The Effect of Dimethyl-Sulfoxide and Sucrose as a Cryoprotectant on the Adenosine Triphosphate and Ultrastructure of Bovine Oocytes Matured *In Vitro*

Yasuhiro Tsuzuki*, Mio Hisanaga, Koji Ashizawa and Noboru Fujihara[1]
Laboratory of Animal Reproduction, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, Japan.

ABSTRACT : The present study was undertaken to assess the influence of dimethyl-sulfoxide plus sucrose solution as a cryoprotectant on the adenosine triphosphate (ATP) content, the ultrastructure and the embryonic development of bovine oocytes matured *in vitro*. We measured the amount of ATP in cumulus cells enclosed oocytes (CO) or denuded oocytes (DO) equilibrated with or removed from the cryoprotectant (1.5 M DMSO + 0.25 M sucrose + 20% fetal bovine serum in physiological saline). As a result, the ATP contents in both CO and DO, equilibrated with the cryoprotectant, were significantly lower ($p<0.05$) than that of the each control group. However, ATP content of DO was recovered to the level of the control group after removal of the cryoprotectant, but failed to restore for CO. In the observation of the ultrastructure by a transmission electron microscope, all of the mitochondria in the ooplasm of CO and DO equilibrated with the cryoprotectant were swollen with peripherally located cristae following decreased electron density. However, a large proportion of these swollen mitochondria were restored to the normal shape which can be observed usually in the control group after removal from the cryoprotectant. To the contrary, the morphology of many mitochondria of the cumulus cells in CO were not recovered to that of the control group after removal of the cryoprotectant. CO with removed cryoprotectant had significantly lower embryonic development up to the blastocysts stage ($p<0.05$) after *in vitro* fertilization compared with that in the control group. These results suggest that the addition and removal of a cryoprotectant has a negative effect for the ATP content of cumulus enclosed oocytes. One of the factor(s) causing the lower embryonic development after removal of cryoprotectant, may be associated with ATP metabolism. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 10 : 1353-1359)*

Key Words : Cryoprotectant, ATP, Ultrastructure, Bovine Oocytes

INTRODUCTION

Frozen-thawed bovine oocytes can be developed to the normal calf after *in vitro* fertilization and embryo transfer (Otoi et al., 1996). However, the oocytes from farm animals showed limited progress due to unsuitable freezing procedure (Zhao et al., 1997). In general, cellular damage that results from freezing can be circumvented by use of permeable organic solutions acting as cryoprotectants (Friedler et al., 1988). It is one of the factors which influence the ultrastructure of mouse and human oocytes or the development of bovine oocytes matured *in vitro* (Schalkoff et al., 1989; Lim et al., 1999). However, the precise nature of equilibration of cryoprotectant to the mammalian oocytes is not fully understood.

Some cryoprotectants such as dimethyl-sulfoxide (DMSO) or hydroxyethyl starch can decrease the content of adenosine triphosphate (ATP), which is needed for the maturation and the development of mammalian oocytes (van Blerkom et al., 1995; Horiuchi et al., 1998), in human blood cells and HeLa cells (Rittmeyer and Nydegger, 1992; Forman et al., 1999). No paper has been published concerning effects of cryoprotectants on ultrastructure and the metabolism of the bovine oocytes, suggesting that further investigation will be needed.

The present study was undertaken to study the effects of cryoprotectant on the ATP content, the ultrastructure and embryonic development after *in vitro* fertilization of bovine oocytes matured *in vitro*.

MATERIALS AND METHODS

Oocyte maturation

Bovine ovaries were brought to our laboratory immersed in physiological saline (0.9% NaCl, 28-33°C) supplemented with 400 U/ml penicillin G potassium salt and 500 μg/ml streptomycin sulfate (Katayama Chemical Co., Osaka, Japan) within 4 h. The oocytes were aspirated from superficial follicles (2-5 mm in diameter) by 20 G needle (Terumo, Tokyo, Japan) attached to a 5 ml syringe (Terumo) containing a few ml of TCM-199 (Hank's salt, Gibco BRL Products, MD, USA) supplemented with 2% (V/V) calf serum (CS, 56°C, 30 min heat-inactivated, Gibco BRL Products). Cumulus enclosed oocytes (CO) were selected and transferred to the maturation medium consisting of 25 mM Hepes buffered TCM-199 with Earl's salt (Gibco BRL Products), 5% (V/V) CS, 50 μM dimethyl-sulfoxide (DMSO, 134-07, Nacalai Tesque, Kyoto, Japan,

* Address reprint request to Yasuhiro Tsuzuki. Tel: +81-985-58-7195, Fax: +81-985-58-7195, E-mail: a01207u@cc.miyazaki-u.ac.jp.
[1]Animal Resource Science Section, School of Agriculture, Graduate School Kyushu University, Fukuoka 812-8581, Japan.
Received September 26, 2000; Accepted May 24, 2001

Tsuzuki et al., 1998), 0.12 U/ml follicle stimulating hormone (F-2293, Sigma, St Louise, Mo, USA) and antibiotics (100 U/ml penicillin G potassium salt, 100 μg/ml streptomycin sulfate, Katayama Chemical Co. and 100 μg/ml dibekacin sulfate, Meiji Seika Co., Tokyo, Japan). The oocytes were incubated for 23-25 h in an atmosphere of 5% CO_2 in air at 39°C with 100% humidity. To make the cumulus cells denuded oocytes (DO), the cumulus cells were removed from CO by using a vortex mixer (G-65, Scientific Industries Inc, NY, USA) with the strongest stirring (2,600-2,700 rpm.). To determine the number of cumulus cells attached to the oocytes, some CO was loaded with 0.5 ml physiological saline supplemented with 20% (V/V) of heat inactivated fetal bovine serum (FBS, Gibco BRL Products) to the 1.5 ml Eppendorf tube (A-150, Assist, Tokyo, Japan) and mixed for a few min until cumulus cells were removed from the oocytes entirely. Thereafter, some drops of the suspension were introduced to the haemocytometer (Thoma type) to count the number of the cumulus cells.

Exposure of the oocytes to the cryoprotectant

CO and DO were washed with physiological saline supplemented with 20% (V/V) FBS. This was used as a carrier for the cryoprotectant solution, mentioned below. After washing, CO and DO were led to the cryoprotectant composed of 1.5 M DMSO, 0.25 M sucrose and the carrier solution in one step manner, and equilibrated for 20 min at room temperature (20-26°C). To remove the cryoprotectant from CO and DO, they were placed in 1.0 M DMSO + 0.5 M sucrose and 0.5 M sucrose in the carrier solution for 5 min each at room temperature. The oocytes were loaded to the carrier solution for 5 min at room temperature and washed once with the carrier solution. One part of CO and DO were transferred to the Milli-Q water to extrude the ATP from the oocytes; the other parts of CO and DO were fixed for electron microscopy. In addition, to assess the thermal effect for the ATP content, ultrastructure and embryonic development, some CO or DO were placed in the carrier solution for the same period of equilibration with the cryoprotectant.

ATP assay

ATP contents of the oocytes were measured using the method reported by Wishart (1982) with some modification. CO and DO in each treatment group was transferred to 1.5 ml Eppendorf tube, boiled (100°C) for 4 min and stored at in a deep freezer (-65 ~ -72°C). The contents of ATP in each group were determined by firefly bioluminescence using lumicounter (A-237, Advantec, Tokyo, Japan). Further more, some CO and DO treated with carrier solution were also measured for their ATP contents.

Transmission electron microscopy

One of CO and DO in each treatment group were fixed with 3% glutar-aldehyde (Taab Lab. Equip. Lim, Berks, UK) in cacodylate buffer (pH 7.4) for overnight at 4-5°C. Then these oocytes were washed twice with the cacodylate buffer and post fixed with 1.33% osmium tetroxide for 1.5 h at 4-5°C. CO and DO were dehydrated with ethanol series and embedded to Quetol-812 (Nisshin EM, Tokyo, Japan). The sections in each group were cut with a glass knife and mounted on a copper grid. The sections were stained with uranyl acetate and lead citrate for a few minutes each, and observed by transmission electron microscope (TEM, H-800, Hitachi, Tokyo, Japan).

***In vitro* fertilization and the subsequent development**

For this assay, DO was not fertilized due to a decrease in the fertilizability (Matsui et al., 1995). CO in each group were transferred to a drop of BO solution (Brackett and Oliphant, 1975) supplemented with 10 μg/ml heparin and 7.5 mg/ml bovine serum albumin (BSA, Fraction V, Katayama Chemical Co.). Frozen semen from a Japanese Black Bull was thawed in a water bath (37-39°C) for 1 min and diluted 2, 4, 8, 16 and 32 times with BO solution to remove glycerol from the spermatozoa. After centrifugation (600×g, 8 min, at room temperature), sperm deposit was diluted at equal volume with BO solution supplemented with the same concentration of heparin and BSA, mentioned above, to adjust the sperm concentration to 300-315×10^4 cells/ml. Equal volumes of semen were added to the drops which contained the oocytes and incubated for 22-26 h. After fertilization, oocytes were introduced to CR1aa (Rosenkrans and First, 1991) containing 1% CS (V/V) and 50 μM DMSO and incubated for additional 10 days. On the second day after insemination, the oocytes were removed from cumulus cells layer and replaced CR1aa supplemented with 15% CS (V/V) and antibiotics. The medium were replaced with fresh medium every 48±2 h.

Statistical analysis

The contents of ATP were analyzed by student t-test. The cleavage rate and development rate up to the blastocyst stage were analyzed with chi-square test (Snedecor and Cochran, 1980).

RESULTS

ATP content assay

The ATP contents from CO and DO are shown in table 1. CO had 5,937±1,107 (means±SD) of cumulus cells, and the ATP contents of DO was 0.877±0.0767 pmol/DO.

The ATP contents of CO treated with carrier solution were the same as the control group. However, CO

Table 1. The effect of a cryoprotectant* on the ATP content of CO and DO

Treatment	ATP contents	
	(pmol/CO)	(pmol/DO)
Control	5.672±0.3033[a]	0.877±0.0767[a]
Carrier Solution**	5.392±2.4811[ab]	0.895±0.0811[a]
Equilibrated	1.898±0.1151[b]	0.473±0.0984[b]
Removed	1.716±0.2512[b]	0.828±0.1633[ab]

Values are represented for Mean±SE.

* Cryoprotectant consisted of 1.5 M DMSO + 0.25 M sucrose+20% FBS in physiological saline.

** Carrier solution consisted of 20% FBS in physiological saline.

CO Cumulus enclosed oocytes. CO has 5,937±1,107 (Mean±SD) cumulus cells.

DO Denuded oocytes from cumulus cells.

a,b Values with different superscripts are significantly ($p<0.05$) different within a column.

equilibrated or removed from the cryoprotectant were significantly lower ($p<0.05$) than that of the control group. These values showed no significant difference compared to carrier solution group.

In the case of DO group, the ATP contents of DO in carrier and control group were the same. However, the equilibrated DO with the cryoprotectant showed significantly lower ($p<0.05$) than that of the control and carrier solution groups. Adversely, if DO were removed from the cryoprotectant, their ATP contents recovered to the level of control group.

Utrastructural observation by TEM

In the control group, a large proportion of cell organelle was located at the center position of the oocytes except for cortical granulosa. Most of cortical granules (CG) were located under oolemma, although other types of the oocytes have CG in cluster near the oolemma. A lot of microvilli were presented in the perivitelline space which is outside of the plasma membrane of the oocytes, and processed to the zone pellucida in the perivitelline space. Mitochondria were hooded or oval in shape and they have clearly cristae with little higher density than that of the control. A lot of vesicles were dispersed within the cytoplasm. They included the some glanulae or lipid like materials. Smooth endoplasmic reticulum were presented near the mitochondria or vesicle. The cumulus cells were attached to the outside of the zona pellucida, and they have some projections, which some invaded to the zona pellucida. However, no cytoplasm of the oocytes was invaded by these projections of the cumulus cells. The cumulus cells were bound to each other by the same projections which were processed from the neighbor cells. Ball or cuboidal shaped nuclei were seen in the center of each cumulus cell, and peripheral chromatin was observed along the inner nucleus membrane. Mitochondria were dispersed within the cytoplasm, and were oval or prolated in shape having a lot of cristae. The rough or smooth endoplasmic reticulum were observed in the cytoplasm of the cells. Large vesicles containing granulosa or lipid like materials were observed in cytoplasm of the cumulus cells (fig. 1, 2, 3).

On the other hand, in equilibrated CO, oocyte matrix was sightly irregular and lower density compared with control group. The number of CG within oocyte cytoplasm was decreased compared to the control group. Almost of all mitochondria were swollen following lower electron density and their cristae were slightly located to the

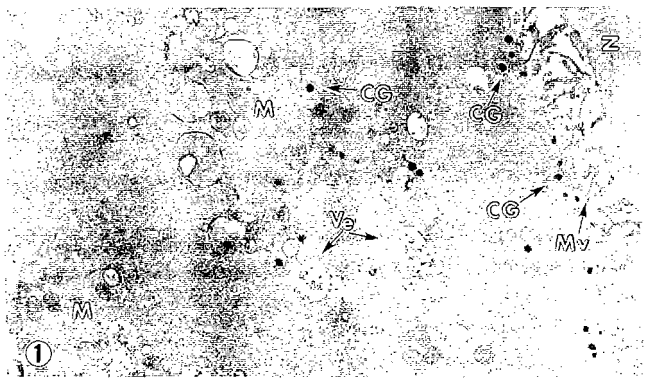

Figure 1. Cumulus cells enclosed oocyte (CO) in control group. Cortical granulosa (CG), mitochondria (M), microvilli (Mv), vesicle and lipid droplet (Ve), zona pellucida (Z).×17,600

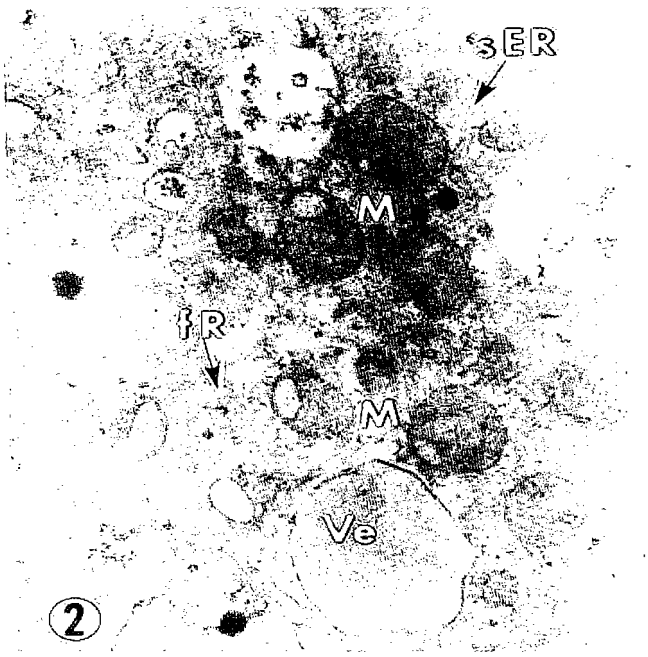

Figure 2. Hooded or oval shape mitochondria (M) of oocyte of CO in the control group. Smooth endoplasmic reticulum (sER), and free ribosome (fR).×16,500

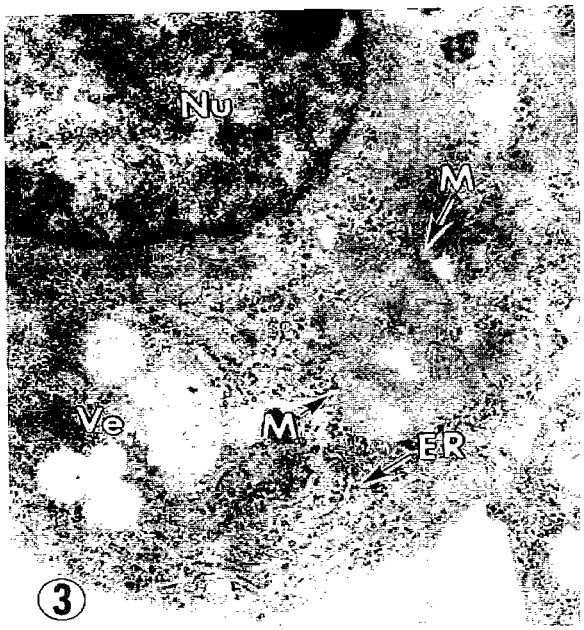

Figure 3. Cumulus cells of CO in the control group. Smooth and rough endoplasmic reticulum (ER), Oval or prolated shaped mitochondria (M), nuclevs (Nu), vesicle and lipid droplet (Ve).×14,300

peripheral site of the mitochondria. Endoplasmic reticulum was also swollen. Some vesicles were fused together. For the observation of cumulus cells, cytoplasm of the cells was drastically higher than that of the control group. All of the endoplasmic reticulum were swollen, and the nucleus membrane was also sightly swollen compared to the control group. The chromatin of the nuclevs was larger than that in the control group. Most of the mitochondria disappeared (fig. 4, 5, 6).

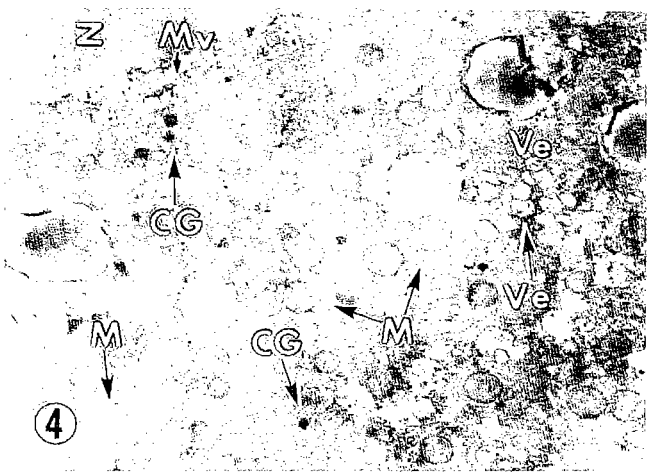

Figure 4. Equilibrated oocyte of CO with the cryoprotectant. Note the swollen mitochondria (M). Cortical granulosa (CG), microvilli (Mv), vesicle and lipid droplet (Ve), zona pellucida (Z).×7,000

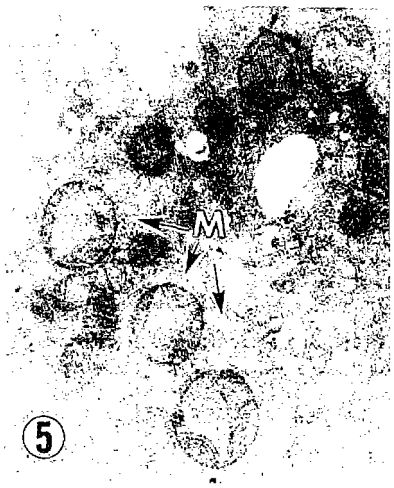

Figure 5. Swollen mitochondria (M) with peripherally located cristae of the oocyte in CO equilibrated with the cryoprotectant.×16,400

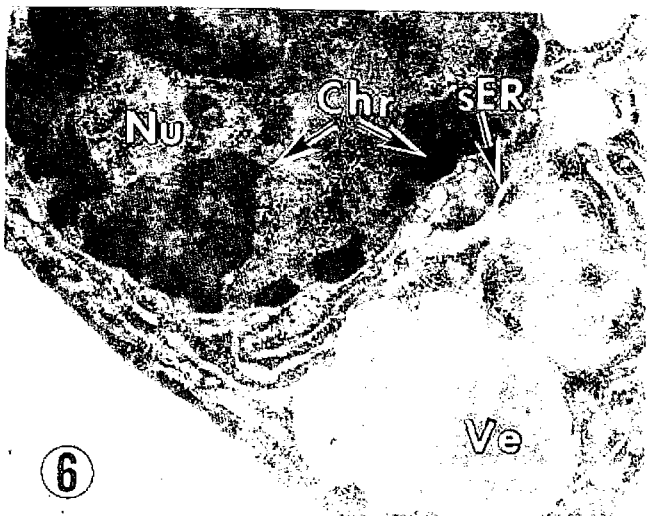

Figure 6. Cumulus cells of the CO equilibrated with the cryoprotectant. Chromosome (Chr), nuclear (Nu), smooth endoplasmic reticulum (sER), vesicle and lipid droplet (Ve). ×14,300

After removal of cryoprotectant, a large proportion of swollen mitochondria of the oocytes were recovered to the normal shape which can be observed in the control group. A lot of CG disappeared from the oocyte cytoplasm. Some vesicles were fused together, an some of the mitochondria of the cumulus cells were swollen with no or few cristae. Most of the endoplasmic reticulum were also swollen. Some of the vesicles were fused together (fig. 7, 8, 9).

The CO in the carrier solution showed similar structure to the control group. However, CG were slightly dispersed

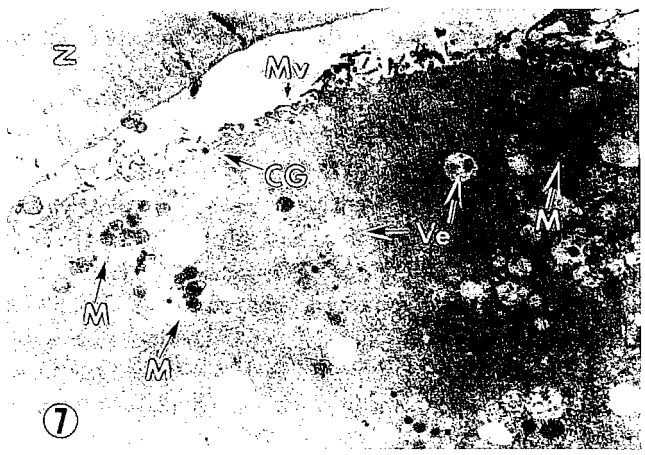

Figure 7. The oocyte of CO removed from the cryoprotectant. Cortical granulosa (CG), mitochondria (M), microvilli (Mv), zona pellucida (Z).×4,200

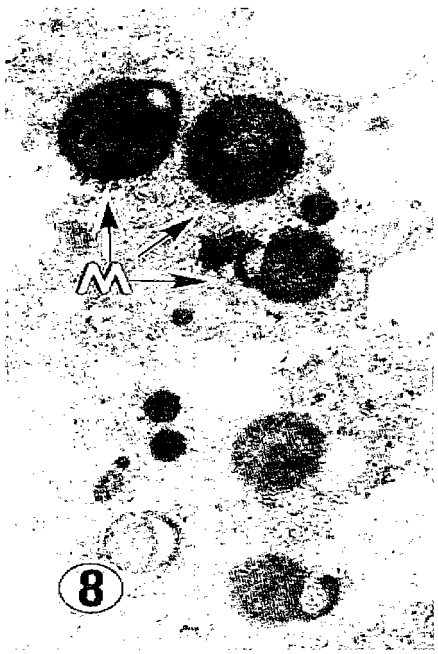

Figure 8. The mitochondria of the oocyte in the removal group.×15,900

within the cytoplasm of the oocytes compared with those in the control group. The number of vesicles was greater than in the control group. For the observation of cumulus cells, the structure was almost similar to that of the control group.

The structural changes of the cell organelle in the DO in each treatment group were observed the same as the oocytes in each treated CO group.

Embryonic development assay

The cleavage rate on the day 3 after insemination from the each group was the almost the same, and no significant differences were found among groups. However, the percent

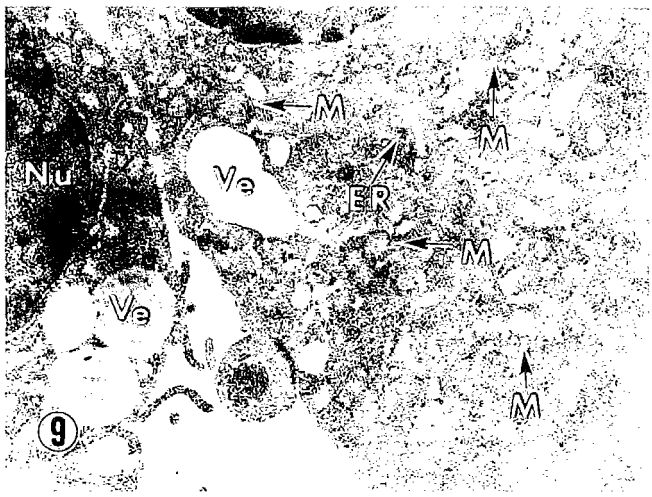

Figure 9. The cumulus cells of CO in the removal group. Note the swollen or prolated shaped mitochondria (M). Swollen endoplasmic reticulum (ER), nuclear (Nu), vesicle and lipid droplet (Ve).×14,300

of development up to the blastocyst stage in removed group was significantly lower ($p<0.05$) than the control group (table 2). In addition, the development rate up to the blastocyst stage in the carrier solution was also significantly lower ($p<0.05$) than that of the control group, and this value was not significantly different compared with the removal group of the cryoprotectant.

DISCUSSION

In the present study, although the oocytes were matured with 50 μM DMSO, the ATP content of DO were not lower than that in previous reports. The ATP content of the control group was 0.877 pmol/DO in the present study. Rieger (1997) reported that the ATP content of bovine oocytes matured *in vitro* was 0.85 pmol/denuded oocyte. On the other hand, Forman et al. (1999) indicated that the ATP content of HeLa cells cultured with DMSO at 0.1% concentration was suppressed. We used 50 μM DMSO during *in vitro* maturation to improve the nuclear maturation

Table 2. The effect of addition or removal of a cryoprotectant on the embryonic development of bovine oocytes matured *in vitro*

Treatment	N	Cleavage rate of bovine oocytes from 2 to 16 cells stage %	The number of the development up to the blastocyst stage %
Control	76	50.0	17.1[a]
Carrier	90	51.1	5.6[ab]
Removed	91	47.3	2.2[b]

a,b; Values with different superscripts are significantly ($p<0.05$) different within a column.

of the oocytes (Tsuzuki et al., 1998). In the light of this, it is suggested that the addition of DMSO at the 50μM concentration to the maturation medium may not suppress the ATP contents of the bovine oocytes *in vitro* The ATP contents of DO equilibrated with the cryoprotectant were significantly decreased ($p<0.05$) compared with those in the control groups. However, the ATP content of DO removed from the cryoprotectant recovered to the level of that in the control group. Again, Forman et al. (1999) indicated that more than 0.1% DMSO in the cultural medium can suppress the ATP convent of the HeLa cells. We used the DMSO and sucrose combination as a cryoprotectant. It is well known that although sucrose cannot penetrate to the plasma membrane of the cells, DMSO can penetrate to the inside of the cells (Friedler et al., 1988). Therefore, the decrease of the ATP content in DO may be caused by the exposure to 1.5 M DMSO, a composition of the cryoprotectant in the present study. However, the decrease of the ATP contents in the DO can be lifted by the removal of the cryoprotectant. This indicates that the suppressive effect of the DMSO for ATP may be restricted to the exposure period for DO. On the contrary, the decreased ATP content of the CO exposed to the cryoprotectant was not recovered to the level of that in the control group. This decrease seemed to be caused by the cumulus cells, not oocytes. It is suggested that the sensitivity for the DMSO may differ between the oocyte and the cumulus cells.

In general, ATP is generated by the mitochondrial oxidative system, and the suppression by DMSO may continue for the cumulus cells, not the oocytes, after removal of the cryoprotectant. These changes might influence the further development of the embryos. Matsui et al. (1995) indicated that cumulus cells were in important factor for the sperm penetration. Therefore, it is considered that suppression of ATP in cumulus cells in the present study might be one of the factors to induce the decrease of embryonic development.

Ultrastructural aspect of control group was almost the same as reported earlier (Schmidt et al., 1995; Zhao et al., 1997). However, few oocytes which had peripherally arranged cell organelle were observed. de-Loos et al. (1992) indicated that the oocytes which had a cortical arranged cell organelle seemed to be delayed in their cytoplasmic maturation. Therefore, the presence of cortical arranged cell organelle might cause the delay of the maturation *in vitro*, and this suggests the *in vitro* maturation system in this study may not be fully appropriate for the oocytes.

From the observation of the equilibrated oocytes with the cryoprotectant by TEM, the almost all mitochondria of the cytoplasmic of the oocytes were swollen and their cristae were located in peripheral sites within the mitochondrial inner membrane. However, after removal of the cryoprotectant, the large proportion of these mitochondria recovered to those of non treated (control) group. The swollen mitochondria were reported in rhesus kidney cells cultured with DMSO (Malinin, 1973) or bovine trophectoderm cells developed *in vivo* for 13 days after insemination and not removed from glycerol solution after freeze-thaw (Mohr and Trounson, 1981). The mitochondria of *Oedogonium cardiacum* were also swollen by the addition of the 5 or 7.5% DMSO (Foissner, 1986). These changes were considered to be involved in the changes of the electron transport chain of mitochondria drastically affected by DMSO (Malinin, 1973). However, antimycin A, a inhibitor for the enzyme between the cytochrome b and c_1 in the electron transport chain of mitochondria, could not induce the structural change of the mitochondria of *Oedogonium cardiacum* when exposed to DMSO (Foissner, 1986). With these reports, it is postulated that the swelling of the mitochondria of the oocytes or cumulus cells in the present study might not have occurred from the inhibition of the cytochrome b and c_1 by the exposure of DMSO. In this regard, Baxter and Lathe (1971) indicated that suppression of ATP in rat kidney might be inhibited of glycolysis by DMSO. With these reports, it is postulated that the decrease the ATP content in the present study may be caused by the inhibition of glycolysis, not electron transport of the mitochondria, by the exposure to DMSO.

The development rate of the CO equilibrated with and removed from the cryoprotectant was significantly lower than that of control. In humans, higher ATP content is associated with better embryonic development (van Blerkom et al., 1995). Therefore, lower level of the ATP in CO might be one of the reasons to induce the lower embryonic development.

Carrier solution treated CO and DO showed the same level of ATP contents, however the development rate up to the blastocyst stage was lower ($p<0.05$) than control group. On the other hand, cortical granules were slightly dispersed within the cytoplasm of the oocytes, and increase of the small vesicles was seen in the ooplasm of CO and DO treated with carrier solution. It is well known that cortical granules can play a significant role to avoid polyspermy. For the mouse, cooling the oocytes can cause the polyspermy (Parks and Ruffing, 1992). Therefore, the possible reason for the decrease in the embryonic development might be that the dislocation of the cortical granules at the ooplasm resulted in polyspermy.

In conclusion, the addition of the cryoprotectant (1.5 M DMSO plus 0.25 M sucrose can cause a temporary decrease in the ATP production with swelling of the mitochondria, and the removal of the cryoprotectant can recover the ATP content of DO, but not CO. The ATP metabolism may be suppressed drastically for the cumulus cells by the exposure to the cryoprotectant.

ACKNOWLEDGMENTS

This study was supported by a research grant for food and meat products from the Ito Memorial Research Foundation, Tokyo, Japan. We also thank the Livestock Improvement Association of Miyazaki Prefecture, and Miyazaki Prefectural Meat Inspection Center of Miyakonojo-Division, for providing frozen semen and the ovaries.

REFERENCES

Baxter, S. J. and G. H. Lathe. 1971. Biochemical effects on kidney of exposure to high concentrations of dimethyl sulphoxide. Biochem. Pharm. 20:1079-1091.

Brackett, B. G. and G. Oliphant. 1975. Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod. 12:260-274.

de-Loos, F., P. van Maurik, T. van Beneden and T.A.M. Kruip. 1992. Structural aspects of bovine oocyte maturation *in vitro*. Mol. Reprod. Dev. 31:208-214.

Foissner, I. 1986. Septation and fragmentation in *Oedogonium* mitochondria as different and independent effects of dimethyl sulfoxide (DMSO) treatment. Experientia 42:958-960.

Forman, S., J. Kas, F. Fini, M. Steinberg and T. Ruml. 1999. The effect of different solvents on the ATP/ADP content and growth properties of HeLa cells. J. Biochem. Mol. Toxicol. 13:11-15.

Friedler, S., L. C. Giudice and E. J. Lamb. 1988. Cryopreservation of embryos and ova. Fertil. Steril. 49:743-764.

Horiuchi, T., Y. Yamauchi and M. Yamada. 1998. ATP in hamster embryos developing from 2-cell to blastocyst stage *in vitro* and at the 2-cell block. J. Mamm. Ova Res. 15:31-36.

Lim, J. M., J. J. Ko, W. S. Hwang, H. M. Chung and K. Niwa. 1999. Development of *in vitro* matured bovine oocytes after cryopreservation with different cryoprotectants. Theriogenology 51:1303-1310.

Malinin, G. I. 1973. Cytotoxic effect of dimethylsulfoxide on the ultrastructure of cultured rhesus kidney cells. Cryobiology 10:22-32.

Matsui, M., Y. Takahashi, M. Hishinuma and H. Kanagawa. 1995. Effects of supplementation of the maturation media with insulin on *in vitro* maturation and *in vitro* fertilization of bovine oocytes. Jpn. J. Vet. Res. 43:145-153.

Mohr, L. R. and A. O. Trounson. 1981. Structural changes associated with freezing of bovine embryos. Biol. Reprod. 25:1009-1025.

Otoi, T., K. Yamamoto, N. Koyama, S. Tachikawa and T. Suzuki. 1996. A frozen-thawed *in vitro*-matured bovine oocytes derived calf with normal growth and fertility. J. Vet. Med. Sci. 58:811-813.

Parks, J. E. and N. A. Ruffing. 1992. Factors affecting low temperature survival of mammalian oocytes. Theriogenology 37:59-73.

Rieger, D. 1997. Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using a scintillation counter to measure the chemiluminescence produced by the luciferin-luciferase reaction. Anal. Biochem. 246:67-70.

Rittmeyer, I. C. and U. E. Nydegger. 1992. Influence of the cryoprotectant agents glycerol and hydroxyethyl starch on red blood cell ATP and 2,3-diphosphoglyceric acid levels. Vox. Sang. 62:141-145.

Rosenkrans, C. F. Jr. and N. L. First. 1991. Cultured bovine zygotes to the blastocyst stage : Effect of amino acids and vitamins. Theriogenology 35:266(Abstr.).

Schalkoff, M. E., S. P. Oskowitz and R. D. Powers. 1989. Ultrastructural observations of human and mouse oocytes treated with cryopreservatives. Biol. Reprod. 40:379-393.

Schmidt, M., P. Hyttel, B. Avary and T. Greve. 1995. Ultrastructure of *in vitro* matured bovine oocytes after controlled freezing in 10% glycerol. Anim. Reprod. Sci. 37:281-290.

Snedecor, G. W. and W. G. Cochran. 1980. Statistical Methods. 7th Ed. The Iowa State University Press, Iowa. pp. 64-82.

Tsuzuki, Y., D. H. Duran, Y. Kuroki, F. Uehara, K. Ashizawa and N. Fujihara. 1998. The effects of dimethyl-sulfoxide on the *in vitro* maturation and fertilization of bovine oocytes and the subsequent development. Asian-Aust. J. Anim. Sci. 11:307-310.

Van Blerkom, J., P. W. Davis and J. Lee. 1995. ATP content of human oocytes and developmental potential and outcome after *in vitro* fertilization and embryo transfer. Hum. Reprod. 10:415-424.

Wishart, G. J. 1982. Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa *in vitro*. J. Reprod. Fertil. 66:457-462.

Zhao, J., M. Hattori and N. Fujihara. 1997. Ultrastructural comparison between immature and *in vitro* matured bovine oocytes cryopreserved in propandiol. J. Mamm. Ova Res. 14:84-94.13591359