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Ultrastructural Comparison between Immature and In Vitro Matured Bovine Oocytes Cryopreserved in Propanediol

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Abstract: *Immature and in vitro matured bovine oocytes were cryopreserved in a solution containing propanediol as a cryoprotectant to compare their developmental ability following the treatment. After cryopreservation, the oocytes were inseminated with frozen-thawed bovine spermatozoa and cultured in vitro for a given period of time to determine the developmental capacity of the embryos. The average rates for cleavage, which were measured 2 days after in vitro fertilization (IVF), were 71.2% for freshly collected immature oocytes, 3.4% for frozen-thawed immature oocytes and 6.8% for cryopreserved in vitro matured oocytes, respectively, though none of the cryopreserved oocytes developed to the blastocyst stage. Following cryogenic storage, the oocytes were processed for transmission electron microscopy (TEM). Freezing and thawing of the immature oocytes induced remarkable ultrastructural changes in oolemma, microvilli, mitochondria and vesicles in ooplasm and cumulus cells surrounding the oocytes, whereas the integrity of cell organelles was relatively better preserved in in vitro matured oocytes. The present results suggest that the cryogenic storage of bovine oocytes, both immature and in vitro matured, induced various kinds of ultrastructural damage which was associated with the low developmental capacity of post freeze-thaw oocytes. On the other hand, in vitro matured oocytes were considered to maintain their structural integrity better than immature oocytes following cryopreservation with propanediol.*

Key words: *Bovine oocytes, Cryopreservation, Propanediol, Ultrastructural damage.*

Following success in the cryopreservation of mouse embryos in liquid nitrogen [1], the protocols were improved and applied to embryos of other species. At least 15 mammalian species have been successfully cryopreserved [2]. Most investigators, however, concentrated their efforts on late-stage embryos [3]. The recent rapid improvement in techniques for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and subsequent *in vitro* culture (IVC) in bovine oocytes has generated new interest in cryogenic storage of oocytes, and thus far some success has been achieved with human oocytes and with mouse oocytes [4–6].

Nevertheless, since oocytes at various stages of development and those from different animal species differ both physiologically and morphologically [7–9], freezing procedures for one species may not be suitable for another, and therefore only limited progress in the cryopreservation of oocytes from farm animals has been achieved. It has been suggested that the reduction in developmental capacity of cryopreserved oocytes may result from cracks in zona pellucida [10, 11], disruption of plasma membrane, extensive disorganisation of the ooplasm and damage to the spindle apparatus [12–14], but the precise ultrastructural damage inflicted on bovine oocytes by cryopreservation has not so far been fully demonstrated.

The purpose of the current study was to investigate (1) subcellular cryoinjury of both frozen-thawed immature and frozen-thawed *in vitro* matured oocytes by transmission electron microscope (TEM) and (2) the developmental capacity of the cryopreserved oocytes following IVF.

Materials and Methods

The experimental procedures employed in this study are shown in the following flow chart.

In vitro maturation of oocytes

(a) Maturation of immature oocytes without cryopreservation: Ovaries were obtained from cows at a

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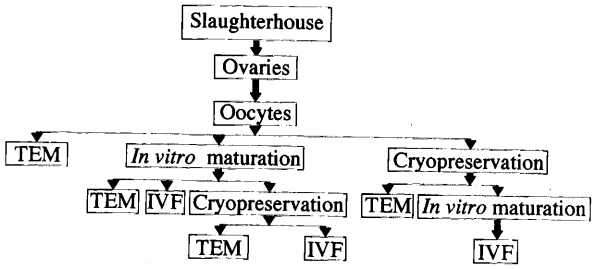

local slaughterhouse and were brought to the laboratory in physiological saline (0.85% NaCl) within 2 h after collection. The cumulus-oocyte complexes (COCs) were collected from follicles 2 to 8 mm in diameter with a 20-gauge needle attached to a 5-ml syringe containing SFRE-2 solution [15] supplemented with 3mM BSA and antibodies (100 mg/ml Gentamicin) and pooled in watch glasses. After being recovered and washed twice in Dulbeco-PBS and three times in maturation medium, ten to fifteen COCs were introduced into a 50-μl drop of maturation medium in 35-mm petri dishes under mineral oil. The oocytes were then incubated for 21 to 23 h at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity.

(b) Maturation of frozen -thawed immature oocytes: Frozen-thawed immature oocytes were washed three times with the maturation medium and introduced into the drops of maturation medium as described above.

In vitro fertilization (IVF)

Semen preparation was carried out according to the method of Ohboshi *et al.* [15]. Briefly, frozen bull semen was thawed in a water bath at 37°C and washed in modified HEPES-TALP as described by Brackett and Oliphant [16]. The sperm suspension was diluted with m-HEPES-TALP containing heparin of 10 μg/ml, theophylline of 5.0 mM and 10 mg/ml BSA to bring about a final concentration of 10×10^6 sperm cells/ml. The oocytes after *in vitro* maturation culture were washed three times in mHEPES-TALP and introduced into 100 μl droplets of the sperm suspension (15 to 20 COCs/drop) and incubated at 39°C for around 6 h in mineral oil in 5% CO_2 in air with maximum humidity.

In vitro culture (IVC)

The medium used for IVC was modified SOF(mSOF) [17]. After 6 h of insemination culture, the presumptive zygotes were washed twice in mSOF supplemented with 1.0% calf serum (CS) and cumulus cells surrounding zygotes were partly removed before being transferred into drops of mSOF. At 48 h after the insemination, the medium was replaced with fresh mSOF supplemented with 5.0% CS and the cumulus cells surrounding the embryos were removed by repeated pipetting, while the cumulus cells attached to the surface of the culture dish were retained. The embryos were cultured for an additional 8 days and the medium was replaced with fresh medium every 48 h. The development of embryos and the number of embryos developing to the blastocyst stage were recorded.

Freezing and thawing

Freezing and thawing of the oocytes were carried out according to the method of Otoi *et al.* [18]. Briefly, COCs were suspended in 25 mM HEPES-TCM 199 containing 5% CS and 1.6 M 1,2-propanediol by three steps at 10-min intervals at room temperature (20–25°C). Following equilibration for 30 min in the final solution, 20 to 25 of the COCs were loaded into 0.25-ml plastic straws (I.M.V. France). The straws were then placed in the cooling chamber of a programmed freezer (FHK ET-1), cooled from 0°C to −5.5°C at the rate of 1°C/min, seeded at −5.5°C, cooled at a velocity of 0.3°C/min to −30°C, and plunged directly into LN_2. The straws were maintained at −196°C for 1 to 20 days.

Thawing was performed by exposing the straws to air for 5 sec and then by plunging into a water bath at 37°C for 30 sec. After thawing, the contents of straws were expelled into a small dish, diluted with HEPES-TCM-199 and CS was added in three steps, decreasing the concentration of cryoprotectant by 0.53 M at 10-min intervals at room temperature (20–25°C).

Preparation of oocytes for transmission electron microscopy (TEM)

The TEM was conducted on (1) control oocytes (both immature and *in vitro* matured) and (2) morphologically normal frozen-thawed oocytes (both immature and *in vitro* matured). The control and frozen-thawed oocytes were fixed in a solution consisting of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature, post-fixed in 1% osmium tetroxide dissolved in 0.1 M cacodylate buffer for 30 min at 0°C. After routine dehydration with a graded series of ethanol, the oocytes were infiltrated with a 1:1 mixture of absolute acetone, Quetol-651 (Nisshin EM Company, Japan) and with absolute Quetol-651 successively. The oocytes were embedded in beem capsules with fresh Quetol-651. Thin sections were cut with glass knives and stained with 1% toluidine blue. Ultrathin sections cut with diamond knife were double-stained with both uranyl acectate and lead citrate, and

examined with a JEM-200CX TEM. Based on the previous observation of bovine oocytes [19–21], the cell organelles in ooplasm such as microvilli, mitochondria, smooth endoplasmic reticulum, cortical granules, and vesicles as well as the cumulus cells surrounding oocytes after cryopresevation were examined in comparison with those of the fresh oocytes in order to determine the various kinds of ultrastructural damage due to freeze-thawing.

Results

In vitro fertilization and in vitro culture

As shown in Table 1, the average rates of cleavage were 71.2% for non-cryopreserved oocytes, 3.4% for cryopreserved immature oocytes and 6.8% for *in vitro* matured-cryopreserved oocytes, respectively. The rate of cleavage of frozen-thawed *in vitro* matured oocytes was slightly higher than that of frozen-thawed immatured oocytes. The development of embryos after IVF of cryopreserved immature oocytes seemed to be slightly faster than that of the frozen-thawed *in vitro* matured oocytes, though no blastocysts were obtained from immature or *in vitro* matured oocytes, while approximately 15% of the oocytes from the non-cryopreserved group developed to the blastocyst stage. On the other hand, when no insemination was conducted, no cleavage of oocytes was observed, suggesting that the parthenogenetic development of oocytes did not occur in the present experimental conditions.

Light-microscopic features of oocytes

In untreated immature oocyte, small vesicles were distributed throughout the entire region of the oocyte and cumulus cells were closely associated with the oocyte (Fig. 1). In the case of *in vitro*-matured oocytes, on the other hand, vesicles were more centralized than those of the immature oocytes and remarkable elongation of cumulus cells was observed (Fig. 2), but the ooplasm of frozen-thawed immature oocytes was clearly different from that of immature and *in vitro*-matured oocytes, showing greatly disorganized cumulus investments

Table 1. Development of frozen-thawed oocytes after *in vitro* fertilization (IVF)

Treatment	No. of oocytes inseminated	No. cleaved on Day 2 (%)	No. of morulae on Day 6 (%)	No. of blast. on Day 8 (%)
Treatment 1[a]	59	42 (71.2)	11 (18.6)	9 (15.3)
Treatment 2[b]	58	2 (3.4)	0 (0)	0 (0)
Treatment 3[c]	59	4 (6.8)	0 (0)	0 (0)
Treatment 4[d]	20	0 (0)	0 (0)	0 (0)

[a] Immature oocytes were cultured for 22 h (Control). [b] Frozen-thawed oocytes were cultures for 22 h. [c] *In vitro* matured oocytes were frozen and thawed. [d] Frozen-thawed *in vitro* matured oocytes were cultured without insemination.

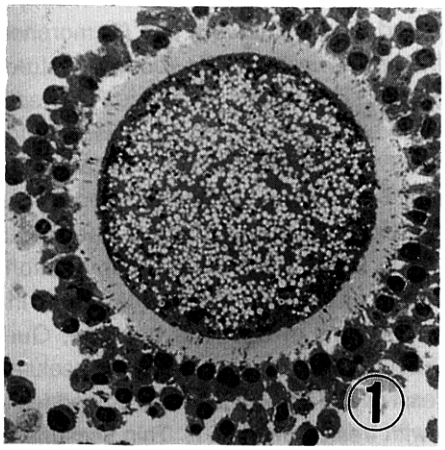

Fig. 1. Freshly collected immature oocyte (× 320).

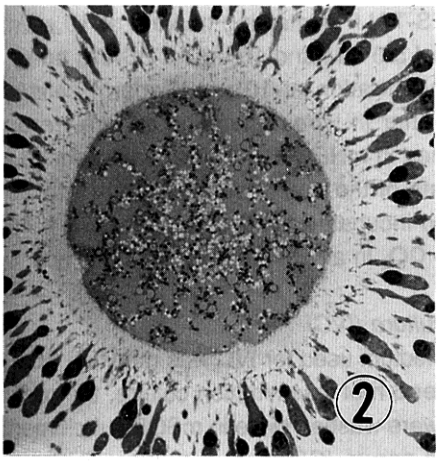

Fig. 2. *In vitro* matured oocyte (× 320).

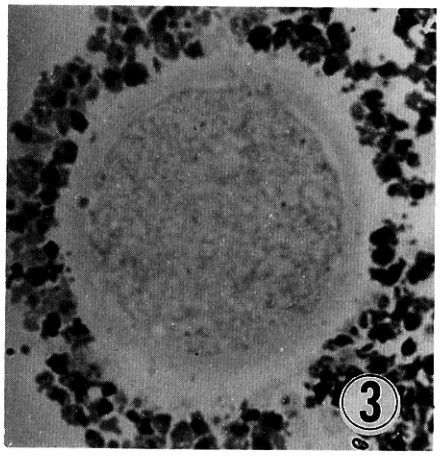

Fig. 3. Frozen-thawed immature oocyte (× 320).

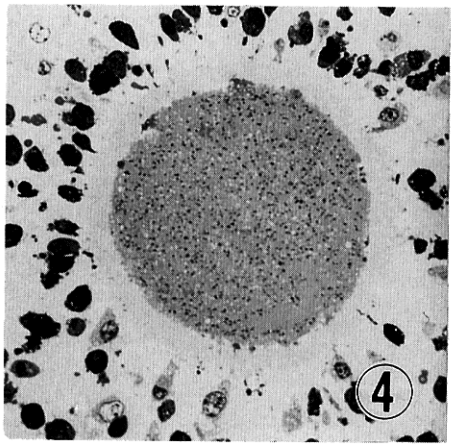

Fig. 4. Frozen-thawed *in vitro* matured oocyte (× 320).

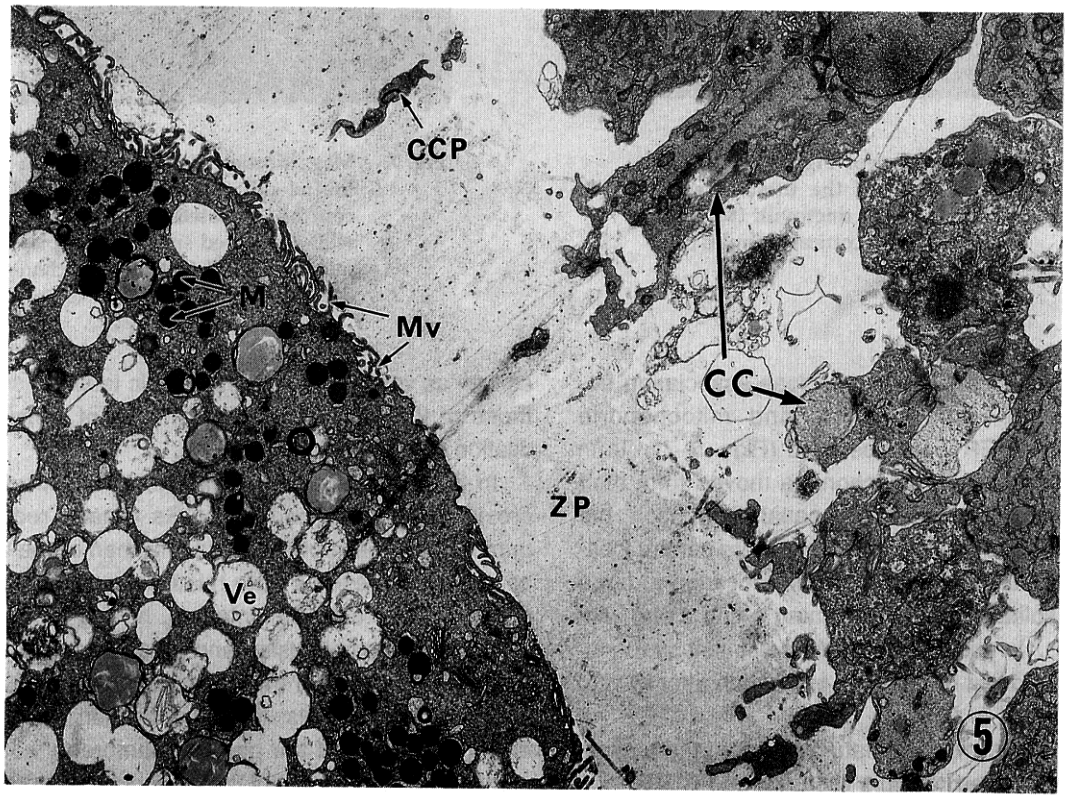

Fig. 5. Electron micrograph of Fig. 1. *Note.* The evenly distributed vesicles (Ve) and peripheral mitochondrial (M) cluster. The zona pellucida (ZP) is transversed by cumulus cell projections (CCP) (× 5,000).

(Fig. 3). Frozen-thawed *in vitro*-matured oocyte also showed slightly different ooplasm from the above mentioned three types of oocytes, although damage to cumulus investments was certainly observed (Fig. 4).

Ultrastuctural features of the treated oocytes

In immature oocytes evenly distributed vesicles, peripheral mitochondrial cluster, well developed smooth endoplasmic reticulum (sER), large peripheral clusters

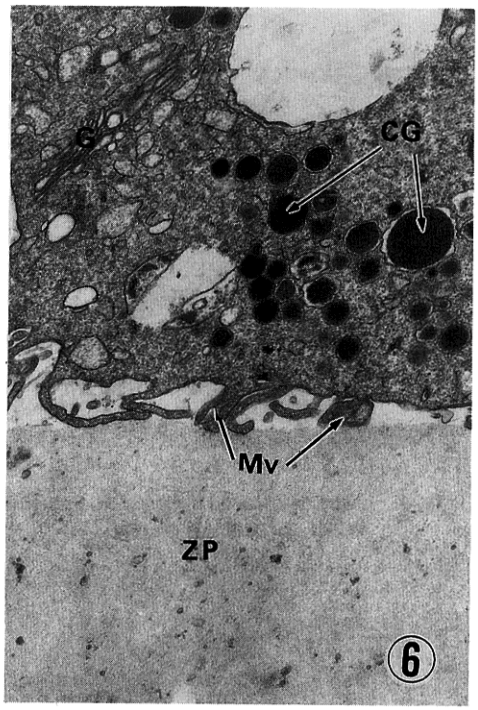

Fig. 6. Magnification of Fig. 5. *Note.* Ooplasm showing the intact plasma membrane with microvilli (Mv) and large clusters of cortical granules (CG) (× 15,000).

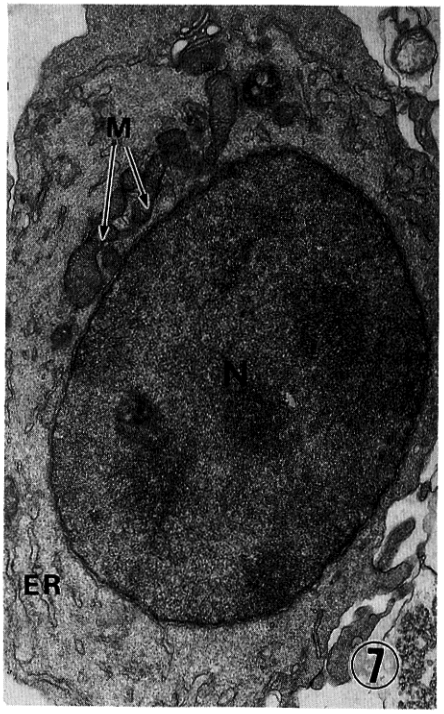

Fig. 7. Magnification of Fig. 5. *Note.* Cumulus cell (CC) with large and centrally located nucleus. The well-developed smooth endoplasmic reticulum (sER) and mitochondria (M) (× 12,500).

of cortical granules (CG) and a close association between vesicles, lipid droplets and mitochondria throughout the sER were observed (Figs. 5–7). In *in vitro* matured oocytes, mitochondria moved from their peripheral location to an even spatial distribution, the vesicles tended to migrate towards a more central location and the CG dispersed from their location in large clusters to solitary positions forming a line along with the oolemma. In addition, cumulus investment surrounding oocytes showed remarkable elongation after *in vitro* maturation (Figs. 8–10).

In the frozen-thawed immature oocytes, the plasma membrane exhibited multiple ruptures and CG tended to flow out of the ooplasm. The vesicles showed a higher degree of confluency and significant variation in size. A large number of such vesicles were also observed in the perivitelline space. Mitochondria were located peripherally in ooplasm and their cristae disappeared almost completely. The sER and mitochondria were noticeably dilated (Figs. 11, 12). The cumulus investment in the frozen-thawed immature oocytes contained numerous vacuoles in cytoplasm. Mitochondria as well as vesicles had characteristic features similar to those of the ooplasm, showing signs of considerable dilation and confluency (Fig. 13).

In the frozen-thawed *in vitro* matured oocytes, microvilli were perturbed and CGs greatly reduced in number, but the rupture of plasma membrane and the confluency of vesicles were relatively modest, and mitochondria were better maintained, having a continuous membrane, normal cristae and a dense matrix, in comparison with those of the immature oocytes (Figs. 14, 15). In cumulus cells, the vacuolization was reduced and the integrity of the plasma membrane was better preserved, though most of the mitochondria were shrunk and the sER was perturbed (Fig. 16).

Discussion

In the present study, the frozen-thawed oocytes showed significantly lower developmental capacity than the unfrozen control oocytes. After cryogenic storage, only 3.4% of immature oocytes and 6.8% of *in vitro*-matured oocytes cleaved after IVF, respectively, and

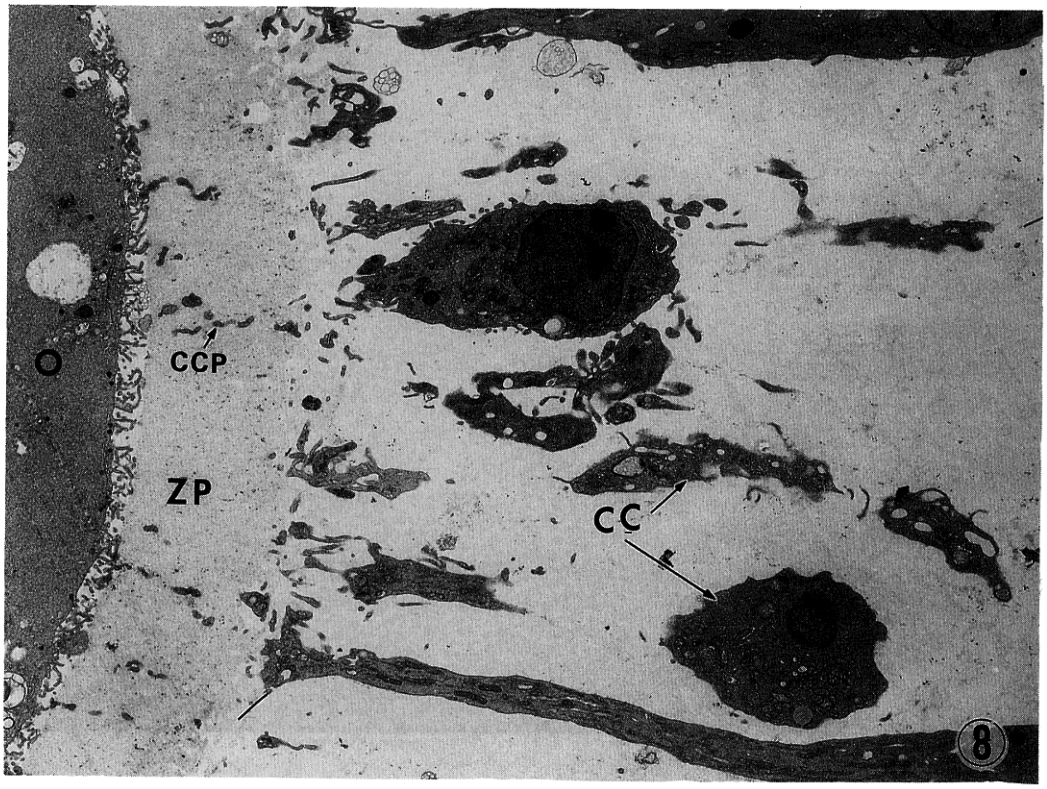

Fig. 8.

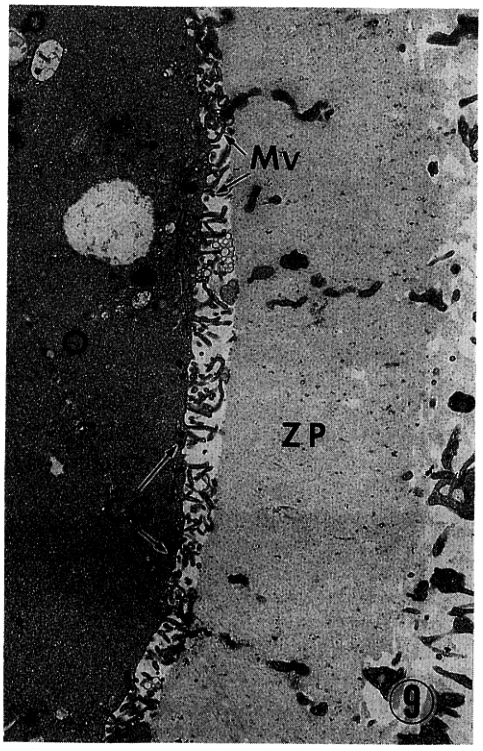

Fig. 9.

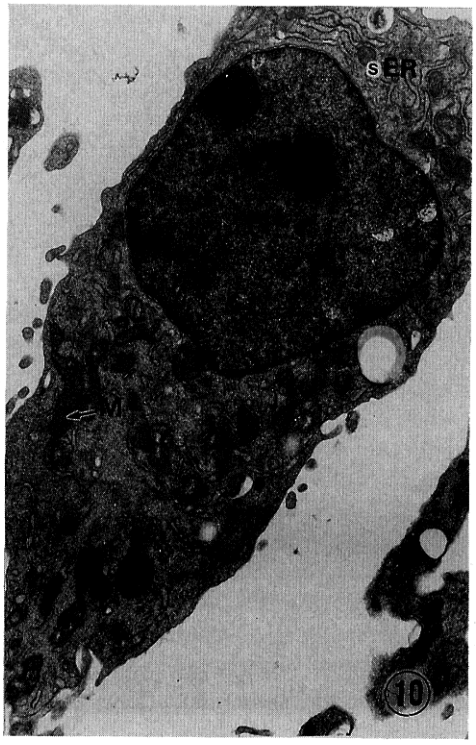

Fig. 10.

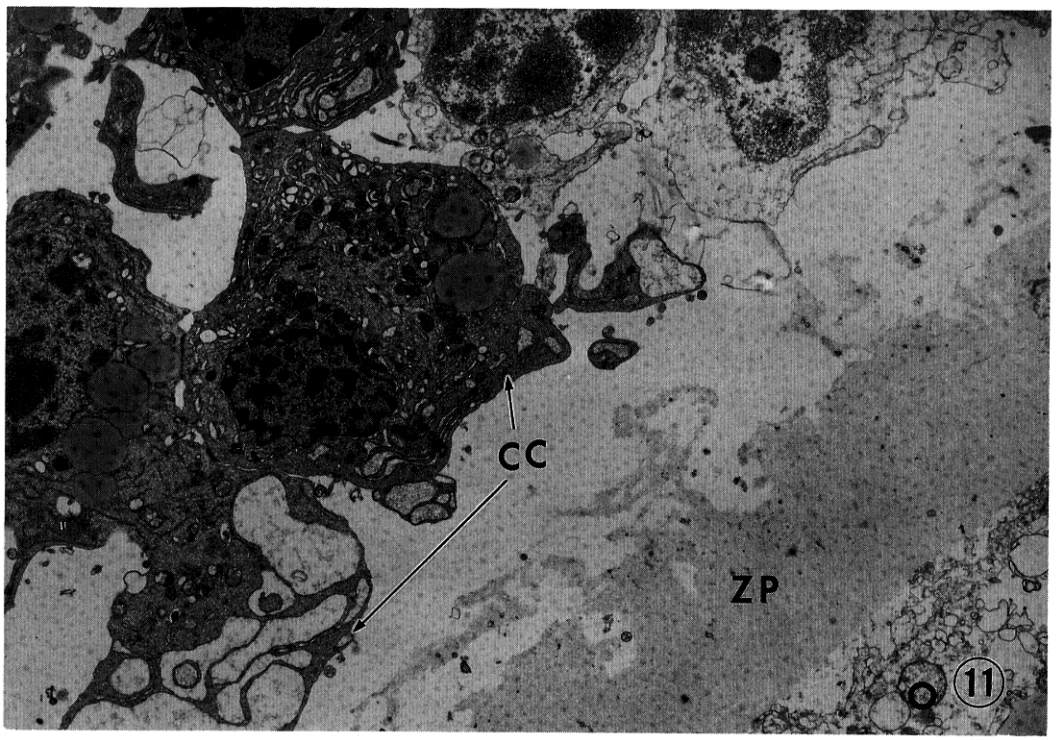

Fig. 11.

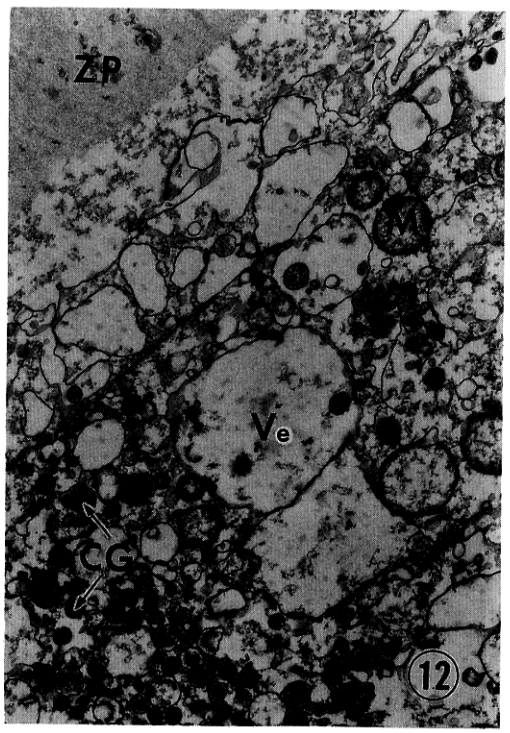

Fig. 12.

Fig. 8. Electron micrograph of Fig. 2. *Note.* The remarkable elongation of cumulus cells (CC) and the zona pellucida (ZP) transversed by cumulus cell projections (CCP) (× 3,000).

Fig. 9. Magnification of Fig. 8. *Note.* The well-developed oolemma (O), microvilli (Mv) and zona pellucida (ZP). Cortical granules (CG) tend to form a line along the oolemma. Microvilli (Mv) erect vertically on the surface of oolemma (× 4,500).

Fig. 10. Magnification of Fig. 8. *Note.* Cumulus cell (CC) containing numerous mitochondria (M) with well-developed cristae and smooth endoplasmic reticulum (sER) (× 10,000).

Fig. 11. Electron micrograph of Fig. 3. *Note.* The ruptures of plasma membrane of oolemma, degradation of cumulus cells (CC) and decondensation of nuclei (N) (× 7,000).

Fig. 12. Magnification of Fig. 11. *Note.* The degenerated oolemma. Cortical granules (CG) tend to flow out of oolemma. Vesicles (Ve) show high degree of confluency. Mitochondria (M) are swollen (× 16,000).

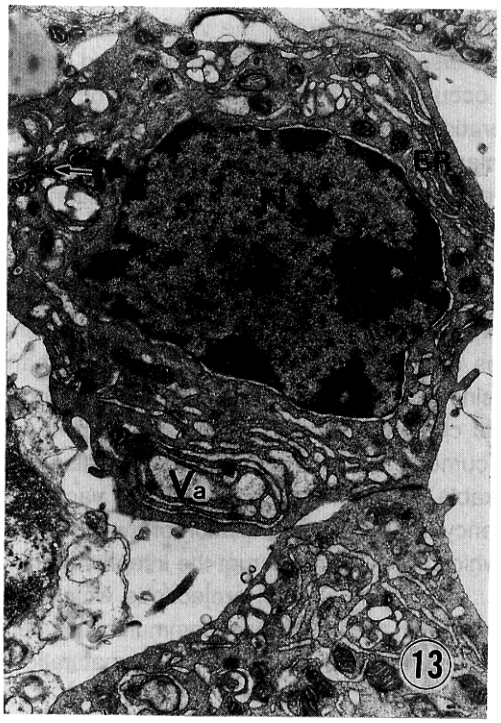

Fig. 13.

none of the treated oocytes developed to the morulae or blastocyst stages, while the cleavage rate for the control group was 71.2% and the rate of blastocyst yield was approximately 15%. These results were in good agreement with previous reports, showing that the current freezing and thawing procedures only allowed subsequent fertilization and development at a very reduced rate [7, 22, 23]. These findings were in contrast with those of Schroeder *et al.* [24] who found that the developmental capacity of mouse embryos was almost the same both for frozen-thawed and freshly collected oocytes. This suggests that bovine oocytes were more sensitive to cryogenic storage than mouse oocytes, and that the freezing procedure used for mouse oocytes may not necessarily be suitable for bovine oocytes.

Fig. 13. Magnification of Fig. 11. *Note.* Cumulus cells (CC) showing numerous vacuoles (Va) and degenerated nuclei (N) (× 12,000).

Fig. 14. Electron micrograph of Fig. 4. *Note.* Dispersed cumulus cells (CC) with high electron density and decondensed nuclei (× 4,500).

Fig. 14.

The ultrastructural investigation in the present study revealed that bovine oocytes were very sensitive to cryopreservation. As mentioned above, the frozen-thawed bovine oocytes lost the integrity of the cell structure as compared with unfrozen oocytes, which had ultrastructural characteristics in accordance with earlier descriptions of bovine oocytes [19–21]. Following freezing and thawing, the confluency between adjacent vesicles and peripherally displaced vesicles was clearly observed, and this has previously been reported in bovine embryos [13] and in bovine *in vitro* matured oocytes when cryopreserved with glycerol as a cryopretectant [25]. Because numerous vesicles in the oocytes containing lipid droplets were considered to constitute a category of oocytes highly vulnerable to freezing [25], the vulnerability of vesicles is also speculated to be a reason for increasing the confluency of vesicles during cryopreservation. On the other hand, it has been reported that the shrinkage of oocytes occurred due to dehydration during freezing and thawing [26] and the confluency of vesicles might be induced by the compression during oocyte shrinkage. In the present study, peripheral displacement of the vesicles and mitochondria was also observed in the frozen-thawed oocytes. It might occur upon reexpansion of the oocytes in the equilibration period [25], and this was considered to be an explanation of the rupture of the plasma membrane and the disappearance of microvilli. In addition, marked dilation of mitochondria and sER was observed in the frozen-thawed immature oocytes, although these cell organelles remain unaffected by freezing and thawing of bovine embryos [13]. This suggests that unfertilized oocytes may be more sensitive to freezing than embryos since the oocyte is likely to be more vulnerable to osmotic changes as well as freezing. On the other hand, cumulus cell surrounding oocytes also showed remarkable subcellular changes such as vacuolization, confluency of vesicles and dilation of mitochondria and sER, which appeared to disrupt the integrity of the cytoplasm. Most cumulus cell projections which may be associated with the intercellular communication between cumulus cells and oocytes during oocyte maturation were interrupted [21].

The present TEM study shows that the structural

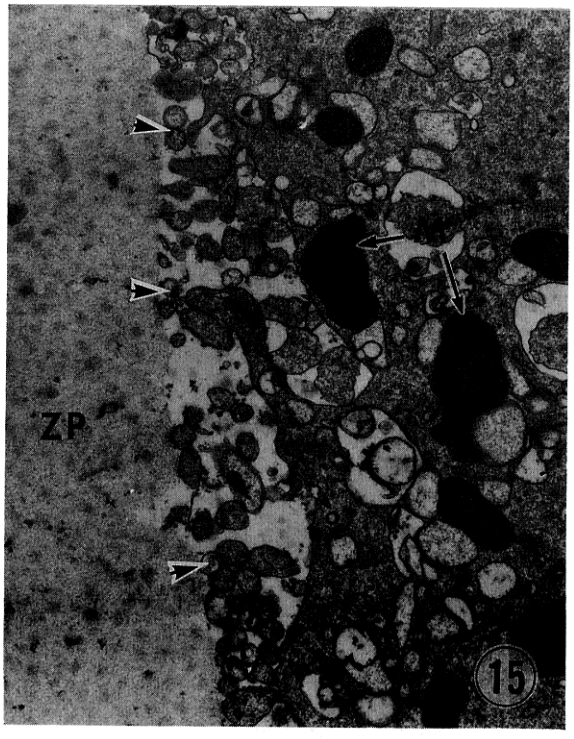

Fig. 15. Magnification of Fig. 14. *Note.* Empty ooplasm in oolemma or microvilli (arrow head) and shrunk mitochondria (M), (× 15,000).

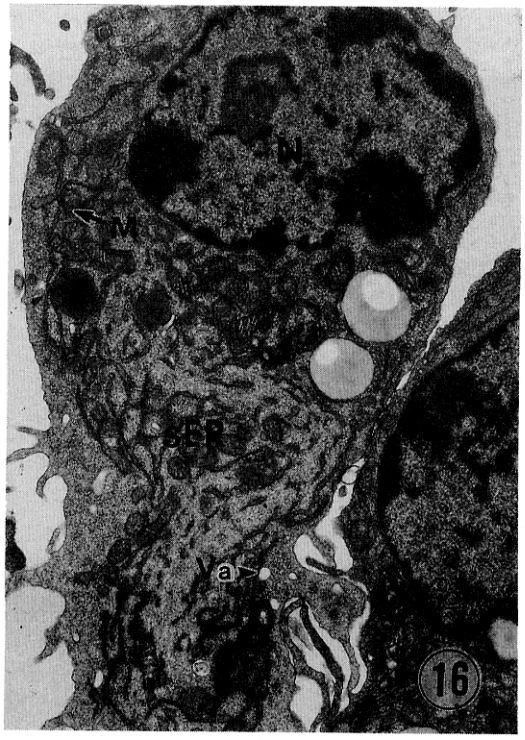

Fig. 16. Magnification of Fig. 14. *Note.* Cumulus cell (CC) showing decondensation of nuclei (N), degenerated smooth endoplasmic reticulum (sER) and few vacuoles (Va) (× 11,000).

integrity of frozen-thawed *in vitro* matured oocytes was better maintained than that of treated immature oocytes, i.e. the vesicles as well as cell organelles such as mitochondria, sER and microvilli were less changed. It may be presumed that the immature oocytes were more sensitive to cryopreservation than the *in vitro* matured oocytes. These findings are in accordance with the statements of Fuku *et al.* [22], but in *in vitro* matured oocytes after freezing and thawing, CGs were rarely observed, while clusters of CGs were preserved in the treated immature oocytes. The release of CGs in bovine oocytes was previously reported by Fuku *et al.* [7], and in mouse oocytes by Johnson *et al.* [11] and Vincent *et al.* [27]. It has been reported that the changes in the zona pellucida were associated with the reduction in the rate of fertilization of oocytes in mouse, and zona hardening was correlated with a reduced number of CGs in the ooplasm in aging mouse oocytes [11, 12]. The loss of CGs could play a role in the modification of zona pellucida during cryopreservation in mouse oocytes. It remains unclear whether CGs in bovine oocytes may have the same effect on zona pellucida as those in mouse oocytes.

In the present study, 1,2-propanediol was used as a cryoprotectant since it was reported to be a more appropriate cryoprotectant for *in vitro* matured oocytes than DMSO as well as glycerol [28, 29], but the present ultrastructural investigation suggests that damage to the immature oocytes was serious and 1,2-propanediol might not necessarily be an appropriate cryoprotectant for immature oocytes. This suggestion is consistent with the observation and interpretations of Herrler *et al.* [28] who reported that for immature bovine oocytes, DMSO/sucrose was superior to all other cryoprotectants.

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