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Analysis of Fertilizability of Bovine Oocytes Cryopreserved in Various Cryoprotectants after In-Vitro Maturation and their Chromosomes at the First Cleavage Division

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Abstract: *The purpose of the present experiment was to determine the fertilizability of bovine oocytes frozen in various cryoprotectants: 1.6 M 1,2-propanediol (PROH)+0.2 M sucrose, 1.6 M dimethylsulfoxide (DMSO)+0.2 M sucrose, 1.6 M glycerol (GL)+0.2 M sucrose, 0.8 M PROH+0.8 M DMSO, and 0.8 M PROH+0.8 M GL. The incidence of morphologically normal oocytes was significantly higher in the 0.8 M PROH+0.8 M DMSO group (56.0%) than in other groups (P<0.05). At 48 h after insemination, the number of eggs that cleaved into the 2-cell stage, in both the 0.8 M PROH+0.8 M DMSO and 1.6 M PROH+0.2 M sucrose groups, was significantly greater than in other groups (P<0.05). By analyzing the chromosomes of first-cleavage eggs, which were fertilized* in vitro *after freezing and thawing, it was found that the incidences of polyploids were higher in all of the frozen-thawed groups than in the control group, but among them there was no significant difference. The frequency of eggs with chromosomes having structural aberrations did not increase in any of the treated groups.*

Key words: *Bovine oocyte, Cryoprotectant, Fertilizability, Chromosome analysis, First cleavage.*

Cryopreservation of mammalian oocytes is one of the most important techniques for gene preservation. With this technique and the *in-vitro* fertilization technique, individuals can be produced from cryopreserved oocytes. After Whittingham [1] reported that mouse oocytes can be successfully cryopreserved and developed until they are live-born young, via the *in-vitro* fertilization and sequent embryo-transfer, many researchers have attempted to freeze mammalian oocytes. Lim *et al.* [2] have demonstrated that some bovine oocytes frozen at the mature stage with glycerol could develop into the blastocyst stage after thawing and insemination. Otoi *et al.* [3] have also reported pregnancies of bovine embryos, which were derived from oocytes frozen with 1.6 M PROH.

Nevertheless, the fertilization rates [3–6] and the developmental capacities [2, 3, 5, 7, 8] in frozen-thawed mammalian oocytes were significantly lower than in those of untreated oocytes. Carroll *et al.* [9] have proposed that the decreased fertilization rate in frozen-thawed mouse oocytes may be caused by some changes in the structure of the zona and/or vitellus although the treatments of freezing and thawing do not impair the mechanism of the block to polyspermy. On the other hand, Glenister *et al.* [4] have reported an increase in polyploids during the first-cleavage division in mouse embryos derived from oocytes frozen-thawed and fertilized *in vitro*. The increase in polyploids may be derived from digyny in the oocytes [9].

Shaw *et al.* [10] have reported that chromosomal aberrations were produced in 2-cell mouse embryos frozen in solution containing 1.5 M or 3.0 M DMSO. Johnson and Pickering [11] have also demonstrated that prolonged exposure of mouse oocytes to DMSO leads to disassembly of the spindle and dispersion of the chromosomes, but to the present there have been no reports concerning chromosomal aberrations in cryopreserved bovine oocytes.

In the present study, we examined the fertilizability and developmental capacity of *in-vitro* matured bovine oocytes frozen with various cryoprotectants. Furthermore, we analyzed chromosomal abnormalities during the first cleavage division in the bovine embryos derived from the frozen-thawed oocytes.

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Materials and Methods

In vitro maturation of oocytes

Within a 2 h period, bovine ovaries were transported from the Utsunomiya city local slaughterhouse to our laboratory, in 0.85% saline containing antibiotics (0.1 mg/ml streptomycin and 100 I.U/ml penicillin), at temperatures of 30 to 35°C. Immature oocytes were aspirated from 2 mm to 8 mm follicles with 18-G needles attached to 5-ml syringes, with Dulbecco's modified phosphate buffer saline (D-PBS) solution containing 0.3% bovine serum albumin (BSA). After washing twice with maturation medium (25 mM Hepes TCM 199 with Earle's salts) supplemented with 5% calf serum (CS) and 0.1 μl/ml antibiotics (0.1 mg/ml streptomycin and 100 I.U/ml penicillin), the cumulus-oocyte complexes were introduced into the maturation medium. They were cultured for 20–22 h in 2% CO_2 and an air at 38.5°C by the method of Kajihara *et al.* [12].

Freezing and thawing

After incubation for 20–22 h, the oocytes were washed twice in D-PBS supplemented with 0.3% BSA. Then 1 or 2 layers of the cumulus cells surrounding each oocyte were left by the pipetting. D-PBS solution, enriched with 10% fetal bovine serum, was used as a carrier of the cryoprotectants. Cryopreservation was done according to the method of Otoi *et al.* [5], but the composition of cryoprotectants and the seeding temperature were modified by the authors. At room temperature, the oocytes were treated in 3 steps at 10 min intervals, as Fig. 1 shows, and then they were placed in various final solutions containing 1.6 M PROH+0.2 M sucrose, 1.6 M DMSO+0.2 M sucrose, 1.6 M GL+0.2 M sucrose, 0.8 M PROH+0.8 M DMSO or 0.8 M PROH+0.8 M GL. After 10 min of equilibration at room temperature in the final solution, the oocytes were loaded into plastic straws. The straws were then placed directly into a cooling chamber of a programmed freezer (Fujihira Co. ET-1) of 0°C, and cooled from 0°C to −7°C at the rate of −1°C/min. They were seeded at −7°C, cooled to −30°C at the rate of −0.5°C/min, plunged into liquid nitrogen and stored for 2–4 weeks. The straws were then removed from the liquid nitrogen, exposed to air for 5 sec, and thawed in water at 37°C. After thawing, the oocytes were put into PBS solution (containing 0.5 M sucrose) for 15 min, to eliminate the cryoprotectants. After washing in D-PBS supplemented with 0.3% BSA, the oocytes were washed twice in TCM-199 supplemented with 5% CS. They were then cultured in the maturation medium for 1 h, in 2% CO_2 and air at 38.5°C.

In-vitro fertilization and chromosome preparation

The *in vitro* fertilization was basically performed according to Hanada's method [13]. Frozen-thawed bovine semen from a Japanese black bull was suspended in BSA-free BO [14] solution containing 10 mM caffeine. The spermatozoa were washed twice by centrifugation at 485 × g for 5 min, and resuspended in BSA-free BO solution. The sperm suspension was then diluted to the ratio of 1:1, with BO solution containing 2.5 mg/ml BSA and 1.7 U/ml heparin, by the method of Niwa and Ohgoda [15]. The frozen-thawed oocytes were trans-

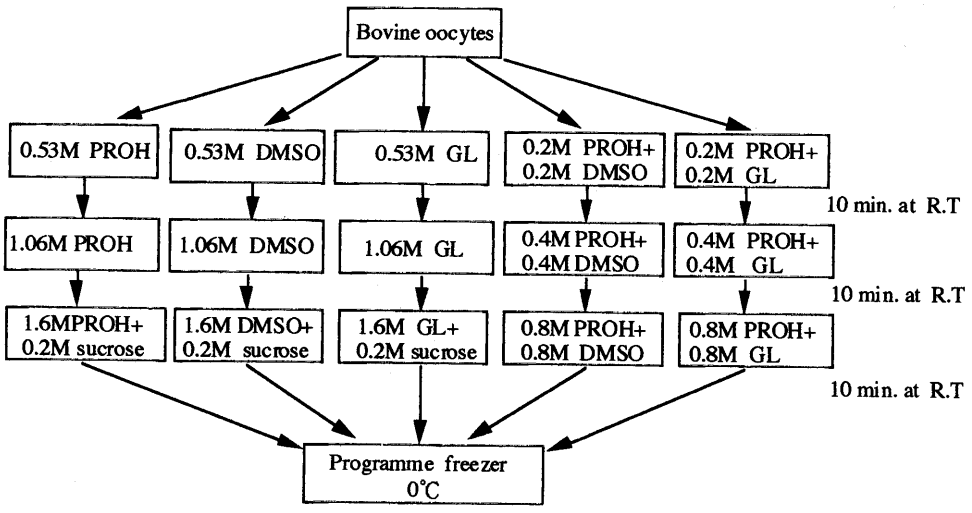

Fig. 1. Procedure of freezing bovine oocytes by 3-step treatments with various cryoprotectants.

ferred into the sperm suspension. The oocytes were then incubated with spermatozoa for 5 h, washed twice in TCM-199 medium, and incubated in TCM-199 medium for 7–9 days. At 20 h after insemination, some eggs were placed in a medium containing 100 ng/ml vinblastine for 8 h, according to our previous report [16]. Chromosome preparations of the 1-cell embryos at the metaphase stage were made by the method of Yoshizawa *et al.* [17]. For comparison of *in vitro* fertilization and development conditions, fresh oocytes were matured and fertilized *in vitro* as a control group.

The data obtained were analyzed by the Fisher's test.

Results

All of the oocytes of normal shape were frozen with various cryoprotectants. The oocytes which had normal round cytoplasm after thawing were defined as morphologically normal. Irregular shaped oocytes or light colored cytoplasm oocytes were considered to be abnormal. The numbers of morphologically normal bovine oocytes frozen-thawed with various cryoprotectants are shown in Table 1. The incidence of normal oocytes in the 0.8 M PROH+0.8 M DMSO group was significantly higher (56.0%) than those of other groups (35.5–46.5%, $P<0.05$). The 1.6 M PROH+0.2 M sucrose group also had a higher incidence of morphologically normal oocytes than in the 1.6 M DMSO+0.2 M sucrose, 1.6 M GL+0.2 M sucrose and 0.8 M PROH+ 0.8 M GL ($P<0.05$) groups.

Table 2 shows the developmental ability of oocytes inseminated after freezing and thawing. At 48 h after insemination, the percentages of eggs divided into 2 cells in the 0.8 M PROH+0.8 M DMSO, and in the 1.6 M PROH+0.2 M sucrose groups were significantly higher than those in other treated groups ($P<0.05$), although they were significantly lower than that in the control group, but between the 0.8 M PROH+0.8 M DMSO and 1.6 M PROH+0.2 M sucrose groups there was no significant difference in the percentage of eggs divided into 2 cells. Furthermore, only a few embryos in these groups developed to the blastocyst stage, compared to the control group.

Table 3 shows the results of chromosomal analysis in first-cleavage eggs fertilized *in vitro* after freezing and thawing. Eggs with two or more pronuclei, or two or more metaphase groups were judged as the fertilized eggs. The fertilization rate was higher in the 0.8 M PROH+0.8 M DMSO (63.0%) group than the other treated groups, and it was similar to that in the control group. The proportion of eggs that remained at the pronuclear stage was significantly ($P<0.05$) higher in the 1.6 M DMSO+0.2 M sucrose group and 1.6 M PROH+0.2 M sucrose group, than in the 0.8 M DMSO+0.8 M PROH and control groups. The rates of eggs with mitotic metaphase figures in the total of fertilized eggs ranged from 61.9 to 85.3% in the treated groups. The rate in the 1.6 M DMSO+0.2 M sucrose group was significantly lower than those in the control and 0.8 M PROH+0.8 M DMSO groups. A significantly higher proportion of fertilized eggs with normal diploid chromosomes ($P<0.05$) was observed in the 0.8 M

Table 1. Numbers of morphologically normal bovine oocytes after freezing and thawing

Cryoprotectants	No. of oocytes		
	Frozen	Recovered (%)	Normal (%)
1.6 M PROH + 0.2 M sucrose	512	497 (97.1)	231 (46.5)[a]
1.6 M DMSO + 0.2 M sucrose	472	464 (98.3)	178 (38.4)[b]
1.6 M GL + 0.2 M sucrose	284	272 (95.8)	105 (38.6)[b]
0.8 M PROH + 0.8 M DMSO	422	411 (97.4)	230 (56.0)[c]
0.8 M PROH + 0.8 M GL	179	170 (95.0)	60 (35.3)[b]

Values with different superscripts show significant differences ($P<0.05$).

Table 2. Results of *in-vitro* culture of bovine oocytes fertilized *in vitro* after freezing with various cryoprotectants

Cryoprotectants	No. of eggs cultured	No. of embryos developed into	
		≥2 cells (%)	Blastocyst (%)
1.6 M PROH + 0.2 M sucrose	231	63 (27.4)[a]	2 (3.2)[a]
1.6 M DMSO + 0.2 M sucrose	159	23 (14.5)[b]	0
1.6 M GL + 0.2 M sucrose	105	20 (19.0)[b]	0
0.8 M PROH + 0.8 M DMSO	227	74 (32.6)[a]	3 (4.1)[a]
0.8 M PROH + 0.8 M GL	58	6 (10.3)[b]	0
Control	198	104 (52.5)[c]	18(17.3)[b]

Values with different superscripts show significant differences ($P<0.05$).

Table 3. Incidences of mitosis and chromosomal aberrations in first-cleavage bovine eggs fertilized *in vitro* after freezing and thawing

	Control	1.6 M PROH + 0.2 M sucrose (%)	1.6 M DMSO + 0.2 M sucrose (%)	0.8 M PROH + 0.8 M DMSO (%)
No. of eggs				
Analyzed	56	72	43	54
Fertilized	36 (64.3)	39 (54.2)	21 (48.8)	34 (63.0)
At pronuclear stage	4 (11.1)a	11 (28.2)^b	8 (38.1)^b	5 (14.7)^a
In mitosis	32 (88.9)^a	28 (71.8)	13 (61.9)^b	29 (85.3)^a
2n*	30 (93.8)^a	22 (78.6)	10 (76.9)^b	24 (82.8)^a
3n*	2 (6.3)	4 (14.3)	2 (15.4)	4 (13.8)
4n*	0	2 (7.1)	1 (7.7)	1 (3.4)
Unfertilized	20 (35.7)	33 (45.8)	22 (51.2)	20 (37.0)
At pronuclear stage	10 (50.0)	15 (45.5)	9 (40.9)	9 (45.0)
In mitosis**	10 (50.0)	18 (54.5)	13 (59.1)	11 (55.0)
Structural aberration	0	1 (1.4)	0	0

*2n: normal diploid, 3n: triploid, 4n: tetraploid, **haploid. Values with different superscripts show significant differences ($P<0.05$).

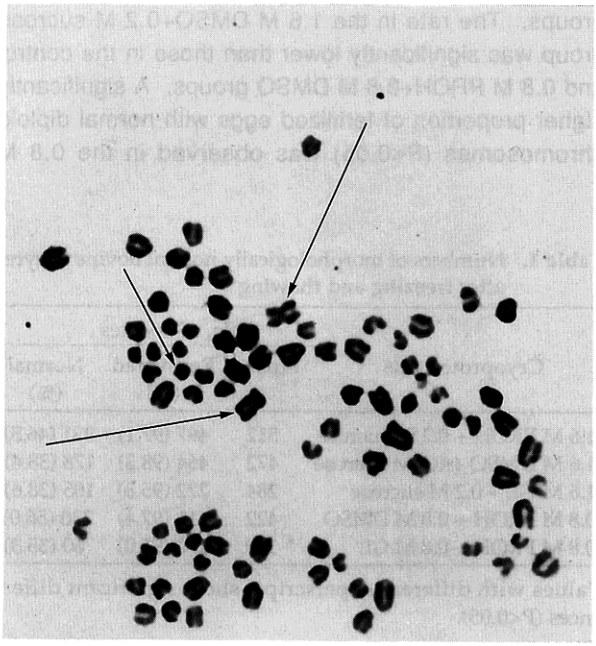

Fig. 2. A triploid metaphase plate (n=90, XXY) at the first cleavage division of a bovine egg fertilized *in vitro* after freezing and thawing (Long arrow: X chromosome, short arrow: Y chromosome).

PROH+0.8 M DMSO group (82.8%) than in the 1.6 M DMSO+0.2 M sucrose group. Many haploids were observed in the 1.6 M DMSO+0.2 M sucrose group, the 0.8 M PROH+0.8 M DMSO group, the 1.6 M PROH+0.2 M sucrose group and the control group. The incidence of polyploids also was high in all of the treated groups, but there was no significant difference between the control group and each treated group, or among the treated groups. A metaphase figure of a triploid embryo derived from a frozen-thawed oocyte fertilized *in vitro* is shown in Fig. 2. A haploid egg with a chromosome having a structural aberration (chromatid delation) was found in the 1.6 M PROH+0.2 M sucrose group.

Discussion

Otoi *et al.* [5] have reported that PROH was superior to GL and DMSO in producing a higher proportion of morphologically normal bovine oocytes, after the thawing of frozen oocytes. The present study showed that the proportion of morphologically normal oocytes treated in the mixture of 0.8 M PROH and 0.8 M DMSO (56.0%) was significantly higher than those in other treated groups and the result with PROH reported by Otoi *et al.* [5]. The proportions of morphologically normal oocytes in the 1.6 M DMSO+0.2 M sucrose and 1.6 M GL+0.2 M sucrose groups (38.4% and 38.6%, respectively), were higher than the values in GL (28.6%), and DMSO (32.6%), reported by Otoi *et al.* [5]. On the other hand, the survival rate after thawing was significantly higher in mouse morulae treated with vitrification solution supplemented with sucrose than in those treated with sucrose-free vitrification solution. Sucrose may reduce therefore toxicity associated with Ficoll in vitrification solution [18]. These results suggest that the toxicity of DMSO may be decreased by mixing it with PROH, or by supplementing it with some sucrose.

Hernandez-Ledezma and Wright [19] have also re-

ported that PROH was better than GL, DMSO, or a PROH+GL combination for cryopreservation of mouse oocytes in regard to the rate of development of zygotes to the 2-cell stage. The proportions of oocytes cleaved beyond the 2-cell stage 48 h after insemination in the 1.6 M PROH+0.2 M sucrose and 0.8 M PROH+0.8 M DMSO groups (27.4% and 32.6%) were significantly higher than those in the other treated groups. On the other hand, the result in the 1.6 M GL+0.2 M sucrose group (19.0%) was similar to that reported with 1.6 M GL (20.6%) by Otoi *et al.* [5], but the fertilization rate in the 1.6 M DMSO+0.2 M sucrose group (14.5%) was lower than that in 1.6 M DMSO (18.3%) reported by Otoi *et al.* [5]. Carroll *et al.* [9] have proposed that the decreased fertilization rate in frozen-thawed mouse oocytes may be caused by some changes in the structure of the zona and/or vitellus, although the freezing and thawing treatments do not impair the mechanism blocking polyspermy. Zona-drilling to bypass the zona pellucida has improved the fertilization rate in frozen-thawed mouse oocytes [20]. If zona-drilling is applied to frozen-thawed bovine oocytes, the fertilization rate may be improved.

Lim *et al.* [2] have reported that *in-vitro* matured bovine oocytes could be successfully cryopreserved by GL, but that the proportion of oocytes that developed to the blastocyst stage after thawing and fertilization was very low (2.5%). Otoi *et al.* [5] have demonstrated that 2-cell embryos developed to the blastocyst stage were 6.1% in the 1.6 M PROH group, 23.1% in the GL group and 5.0% in the DMSO group, respectively. In our results, olny a few 2-cell embryos in two groups (1.6 M PROH+0.2 M sucrose and 0.8 M PROH+0.8 M DMSO) developed to the blastocyst stage (3.2% and 4.1%). These low rates of development to the blastocyst stage suggest that declining ability of frozen-thawed bovine oocytes to develop into blastocysts may reflect the worst effect of cryoprotectants during early cleavage stages.

A higher incidence of polyploidy has been shown in mouse eggs fertilized *in vitro* than in those fertilized *in vivo* [21, 22], in first-cleavage mouse embryos obtained from frozen-thawed oocytes [4], and in bovine embryos fertilized *in vitro* [23, 24]. In *in vitro* fertilization, the high incidence of polyploidy was caused by polyspermy [21–23, 25-28]. Carroll *et al.* [9], however, have reported that the cause of the high incidence of polyploidy was digyny, not polyspermy, in mouse embryos derived from frozen-thawed oocytes. In bovine oocytes also, it has been shown that polyspermic fertilization did not occur in frozen-thawed oocytes beyond the level of non-frozen oocytes [5, 29]. In the present study, the incidences of polyploids were high in all of the treated groups, but there was no significant difference between the control group and each treated group, or among the treated groups.

Shaw *et al.* [10] have reported that the highest incidence of structural aberrations of chromosomes was caused in 2-cell mouse embryos frozen in the 3.0 M DMSO concentration, but that the 4.5 M DMSO concentration did not cause an increase in chromosomal damage. Furthermore, the prolonged exposure of mouse oocytes to DMSO might lead to disassembling of the spindle and dispersion of the chromosomes [11], but the present study showed that there was no increase in structural aberrations of chromosomes in any treated group.

For freezing of *in vitro* maturated bovine oocytes, we recommend the mixture of 0.8 M PROH and 0.8 M DMSO as a cryoprotectant.

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