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Cryopreservation of Recipient Oocytes Collected from Korean Native Cattle: Incidence of the Zona Hardening of Recipient Oocytes Collected from Korean Native Cattle at the Different Stages of Cryopreservation[1]

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복제 한우 생산을 위한 수핵난자의 동결에 관한 연구: 상이한 동결과정 중 한우 수핵난자에서 일어나는 투명대 경화

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요 약

핵 이식에 공여되는 수핵난자의 효과적인 동결보존을 위하여 한우 성숙난자를 1.0 M dimethylsulfoxide (DMSO) 또는 1.0 M glycerol이 함유된 동결보호제를 이용하여 처리하거나, 동결보호제 처리 후 완만동결법을 이용한 동결융해를 시행하여 상기 실험처리로 야기되는 투명대 경화현상을 관찰하였다. 도축장 유래의 난소에서 미성숙난자를 채취한 후 10% 소 태아혈청을 함유한 TCM-199을 이용하여 22~24 시간 동안 체외성숙 배양을 이행하였다. 배양 후 작출된 성숙난자를 각각의 동결보호제로 처리, 혹은 처리 후 동결융해한 후 protease 를 이용하여 투명대의 경화현상 발생의 빈도를 조사하였다. 또한 동결란을 동결정액을 이용한 체외수정에 공여한 후 정자 침입능을 조사하였다. 동결보호제로 처리한 난자에 있어서 보호제의 종류와 관계없이 투명대 경화현상이 유의적 ($P<0.05$) 으로 증가하였으나 이후의 동결융해 처리에 의한 추가적인 경화 현상의 발생은 증가하지는 않았다. 또한 투명대 경화현상의 발생양상을 동결보호제 처리 후 10분 간격으로 측정한 결과 DMSO의 경우 처리 후 10분, glycerol의 경우 처리 후 20 분 후부터 유의적으로 증가하였다. 체외수정 후 동결난자의 투명대 경화현상을 검토한 결과 신선란과의 유의적 차를 발견할 수 없었으며, 수정율 및 난자 1 개당 침입한 정자의 수는 동결란에서 유의적으로 증가하지 않았다. 본 연구의 결과 동결난자의 투명대 경화현상은 동결보호제 처리과정에서 이미 일어나지만, 이러한 투명대 경화현상이 난자의 동결보존 후 수정능에는 현저한 영향을 미치지 않는다는 사실이 규명되었다.

(Key words : bovine oocyte, cryopreservation, zona hardening, DMSO, glycerol)

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INTRODUCTION

Development of a cryopreservation technique for mammalian oocytes is important for efficiently providing recipient oocytes for nuclear transfer systems using embryonic cells or various somatic cells. In numerous studies (Kubota et al., 1998; Ito et al., 1999), cryopreserved bovine oocytes was used as a recipient cytoplast in the nuclear transfer system. However, several studies pointed out that developmental capacity of frozen-thawed oocytes was significantly decreased compared with fresh oocytes (Lim et al., 1991). Different biological events accompanied cryopreservation influence the survival of oocytes after freezing and thawing (Le and Massip, 1999: Vajta et al., 1999; Zeron et al., 1999) and, based on this information, many factors resulted in freezing damage were further examined in bovine oocytes (Saunders and Parks, 1999; Zeron et al., 1999; Fuku et al., 1992).

Excessive sperm entry into the cytoplasm of oocytes can be completely blocked by the hardening of zona pellucida immediately after the penetration of a single spermatozoon (Gwatkin et al., 1973). The hardening is also occurred when oocytes were exposed to a number of factors; culturing in serum-free medium (Zhang et al., 1991), treating with DMSO (Johnson, 1989), ethanol (Gulyas and Yuan, 1985), calcium ionophore A23187 (Moller and Wassarman, 1989) and propanediol (Matson et al., 1997), exposing to low temperature (Johnson et al., 1988), and fertilizing *in vivo* or *in vitro* (DeMeestere et al., 1997). Cryopreservation procedure also induces the hardening (Carroll et al., 1990) and this biological event negatively affects the fertilization and developmental capacity of oocytes (Carrol et al., 1989). In our previous study (Lim et al., 1992), lower fertilization and developmental competences were found in frozen oocytes than in fresh oocytes, and we hypothesized that the zona hardening might be one of the factors responsible for their low developmental competence.

Therefore, this study was carried out to examine how the zona hardening occurs after the cryopreservation of bovine oocytes and whether this affects the fertilization capacity of frozen-thawed oocytes.

MATERIALS AND METHODS

1. Preparation of Oocytes

Cumulus-enclosed oocytes aspirated from 3 to 5 mm follicles were washed three times with a washing medium[1] and co-cultured with 2.5×10^6 granulosa cells/ml in one well of four-well multidish (Nunclon, Roskilde, Denmark) containing 1 ml maturation medium[2] in a 39℃ CO_2 incubator under 5% CO_2 in air. After 22 to 24 h of maturation culture, the oocytes were denuded by gentle pipetting and allocated into each experimental group.

2. Frozen-Thawed Procedures

As a cryoprotectant, either 1.0 M glycerol or 1.0 M DMSO in phosphate buffered saline supplemented with 4 mg/ml BSA and antibiotics (PBSB) was used. PBSBs supplemented with three concentrations (0.7, 0.3 and 0.0 M) of glycerol or DMSO in PBSB and with 0.5 M sucrose were used for the removal of the cryoprot-

[1] TCM-199 (Gibco Laboratories, New York, USA) supplemented with 3mg/ml bovine serum albumin (BSA; essentially globulin free, Sigma Chemical, St. Louis, USA), 2mM $NaHCO_3$ (Ishizu-seiyaku, Osaka, Japan), antibiotics (100 iu/ml penicillin and 100 μg/ml streptomycin), and 25 mM Hepes (Sigma).

[2] TCM-199 supplemented with 10% fetal calf serum (FCS; Sanko-junyaku, Tokyo, Japan; Wheaton, USA), antibiotics and 13mM $NaHCO_3$.

ectants from the oocytes after thawing. Oocytes were placed in each cryoprotectant and loaded into a 0.25-ml French straws (IMV, L'Aigle, France) within the 30 min after the first exposure to the cryoprotectants. The straws were then seeded by a pre-cooled forceps and placed into a programmable freezer (FHK, Tokyo, Japan) at −5.1℃. The straws were kept at this temperature for 15 min and cooled at a rate of −0.5℃/min to −32.5℃. The straws were then placed 1 to 2 cm above the surface of liquid nitrogen (LN_2) for 10 sec and directly plunged into LN_2.

At 2 to 3 weeks after cryopreservation, the straws were placed in air for 5 sec and placed in a water bath at 39℃ for thawing. Oocytes were then expelled from the straws and the cryoprotectants were removed by a three step method described by Lim et al. (1991).

3. *In Vitro* Fertilization

Oocytes were inseminated *in vitro* by the methods of Niwa et al. (1988). Briefly, oocytes were washed three times in a medium Brakett and Oliphant (BO) supplemented with 20 mg/ml BSA and 20μg/ml pig intestinal mucosal heparin. Eight to 10 oocytes were then introduced into 50-μl of the medium, which had been previously covered with warm paraffin oil in a 35×10mm plastic petridish (Cat. 1008, Nunc, Noskilde, Denmark). The dishes were kept in a CO_2 incubator for 30 min until insemination.

Two 0.5-ml straws of frozen semen obtained from a Korean native cattle bull were thawed in a water bath at 39℃ and the spermatozoa were washed twice in BO supplemented with 10 mM caffeine sodium benzoate by centrifugation at 833-g for a period of 10 min each. A 50-μl of final sperm suspension (4.0×10^6 spermatozoa/ml) was then introduced into 50-μl of the medium containing the oocytes. The mixture gave final concentrations of 10 mg/ml BSA, 10 μl/ml heparin, 5 mM caffeine and 2.0×10^6 spermatozoa/ml.

4. Assay for the Detection of Zona Hardening

Oocytes were washed three times with phosphate buffered saline containing 5% (v/v) FCS and antibiotics(PBSF). Then, four- to five- oocytes were placed into a 50-μl droplet of a 0.5% (w/v) protease (A-6911; sigma) in phosphate buffered saline containing 1% (w/v) PVP at 39℃ and the lysis of zona pellucida was observed under a stereo microscope (SMZ-2B; Nikkon). Zona pellucida was considered as lysed when it had completely disappeared and the oocytes become to attach to the bottom of the dish (Downs et al., 1986). Elapsed time from the placement of oocytes into the droplets of the protease to the completion of zona lysis was measured for evaluating the hardening of the zona.

5. Sperm Penetration Assay

Spermatozoa penetrated into the perivitelline space (PVS) and ooplasm were observed by Hoechest 33342 fluorescent (H-33342) and aceto-orcein, respectively. Inseminated oocytes were washed three times with PBSF and treated with 0.2 μl H-33342/ml in PBSF at 39℃ for 1 h, by a slightly modified method of Conover and Gwatkin (1988). Oocytes were then observed with an inverted microscope equipped with a phase contrast and an 100-W mercury bulb. After observation, oocytes were then fixed, stained with aceto-orcein and observed with a phase contrast microscope.

Experiment 1

Oocytes matured in culture were treated with each cryoprotectant for 30 min or treated and frozen-thawed. Incidence of hardening in each

group of oocytes was then examined the zona solubility.

Experiment 2

Oocytes were treated with each cryoprotectant for 10, 20, 30 or 40 min at the room temperature. Each group of oocytes was then washed three times with PBSF and evaluated the zona hardening.

Experiment 3

Oocytes treated with each cryoprotectant for 30 min were inseminated *in vitro* and examined the sperm penetration rate and the number of penetrated spermatozoa per oocyte at 8 h after insemination. Oocytes with spermatozoa into PVS or ooplasm were considered as being penetrated.

6. Statistical Analysis

All experimental groups were replicated three times each. Data of experiments were calculated by Mann-Whitney's U test or student-t test, which was chosen by Bartlett test, and χ^2 test ($P<0.05$).

RESULTS

Table 1 shows the results of preliminary study for evaluating the maturation of oocytes co-cultured with 2.5×10^6 granulosa cells for 22~24 h. Almost all of the oocytes (85/87=97.7%) had expanded cumulus cells and 91.8% of the oocytes reached at metaphase II.

Incidence of zona hardening in oocytes either treated with cryoprotectant or treated and frozen-thawed was shown in Table 2 (Experiment 1). There was a significant increase in the incidence of hardening in treated oocytes compared with that of control ($P<0.05$). However, freez-

Table 1. Maturation of cumulus-enclosed oocytes after co-cultured with granulosa cells for 22~24 h

No. (%) of oocytes		Immature oocytes at the stage of						No. of abnormal oocytes
Cultured	With expanded cumulus	Matured (at M II)	GV	GVBD	MI	AI	TI	
87	85(97.7)	78(91.8)*	1	2	1	1	2	1

GV=germinal vesicle, GVBD=germinal vesicle breakdown, MI=metaphase I, AI=anaphase I, TI=telophase I, M II=metaphase II

*Percentage of the number of oocytes with expanded cumulus cells and first polar body.

Table 2. Incidence of zona hardening in bovine mature oocytes either treated with different cryoprotectants or treated and frozen-thawed

Groups	Glycerol(1.0 M in PBS)		DMSO(1.0 M in PBS)	
	No. of oocytes examined	Time for dissolution (sec, mean±SEM)	No. of oocytes examined	Time for dissolution (sec, mean±SEM)
Control*	30	94.5 ± 2.1^a	34	87.4 ± 3.7^a
Treated	30	116.7 ± 4.1^b	30	101.2 ± 3.6^b
Frozen+	32	114.3 ± 6.5^b	34	101.8 ± 3.7^b

*Oocytes treated with PBSF for 30 min

$^{a-b}$: Different superscript in each parameter are significantly different, $P<0.05$.

Table 3. Hardening of the zona pellucida of bovine oocytes matured in culture and treated with the different kinds of cryoprotectant for various durations

Duration of treatment (min)	Glycerol(1.0 M in PBS)		DMSO (1.0 M in PBS)	
	No. of oocytes examined	Time for dissolution (sec, Mean±SEM)	No. of oocytes examined	Time for dissolution (sec, Mean±SEM)
0	52	85.2±2.7[a]	52	85.2±2.7[a]
10	50	88.8±2.3[a]	56	102.7±3.0[b]
20	49	104.4±4.1[b]	50	101.4±2.9[b]
30	63	116.2±3.3[c]	55	101.0±3.1[b]
40	48	111.9±4.6[c]	56	102.1±2.8[b]

Oocytes treated with each cryoprotectant for 30 min and then frozen with the same cryoprotectant.

[a-c]: Different superscripts in each column are significantly different ($P<0.05$).

Table 4. Zona penetration of spermatozoa in bovine oocytes matured in culture and treated with the different kinds of cryoprotectant for 30 min at 8 h after *in vitro* insemination

Groups	No. of oocytes		No. of oocytes penetrated with n spermatozoa		Average No. of sperm penetrated (Mean±SEM)
	Examined	Penetrated(%)	n=1(%)	n≥2(%)	
Control	53	45(84.9)	27(60.0)	27(60.0)	1.30±0.13[a]
Glycerol	49	36(73.5)	25(69.4)	25(69.4)	1.08±0.14[ab]
DMSO	56	36(64.3)	29(80.6)	7(19.4)	0.76±0.08[b]

[a-b]: Different superscripts in the same column are significantly different ($P<0.05$).

ing and thawing after the treatment did not significantly induce the zona hardening of oocytes.

Table 3 shows the incidence of zona hardening in oocytes treated with each cryoprotectant for various durations (Experiment 2). The zona hardening of oocytes treated with glycerol was significantly found from 20 min after the treatment. On the contrary, the zona hardening was significantly found from 10 min when oocytes were treated with DMSO.

The number of spermatozoa penetrated into the oocyte cytoplasm at 8 h after insemination were shown in Table 4 (Experiment 3). No significant differences in the number of oocytes penetrated were found among the treatments, although the value was slightly higher in the control group than in the treatment groups. Furthermore, the number of penetrated spermatozoa per oocyte was not significantly decreased in oocytes treated with either DMSO or glycerol than in oocytes with no treatment.

DISCUSSION

The results of this study demonstrate that both the treatment of glycerol and DMSO induce the zona hardening of bovine oocytes matured in culture. There are many reports on the relationship between DMSO and zona hardening in other species (Vincent et al., 1990; Kohno et al., 1989). However, the effect of glycerol on the hardening in bovine oocytes has not been studied up to the present.

Results of the Experiment 1 clearly demonstrated that the zona hardening of bovine oocytes already occurred during the equilibration

with the cryoprotectants before freezing. Carroll et al.(1989) reported in the mouse that, although the major changes in the zona pellucida which limit fertilization occurs during the complete freeze-thaw cycle, this alteration is not resulted from the manipulations before and after freezing. On the contrary, Vincent et al. (1991) reported that the zona hardening occurs when oocytes are equilibrated with DMSO. Similar results with Vincent et al. (1991) were obtained in our present experiment and our results additionally inform that glycerol also induce the hardening during the procedure of cryoprotectant equilibration. All of inconsistencies on the zona hardening may be resulted from species specificity. In addition, a degree of zona hardening is dependent upon the conditions, to which the oocytes are exposed (Johnson et al., 1989) and, if our procedure are properly adjusted, the solubility may not severely change during the whole cryopreservation procedure.

The rates of sperm penetration in the control and treated oocytes were similar. It indicates that the incidence of zona hardening in oocytes slowly frozen with either DMSO or glycerol does not decrease the fertilization capacity of oocytes. Other factors, such as the abnormalities in cytoplasm, chromosome and spindle may be more responsible for the low fertilizability of oocytes frozen at metaphase II. On the other hand, this paradoxical results on zona hardening found in this study and sperm penetration may be related to incomplete zona hardening, which can occur in the suboptimal *in vitro* culture condition. The quantity of cortical granule exocytosis relates to the completion of zona hardening (Cran and Esper, 1990), oocytes matured *in vitro* may have a limited number of cortical granule in their peripheral cytoplasm due to the incompletion of cytoplasmic maturation.

It was reported that DMSO stabilize and assembles microtubule system (Katsuda et al., 1988), and glycerol binds to microtubule proteins, which causes the rearrangement of microtubules (Detrich et al., 1976). Since the cytoskeletal system involves in the release of cortical granules and the induction of zona hardening (Burgoyne and Cheek, 1987; Sato et al., 1990), we suppose that the action of glycerol and DMSO affects the zona hardening through the cytoskeletal system.

Time required for the hardening was different according to the type of cryoprotectants. We suppose that it relates to a different membrane permeability. DMSO, which is smaller molecular weight and have a stronger affinity to water molecule than glycerol, can diffuse more easily to the cytoplasm across the membrane than glycerol and affects rapidly on the changes in zona pellucida.

SUMMARY

The incidence of zona hardening and the sperm penetration of oocytes either treated or frozen with glycerol or DMSO was examined in mature oocytes retrieved from Korean native cows. Zona hardening occurred during the treatment with the cryoprotectant before freezing and thawing, no additional hardening was induced by subsequent freezing procedure. The hardening of oocytes treated with DMSO and glycerol decreased significantly at 10 min and 20 min after the treatment, respectively. However, the zona penetration of spermatozoa did not differ significantly between control and treated groups of oocytes when examined at 8 h after *in vitro* insemination. We concluded that the zona hardening was occurred when bovine oocytes were treated with the cryoprotectants before freezing, but it did not affect significantly sperm penetration of frozen oocytes.

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