,000g for 20 min, and then the lipid layer within the cytoplasm was removed from some of the centrifuged zygotes as described above. These treated zygotes were subsequently cultured with cumulus cells in the culture medium. The frequencies of cleavage and development to blastocysts of the zygotes in these treated and control (intact) groups were evaluated on days 2 and 7 to 9, respectively.

Experiment 2

The influence of the force of centrifugation on the developmental potential of frozen-thawed embryos which developed to the 8-cell stage following centrifugation treatment at the one-cell stage was examined. Zygotes were centrifuged at 8000, 12,000, or 16,000g for 20 min and subsequently cultured with cumulus cells in the culture medium. After incubation, the embryos that developed to the 8-cell stage were selected on day 2 and then frozen with 1.8 M EG containing 0.05 M T and 5% PVP by the one-step method. The frozen embryos in each treatment were thawed and then cultured with cumulus cells. The survival and development to blastocysts of the frozen-thawed 8-cell embryos in these treated and control (intact) groups were evaluated on days 3 and 7 to 9, respectively.

Experiment 3

The effect of lipid removal on the freezing tolerance of bovine 8-cell embryos was examined. Since we found that the centrifugation treatment at 16,000g was more effective for the developmental capacity of frozen-thawed embryos in the second experiment, zygotes were centrifuged at 16000g for 20 min. In some of the centrifuged zygotes, lipids from the cytoplasm were removed as described above. The embryos that developed to the 8-cell stage following each treatment were selected on day 2 and then frozen with 1.8 M EG containing 0.05 M T and 5% PVP by the one-step method. The survival and development to blastocysts of the frozen-thawed 8-cell embryos in these treated

TABLE 1
Developmental Capacity of Bovine Zygotes after Various Treatments

Treatments	No. of zygotes examined	No. of zygotes cleaved (%)	No. of zygotes developed to (%)	
			Blastocyst	E./H. blastocyst[a]
Control[b]	138	105 (76.1)	58 (42.8)	53 (38.4)
Centrifugation	146	112 (76.7)	51 (34.9)	44 (30.1)
Lipid removal	122	103 (84.4)	50 (41.0)	39 (32.0)

Note. Three experiments were performed in each group.

[a] E./H. blastocyst, expanded/hatched blastocyst.

[b] Control, zygotes that were simply cultured and are not treated in any way.

and control (intact) groups were evaluated on days 3 and 7 to 9, respectively.

In experiments 2 and 3, *in vitro* developmental capacity into blastocysts of the fresh intact 8-cell embryos was also evaluated concurrently.

Count of Cell Numbers

As a supplement to the third experiment, 10 to 20 frozen-thawed embryos which developed into blastocysts on day 8 were collected randomly from each treatment group, treated with 0.25% pronase (Sigma) in culture medium for 1–2 min to dissolve the zona pellucida, rinsed in fresh medium, and then incubated in DPBS containing bisbenzimide (Hoechst 33342, 10 μg/ml; Sigma). After 20 min of incubation, the embryos were pressed onto a glass slide and their cell nuclei were then counted under a fluorescent microscope (Optiphot, Nikon, Tokyo, Japan).

Statistical Analysis

The treatment effects were compared by χ^2 analysis and the Fisher exact probability test. Duncan's multiple range test was used to compare the mean cell numbers of the blastocysts. Differences at a probability value (P) of 0.05 or less were considered significant.

RESULTS

Experiment 1

As shown in Table 1, there was no significant difference among the treatment groups with respect to the rates of cleavage and development to both the blastocyst and the expanded/hatched blastocyst stages of the zygotes.

Experiment 2

As shown in Table 2, there were no significant differences among the treatment groups

TABLE 2
Developmental Capacity of Frozen-Thawed Bovine Embryos Following Centrifugation at Different Forces

Centrifugation (g)	No. of embryos examined	No. of embryos survived (%)	No. of embryos developed to (%)	
			Blastocyst	E./H. blastocyst[a]
Control[b]	78	53 (67.9)	19 (24.4)	13 (16.7)
8,000	84	63 (75.0)	20 (23.8)	13 (15.5)
12,000	70	54 (77.1)	21 (30.0)	11 (15.7)
16,000	78	61 (78.1)	24 (30.8)	11 (14.1)

Note. Three experiments were performed in each group.

[a] E./H. blastocyst, expanded/hatched blastocyst.

[b] Control, 8-cell embryos that are just cryopreserved (no centrifugation treatment).

TABLE 3

Developmental Capacity of Frozen-Thawed Bovine Embryos Following Various Treatments

Treatment	No. of embryos examined	No. of embryos survived (%)	No. of embryos developed to (%)		Mean cell number of blastocysts (±SD)
			Blastocyst	E./H. blastocyst[a]	
Control[b]	66	42 (63.6)	16 (24.2)[c]	10 (15.2)	87.4 ± 18.6^A
Centrifugation	82	60 (73.2)	34 (41.5)[de]	12 (14.6)	60.5 ± 30.0^B
Lipid removal	67	53 (79.1)	32 (47.8)[e]	12 (17.9)	66.3 ± 23.2^{AB}

Note. Values with different superscripts within a column are significantly different [c, d ($P < 0.05$); c, e ($P < 0.01$); A, B ($P < 0.05$)]. Five experiments were performed in each group.

[a] E./H. blastocyst, expanded/hatched blastocyst.

[b] Control, 8-cell embryos cryopreserved without any other treatment (neither centrifugation nor lipid removal).

with respect to the rates of survival and development to both the blastocyst and the expanded/hatched blastocyst stages of the frozen-thawed 8-cell embryos. However, in the centrifugation groups, the rates of survival and development to the blastocyst tended to increase with increasing force of centrifugation, but the rates were not significantly different among the groups.

Experiment 3

As shown in Table 3, there was no significant difference among the treatment groups with respect to the survival rate of the frozen-thawed 8-cell embryos. However, the rates of development to the blastocyst stage of the centrifugation ($P < 0.05$) and lipid removal ($P < 0.01$) groups were significantly higher than those of the control group. Nevertheless, there were no significant differences among the groups with respect to the rate of development to the expanded/hatched blastocyst stage. The mean cell number of frozen-thawed embryos which developed into blastocysts on day 8 was significantly lower ($P < 0.05$) in the centrifugation group and also tended to be lower in the lipid removal group, compared to the control.

In experiments 2 and 3, 62 of 97 fresh intact 8-cell embryos (63.9%) developed to the expanded/hatched blastocyst stage.

DISCUSSION

Several investigators have recently demonstrated that the removal of the intracellular lipid droplets prior to cryopreservation improves the survival of both porcine (10) and IVP bovine embryos (16) at the early cleavage stage and even that of IVP bovine blastocysts (4) at significant levels. However, polarization alone or the partial removal of the lipids from porcine embryos have proven to be inferior to full lipid removal in terms of the chilling tolerance (9). It was also demonstrated that centrifugation treatment prior to cryopreservation did not improve the frequency of development to blastocysts of both frozen-thawed bovine oocytes (12) and embryos at the early cleavage stage (16). It has been suggested, in porcine embryos, that the high concentration and variable distribution of intracellular lipids in the blastomeres may induce heterogeneous intracellular ice nucleation or lipid-phase changes that contribute to the high sensitivity of the embryos to cryopreservation (5). Further, preimplantation mouse embryos, which have few lipid droplets, can be successfully frozen at all stages of development (8, 17). Therefore, although initially high-speed centrifugation for a long period at the zygote stage was attempted to reduce the freezing sensitivity of IVP bovine embryos at the 8-cell stage, the lipid removal by micromanipulation was considered to be indispensable to obtain sufficient freezing tolerance of these embryos.

In the present study, although an obvious difference was not observed among the embryos centrifuged at various speeds in regard to the distribution of the polarized lipids to the blastomeres (data not shown), there was a little

evidence to suggest that the polarization of lipids in the cytoplasm of zygotes by higher centrifugal force may improve the freezing tolerance of bovine 8-cell embryos developed after the centrifugation treatment of the zygotes (Table 2). The developmental capacity into blastocysts of the frozen-thawed 8-cell embryos was improved by the treatments of centrifugation (at $16,000g$ for 20 min) or subsequent lipid removal at the 1-cell stage. However, with respect to the developmental potential to the expanded/hatched blastocyst stage, no difference was found among these treated and intact 8-cell embryos after freezing and thawing (Table 3). It was observed in this study that the frequencies of frozen-thawed embryos forming blastoceles and containing some degenerated blastomeres were relatively higher in the centrifugation and lipid removal groups (data not shown). Such a type of embryo was also regarded as being at the blastocyst stage. It was also found that the mean cell number of frozen-thawed embryos that developed into blastocysts (day 8) tended to be low in both the centrifugation and the lipid-removed groups compared to the control (Table 3). Hence, it appeared that these post-thawed embryos were unlikely to expand or to hatch because of their low cell numbers. The two limitations cited above seem to reflect certain restrictions of the developmental capacity of both treatment groups despite their high development rates into blastocysts compared to the controls after freezing-thawing. Since both the lipid-polarized and the lipid-removed zygotes maintained their normal *in vitro* developmental ability after such treatments (Table 1), the degeneration of the blastomeres and the resultant restriction of their further development *in vitro* are thought to be attributable to their injury during freezing and thawing.

Leibo and Oda (6) reported that when PVP was combined with a relatively low concentration of EG, it enhanced the cryoprotective properties of EG solutions, yielding high survival rates of mouse zygotes and embryos frozen either slowly or rapidly. Although the mechanism of the large polymer PVP (molecular weight, average 30,000) is not clear, Suzuki *et*

al. (13), using biopsied bovine blastocysts, suggested that PVP might play a beneficial role by forming a protective coating around the membrane of the cells during freezing and thawing. In the present study, since small incisions were made at the zona pellucida of the embryos during the lipid aspiration, a cryoinjury of the embryos with the lipid removal treatment (as in the zona-free embryos during freezing and thawing) is expected. Moreover, Suzuki *et al.* (14) demonstrated that 1.8 M EG plus 0.05 M T supplemented with 5% PVP had the most beneficial effect for the slow freezing and thawing of bovine germinal vesicle oocytes. Therefore, this solution was employed for the cryopreservation of bovine 8-cell embryos using a direct exposure and rehydration process as previously described. As a result, even in the control intact 8-cell embryos, a relatively high development rate to expanded/hatched blastocyst stage was observed after freezing-thawing [16.0% (23/144); Tables 2 and 3], but this rate was still significantly lower ($P < 0.01$) than that of the fresh intact 8-cell embryos [63.9% (62/97)].

In conclusion, the present results indicate that centrifugation with or without lipid removal of zygotes has little or no effects on the ability of the bovine 8-cell embryos to develop *in vitro* following cryopreservation, but the quality of the embryos is lower. In the future, the optimum freezing and thawing procedure including cryoprotectants must be developed for the efficient cryopreservation of bovine early stage embryos.

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