# In Vitro Fertilizing Ability of Bovine Oocytes Frozen-Thawed at Immature, Maturing, and Mature Stages

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***Abstract:*** *The effect of the nuclear stage during in vitro maturation (IVM) on the fertilizing ability of frozen-thawed bovine oocytes was investigated. Oocytes with compact cumulus cells were cultured for 0, 6, 12 and 24 h, and were subjected to 2-step freezing in the presence of 10% ethylene glycol and 0.1 M sucrose. After thawing, the oocytes were additionally cultured to give 24 h for a total IVM period, and were inseminated with 3.5 to 5.0 × $10^6$ cells/ml frozen-thawed spermatozoa for 20 h in TALP solution containing 20 μg/ml heparin. In vitro fertilization rates of oocytes frozen-thawed at 0, 6, 12 and 24 h of IVM were 40, 47, 49 and 54%, respectively, all of which were significantly lower than that of nonfrozen control oocytes (77%). A higher frequency of polyspermic fertilization was observed in the oocytes frozen-thawed at 24 h of IVM (53 vs. 18% in nonfrozen control oocytes). Lowering the sperm concentration during insemination from 5.0 × $10^6$ cells/ml to 1.0 × $10^5$ cells/ml did not decrease the rate of polyspermic fertilization in the frozen-thawed oocytes.*

***Key words:*** *Bovine oocytes, Ethylene glycol, Freezing, Polyspermy.*

A possible advantage of oocyte cryopreservation may be to establish ovum banking, but oocytes have been shown to be much more difficult to cryopreserve than zygotes or later stage embryos. Although pregnancies and live calves have been derived from cryopreserved bovine oocytes [1–3], the rate of survival of oocytes has been low [1–7]. Many problems are associated with the cryopreservation of *in vitro*-matured or ovulated oocytes, including spindle disorganization, and loss or clumping of microtubules [8–11]. Such changes resulted in some scattering of chromosomes, or increased polyploidy at fertilization. In addition, it has been suggested that cryoprotectants may induce a premature release of cortical granules, resulting in the hardening of zona pellucida and decrease in fertilization [12, 13]. Therefore, cryopreservation of immature oocytes as an alternative approach has been attempted, but these attempts have had very limited success [1, 5, 14, 15]. Ultrastructural evaluation of cryopreserved bovine [16] and equine [17] oocytes suggests that the damage to immature oocytes due to freezing may be associated with the destruction of intercellular communication between cumulus cells and the oocytes.

During oogenesis, most mammalian oocytes are arrested in the prophase stage of the first meiotic division, with a prominent germinal vesicle (GV). The meiosis of oocytes can resume *in vitro*, characterized by germinal vesicle breakdown (GVBD) in pro-metaphase I. The nuclear stage of maturing oocytes progresses to metaphase I, anaphase I, and telophase I, and reaches metaphase II with the extrusion of the first polar body. Since intercellular communication through gap junctions between cumulus cells and the oocyte may contribute to triggering the meiosis resumption [18, 19], the freeze-sensitivity of oocytes is likely to be affected by their nuclear conditions. In one paper by Lim *et al.* [5], it was reported that the postthaw morphological survival and the cleavage rate following *in vitro* fertilization of immature bovine oocytes after 2-step freezing were lower than those of maturing and mature oocytes.

This study was designed to investigate the *in vitro* fertilizing ability of bovine oocytes following cryopreservation before, during and after *in vitro* maturation (IVM). Monospermic fertilization and pronucleus formation were the criteria to assess their normality. An additional attempt was to reduce the high incidence of polyspermic fertilization in frozen-thawed mature oocytes.

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## Materials and Methods

*In vitro maturation*

Bovine ovaries were obtained from a local slaughterhouse and transported in phosphate-buffered saline (PBS) at 30°C to the laboratory within 4 h of slaughter. Oocytes with compact cumulus cells were recovered by slicing and washing the ovary surfaces. In Experiment 1, oocytes were cultured for 0, 6, 12 and 24 h in 25 mM HEPES-buffered TCM199 supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM sodium pyruvate (Sigma Chemical Co.), 1 μg/ml estradiol-17β (Sigma) and 0.02 Armour Unit/ml FSH (Denka Pharmaceuticals Co.) at 39.0°C in humidified 5% $CO_2$ in air (20 to 30 oocytes per 500 μl of IVM medium under mineral oil). Some of the oocytes were fixed for 24 h in acetic acid/ethanol (1/3, v/v) and stained with 1% orcein (w/v) in 45% acetic acid, after the cumulus cells were removed by repeated aspiration with a fine-bore glass pipette in 500 μl of PBS containing 500 U/ml hyaluronidase (Sigma). Nuclear maturation stages of the oocytes were determined at × 200 magnification. In Experiment 2, oocytes were cultured for 24 h as described above.

*Freezing and thawing*

The cryoprotective medium consisted of 10% (v/v) ethylene glycol (Wako Pure Chemical Co.) and 0.1 M sucrose (Wako) in PBS supplemented with 10% FBS [15]. Oocytes were transferred to the cryoprotective medium at 25°C and then groups of 10 to 15 oocytes were loaded into 0.25-ml straws (FHK). Ten minutes after transferring oocytes into the cryoprotective medium, the straws were placed into the alcohol bath of a programmable freezer (MPF-1000, EYELA) 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. After at least 48 h in liquid nitrogen, the straws were warmed rapidly (5 sec in 25°C air, followed by 15 sec in a 35°C water bath). The contents of the straws were expelled, and the oocytes were immediately transferred into PBS + 10% FBS. The oocytes were washed twice in PBS + 10% FBS for 10 min at 30°C, rinsed in 2.5 ml of IVM medium and then placed into 500 μl of IVM medium under mineral oil. The oocytes were cultured as described above, to give a 24 h total maturation period.

*In vitro fertilization*

Two ejaculates of semen from a Japanese Black bull whose spermatozoa had been frozen in an egg yolk-Tris buffer/glycerol solution were used. Frozen-thawed spermatozoa (about 1.0 ml) were washed twice with HEPES-buffered Tyrode's medium supplemented with 2% BSA (Fraction V; Sigma) by centrifugation at 200 x g and then resuspended in 0.6 to 0.8 ml of the HEPES-buffered Tyrode's medium. The sperm suspension was layered over a glass wool column, and allowed to filter by gravity. Then 10 μl of the filtered sperm suspension was added to 40 μl of IVF medium, where 10 to 25 oocytes with the number of cumulus cells reduced by 30 sec Vortex-mixing were placed. The IVF medium consisted of Tyrode's medium supplemented with 0.6% BSA, 10 mM lactate, 1 mM sodium pyruvate (called TALP) and 20 μg/ml heparin (Sigma). The sperm concentration during insemination was 3.5 to 5.0 × $10^6$ cells/ml (Experiments 1) or 0.1, 0.5, 1.0 and 5.0 × $10^6$ cells/ml (Experiment 2). Twenty hours after insemination, oocytes were fixed and stained as described above, and evaluated for sperm penetration at × 200 magnification. Oocytes with both male and female pronuclei were considered normally fertilized. Oocytes exhibiting asynchrony in pronucleus formation (female pronucleus + decondenced/intact sperm head, or metaphase II/anaphase II plate + male pronucleus, or metaphase II/anaphase II plate + decondenced/intact sperm head) were considered abnormally fertilized. Polyspermic fertilized oocytes were also recorded. Oocytes with 1 pronucleus only were defined as unfertilized, activated oocytes. Degenerated oocytes were those atrophied or fragmented.

*Statistical analysis*

Four replicates were conducted for each group. The proportions of fertilized, unfertilized and degenerated oocytes in total treated oocytes, as well as of fertilized oocytes with both pronuclei and polyspemic fertilized oocytes, were arcsin-transformed before being subjected to one-way analysis of variance. The significance of difference between means was compared by a post-hoc, Fisher's protected least significant difference test [20]. A value of $P<0.05$ was chosen as an indication of statistical significance.

## Results

*Experiment 1*

Eighty-eight percent (23/26) of oocytes at 0 h of IVM had a prominent GV. At 6 h of IVM, 72% of oocytes resumed meiosis, and were in the GVBD stage (23/32). All oocytes at 12 h of IVM resumed meiosis, being in

**Table 1.** Effect of maturation stages of bovine oocytes on *in vitro* fertilization rates after freezing and thawing

| IVM periods before freezing (hr) | No. (%) [%] of oocytes | | | | | |
|---|---|---|---|---|---|---|
| | Treated | Fertilized | | | Unfertilized | Degenerated |
| | | Total: | Normally | Polyspermy | | |
| 0 | 68 | 27 (40)[a] | 9 [33][a] | 7 [26][a] | 12 (18) | 29 (43)[a] |
| 6 | 72 | 34 (47)[a] | 18 [53][bc] | 6 [18][a] | 17 (24) | 21 (29)[b] |
| 12 | 68 | 33 (49)[a] | 9 [27][a] | 11 [33][ab] | 24 (35) | 11 (16)[c] |
| 24 | 70 | 38 (54)[a] | 12 [32][ab] | 20 [53][b] | 13 (19) | 19 (27)[bc] |
| Control | 65 | 50 (77)[b] | 33 [66][c] | 9 [18][a] | 15 (23) | 0 ( 0)[d] |

[a–d]Different superscripts within a column denote significant differences ($P<0.05$). (%): Calculated from treated oocytes, [%]: Calculated from fertilized oocytes.

the GVBD (18%; 7/38), or metaphase I (82%; 31/38) stage. After 24 h of IVM, 81% (22/27) of the oocytes reached the metaphase II stage and the rest of the oocytes (19%, 5/27) were in metaphase I. Therefore, oocyte populations at 0, 6, 12 and 24 h IVM appeared to be representatives of the GV-stage, GVBD-stage, metaphase I-stage and metaphase II-stage, respectively.

*In vitro* fertilization rates of frozen-thawed oocytes as well as nonfrozen control oocytes are shown in Table 1. Overall fertilization rates of frozen-thawed oocytes (40 to 54%) were lower than that of nonfrozen control oocytes (77%; $P<0.05$). These fertilization rates could be re-calculated to be 69% (27/39; 0 h IVM), 67% (34/51; 6 h IVM), 58% (33/57; 12 h IVM), 75% (38/51; 24 h IVM) and 77% (50/65; nonfrozen control), when the results were limited for morphologically non-degenerated oocytes in their whole-mount preparations ($P>0.05$). Polyspermic penetration occurred more frequently in the oocytes that were frozen-thawed at 24 h of IVM (53% of fertilized oocytes vs. 18% of control oocytes). Ploidy of polyspermic fertilized oocytes was similar in all 5 groups (3.43 ± 0.54, 3.17 ± 0.41, 3.55 ± 0.69, 3.40 ± 0.68 and 3.44 ± 0.73 in frozen groups at 0, 6, 12, 24 h IVM and nonfrozen control group, respectively; mean ± SD). The proportion of normally fertilized oocytes with both pronuclei was higher in a group frozen at 6 h of IVM (53%) than in other frozen groups (27 to 33%). Morphological survival of frozen oocytes, assessed by the proportion of non-degenerated oocytes, was lower in the 0 h group (57%) than in the 6, 12 and 24 h groups (71 to 84%).

*Experiment 2*

Fertilization rates of oocytes frozen-thawed after 24 h of IVM as a function of sperm concentration are shown in Fig. 1. In the nonfrozen control group, the use of 5.0 × $10^6$ sperm cells/ml resulted in the highest overall normal fertilization rate (48%, 31/65). The polyspermic

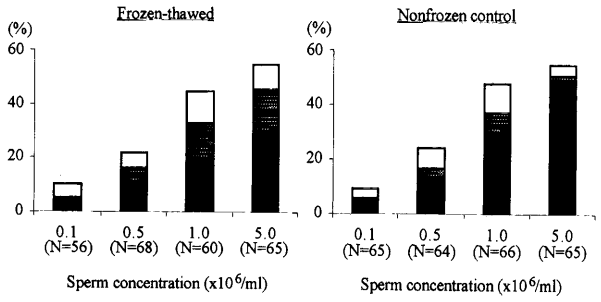


**Fig. 1.** Effect of sperm concentrations during insemination of frozen-thawed oocytes on their fertilization rates. N=number of all treated oocytes. ■: Normal fertilization, ▩: Polyspermic fertilization, □: Abnormal fertilization.

fertilization rate of this group was 6% (2/36) of all fertilized oocytes. Lower sperm concentrations (0.1 to 1.0 × $10^6$ cells/ml) resulted in lower normal fertilization rates (6 to 29%). Compared with nonfrozen oocytes, normal fertilization rates of frozen-thawed oocytes (5 to 31%) were lower throughout the sperm concentrations examined, although total fertilization rates were comparable. The incidence of polyspermic fertilization was not decreased by lowering the sperm concentration (27 to 30% of fertilized oocytes in 0.5 to 5.0 × $10^6$ cells/ml groups).

## Discussion

It has been reported that *in vitro* fertilization rates of frozen-thawed bovine oocytes are significantly lower than nonfrozen control oocytes and the survival of the oocytes as assessed by development into blastocysts is only a few percents of all treated oocytes [1, 3, 6, 7]. Immature oocytes were more sensitive to cryopreservation than *in vitro*-matured oocytes [1, 5]. In the present study, according to the progress of nuclear maturation

in bovine oocytes, mechanical damage caused by freezing became less frequent. In addition, proportions of normally fertilized oocytes in frozen oocytes were lower than in nonfrozen oocytes (33% in 0 h of IVM group and 27% in 12 h of IVM group vs. 66% in the control group), regardless of the comparable polyspermic fertilization rates of the 3 groups, suggesting that cytoplasmic components concerning pronucleus formation, such as certain proteinase and smooth endoplasmic reticulum, may be influenced by the freeze-thaw process for oocytes. These results may indicate that cryopreservation of oocytes at immature and *in vitro*-maturing stages is not optimal, but in a group of oocytes frozen-thawed at 6 h of IVM (71% were in the GVBD stage), the proportion of normally fertilized oocytes with male and female pronuclei was not less than that of the nonfrozen control group (54 vs. 66%). This result suggests that the GVBD stage oocytes can support monospermic fertilization and pronucleous formation once the oocytes have survived cryopreservation. At this maturation stage of oocytes, the positive role of intercellular gap junctions between cumulus cells and the oocyte [18, 19] may be terminated, whereas no harmful effect of the freeze-thaw process on spindles in the metaphase plate [8–11] has yet appeared.

Frozen-thawed oocytes after 24 h of IVM in Experiment 1 had the same fertilizing ability as nonfrozen control oocytes (75 vs. 77%) when the oocytes were limited to the morphologically surviving ones. The high fertilization rate of frozen oocytes may be explained by easier accessibility of sperm cells to vitellus through damaged cumulus-cumulus communication or zona pellucida. A high incidence of polyspermic fertilization in the frozen oocytes was noted, suggesting that the freeze-thaw process for mature oocytes may affect the mechanism regulating the number of sperm to be penetrated, such as cortical granule exocytosis. Ploidy of the polyspermic fertilized oocytes, based on the number of intact sperm heads, enlarged sperm heads or pronuclei, was not influenced by the nuclear stages at freezing and thawing, but if maternal chromosomes were scattered into pieces by the freeze-thaw process for oocytes and the chromosomal fragments could transform into multiple pronuclei during spontaneous activation, such oocytes may have been classified as polyspermic fertilized oocytes. An effort to define paternal pronuclei based on the presence of a sperm tail was not successful because the sperm tail of even the nonfrozen control oocytes with two pronuclei and two polar bodies was not always distinguishable. In earlier reports on cryopreservation of bovine *in vitro*-matured oocytes [5, 6], polyspermic fertilization had not occurred beyond the level of nonfrozen control oocytes. An IVF approach with a decreased sperm concentration (Experiment 2) failed to reduce the high incidence of polyspermic fertilization in the frozen mature oocytes. Further research is needed to clarify this high incidence of polyspermic fertilization in frozen mature oocytes.

In the present study, *in vitro* culture of the presumptive zygotes derived from frozen oocytes was not done. Since only 0.6 to 2.7% of all frozen oocytes have been reported to develop to blastocysts in the exhaustive studies by Otoi *et al.* [3, 6, 7], production of a sufficient number of normally fertilized oocytes after freezing will be a prerequisite for the production of transferable blastocysts and living calves. Recently in a study by Martino *et al.* [21], 10 to 15% of bovine mature oocytes cryopreserved by vitrification in the presence of ethylene glycol on electron microscope grids have been reported to develop to blastocysts under *in vitro* culture conditions where >40% of the nontreated control oocytes could develop to that stage. Developmental competence to blastocysts of frozen oocytes may reflect their metabolic normality during early cleavage stages.

In conclusion, (1) mechanical damage caused by freezing occurred less frequently in maturing and mature oocytes than in immature oocytes. (2) Fertilization rates of oocytes frozen at 4 given maturation periods were similar to that of nonfrozen oocytes, once the oocytes survived cryopreservation. (3) After cryopreservation, the incidence of polyspermic fertilization in mature oocytes was higher than those in immature and maturing oocytes.

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