Butyrolactone-I Reversibly Inhibits but does not Improve the Maturation and Subsequent Development of Sheep Oocytes *In vitro*

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Abstract: Butyrolactone-I (BL-I) which is a selective inhibitor of cyclin-dependent kinases, competitively binds p34^{cdc2} and inhibits meiotic resumption. However, the function of BL-I in improving the synchronization of nuclear and cytoplasmic maturation of mammalian oocytes *in vitro* remains unknown. In the present study, the effects of different concentrations and durations of BL-I treatment on the *In vitro* Maturation (IVM) of sheep oocytes were assessed together with the effects after removal of inhibition for different durations. The developmental competence of ovine oocytes was also determined based on the maturation rate and blastocyst rate of ovine parthenogenetic and *in vitro* fertilized embryos. The ultrastructure of oocytes treated by BL-I was also observed and analyzed by transmission electron microscopy. The germinal vesicle rate was arrested in 62.9% of oocytes treated by *in vitro* culture for 24 h with 150 μmol L^{-1} BL-I. The maturation and blastocyst rates of the treatment groups were 70.83 and 20.33% vs. 63.44 and 24.39% in the control but these differences were not significant. The number of blastocysts produced by IVC for 24 h after removal of inhibition was significantly higher than that for the control (71.26±32.68% vs. 66.73±21.64%, p >0.05). Furthermore, electron micrography analysis also showed some differences with regard to the structure of microvilli, mitochondria and granular cells. The BL-I-induced inhibition of nuclear maturation in ovine oocytes did not appear to play a role in improving their IVM and embryonic development via enhancement of the synchronization of nuclear and cytoplasmic maturation but cytoplasmic maturation was still promoted. In conclusion, BL-I reversibly inhibits IVM of ovine oocytes but does not improve their maturation and subsequent *in vitro* development.

Key words: Sheep, oocyte, *in vitro* maturation, butyrolactone-I, ovine

INTRODUCTION

Livestock oocytes that completely mature *in vitro* and *in vivo* are highly sought in the animal embryonic engineering field but the status of maturation of these oocytes *in vitro* is normally much lower than that *in vivo*. In addition, it has also been widely reported that synchronized maturation of the nucleus and cytoplasm is also necessary so that oocytes can support their subsequent fertilization and embryonic development (Trounson *et al.*, 2001). It has been found that temporal blocking of the meiotic maturation of murine antral follicles may promote the developmental competence of oocytes during their *in vivo* maturation (Nogueira *et al.*, 2003). Normally, meiotic maturation is considered to be much faster than cytoplasmic maturation during *In vitro*

Maturation (IVM) because of the lack of precise control observed *in vivo*. Therefore, cytoplasmic maturation *in vitro* is often not complete. Poor maturation of oocytes has also been known to result in a lack of further embryonic development *in vitro* (Carneiro *et al.*, 2002).

Studies regarding oocyte coculture, antral follicles, meiosis maturation inhibitors and cytoplasmic maturation catalysts have suggested that the synchronization of nuclear and cytoplasmic maturation *in vitro* for mammalian oocytes may be beneficial for improving their further fertilization and early embryonic development. In addition, it has been reported that Butyrolactone (BL-I) can inhibit Maturation-Promoting Factor (MPF) to interrupt meiotic resumption (Mermillod *et al.*, 2000; Hashimoto *et al.*, 2002). Furthermore, BL-I has also been found to improve the developmental potential of other

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livestock but not of ovine immature oocytes *in vitro* (Wu *et al.*, 2002) and the ultrastructure of these oocytes was also found to be affected (Fair *et al.*, 2002; Lonergan *et al.*, 2003). However, the effect of BL-I on IVM of sheep oocytes remains unknown. The present study therefore, examined the optimal incubation concentration and duration of BL-I treatment for ovine oocytes matured *in vitro*, as well as their *In vitro* Fertilization (IVF) and ultrastructural features after BL-I treatment.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. The Tissue Culture Medium 199 (TCM, 199) was from Gibco (Grand Island, NY); BL-I was from Biomol (Plymouth Meeting, PA).

Sheep oocyte collection and IVM: Ovaries collected from slaughtered sheep were transported in Ca^{2+} and Mg^{2+} supplemented physiological saline to the laboratory; this step lasted for 2-4 h and the samples were maintained at 25-28°C during this period. Subsequently, the ovaries were briefly washed twice in physiological saline. Sheep oocytes were collected by slicing the follicles (diameter, 2-5 mm) and oocytes with intact cumulus cells and evenly granulated cytoplasm (Cumulus-Oocyte Complex, COCs) were selected and randomly assigned to each treatment. Each group of 10 COCs was introduced into a 50 μL droplet of medium placed in a plastic dish covered with mineral oil. The COCs were cultured at 38.5°C under 5% CO_2 with high humidity.

Immature ovine oocytes were washed 6 times and cultured in basic culture medium with TCM199, 10 mmol L^{-1} HEPES, 0.6% Bovine Serum Albumin (BSA), 1 μg mL^{-1} E_2, 10% FCS, 0.25 mmol L^{-1} Na-Py, 10 μg mL^{-1} FSH, 10 μg mL^{-1} LH and 25 ng mL^{-1} EGF after they were incubated in BL-I-containing medium.

IVF: Fresh semen from a breeding ram of proven fertility was collected, washed in Synthetic Oviduct Fluid (SOF) and centrifuged twice at 200×g for 5 min. After the second round of centrifugation, the pelleted sperm was layered under 1 mL of fertilization medium (SOF+20% heat-inactivated estrous sheep serum+10 mmol L^{-1} penicillamine+10 mmol L^{-1} hypotaurine+10 μg mL^{-1} heparin+0.5 mol L^{-1} calcium lactate+100 IU mL^{-1} penicillin +100 μg mL^{-1} streptomycin) and incubated for 30 min at 38.5°C for swim-up. Supernatants containing the highly motile spermatozoa were added to the fertilization medium. The final concentration of the spermatozoa was $5×10^6$ mL^{-1}.

For fertilization, OCCs that had been washed 3 times in fertilization medium were placed into each droplet

containing spermatozoa. The maximum number of oocytes per drop was 15. IVF was performed at 38.5°C under 5% CO_2 in air at high humidity for 17-19 h.

In vitro culture: After IVF, presumptive zygotes were denuded of the surrounding cumulus cells by passing them through a fine pipette in SOF with 10 mmol L^{-1} HEPES and 0.3% BSA. They were then segregated to 50 μL culture drops (10 embryos/drop) consisting of SOF supplemented with 2% (v/v) BME-essential amino acids, 1% (v/v) MEM with nonessential amino acids and 10% FCS and incubated at 38.5°C under 5% CO_2 in air at high humidity. Culturing was continued until 6-10 days after fertilization. The cleavage rate was recorded once on day 2 and the blastocyst number was recorded on day 6 after insemination (day 0 = day of IVF).

For estimation of the blastocyst cell number, blastocysts were collected and washed in PBS without BSA or serum. They were subsequently stained for 10 min by using Hoechst 33342 (10 mg mL^{-1} in 2.3% (w/v) sodium citrate). Then, they were washed in PBS, placed on a slide and visualized using a fluorescence microscope.

Determination of oocyte nuclear maturation: COCs were cultured in TCM199+2.2 mg mL^{-1} NaHCO$_3$+10 mmol L^{-1} HEPES+0.6% BSA+0.25 mmol L^{-1} Na-Py+2.75 mmol L^{-1} sodium lactate+100 IU mL^{-1} penicillin+100 μg mL^{-1} streptomycin with or without BL-I (10, 50, 100 and 150 μM). They were then denuded, fixed in acetic acid (90%)-ethanol (3:1, v/v) overnight at 4°C, stained with acetic orcein and observed under a microscope to determine their nuclear stage.

Processing of sheep oocytes for transmission electron microscopy: Immature oocytes, oocytes cultured *in vitro* for 8 h (normal culture group and BL-I-treated group) and oocytes cultured *in vitro* for 24 h (normal culture group and BL-I-treated group) were collected. The oocytes were washed in PBS and fixed in 2.5% glutaraldehyde overnight at 4°C. Then, they were fixed in 1% OsO$_4$ in 0.1 M cacodylate buffer for 60 min. The samples were dehydrated through ascending concentrations of acetone (30, 50, 70, 90, 100 and 100%) and embedded in Epon. Ultrathin sections (80 nm) were collected on copper grids, contrasted with uranyl acetate and lead citrate and examined using a transmission electron microscope (Jeol, JEM-1230, USA). Cytoplasmic analyses were conducted using the midsection of all oocytes.

Statistical analysis: Data were analyzed by ANOVA by using the general linear models in the Statistical Analysis System (SAS Institute, Cary, NC) software to determine differences between the results for the treatments. Differences were considered to be significant when p was <0.05.

RESULTS

IVM of sheep oocytes incubated with different BI-L concentrations: Immature ovine oocytes were incubated in medium with different concentrations of BL-I for 24 h. The integrity of Germinal Vesicles (GVs) was observed after they were stained with acetic orcein. The number of GV-stage oocytes increased as the concentration of BL-I in the medium increased and 42.37 and 62.9% of oocytes were inhibited at the GV stage when incubated with 100 and 150 µM BL-I, respectively. This proportion was significantly different from that for the negative control (p<0.01). Additionally, 150 µM BL-I extensively blocked GV Breakdown (GVBD) (Fig. 1) with few cumulus cells diffusing during incubation.

Effects of incubation duration with BL-I on oocyte maturation *in vitro*: Ovine oocytes were incubated in medium containing 150 µM BL-I for 8, 16 and 24 h. The GVBD of the ovine oocytes was then observed after they were stained with acetic orcein and 60.78, 57.45 and 60% of oocytes were observed to be at the GV stage (Fig. 2) after incubation with BL-I for 8, 16 and 24 h, respectively. The number of oocytes at the GV stage was not significantly different among the experimental groups (p>0.05) but the results for the experimental groups significantly differed from those for the control group (p<0.05).
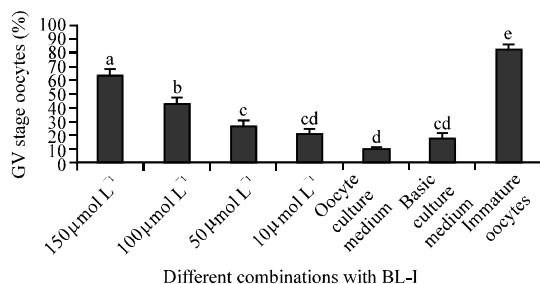

Fig. 1: GVBD of ovine oocytes in different combinations with BL-I (24 h incubation)
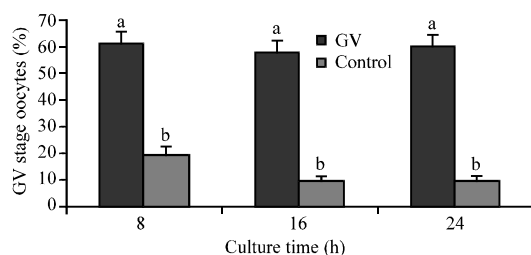

Fig. 2: Effect of different durations of BL-I treatment on GVBD of ovine oocytes

Maturation of ovine oocytes after incubation with BL-I for 8 h: The maturation of ovine oocytes incubated in maturation medium for 16, 20 and 24 h after incubation in 150 µM BL-I for 8 h was analyzed. The proportions of MII-stage oocytes treated with BL-I for 8, 16 and 24 h were 52.27, 65.38 and 70.83% (Fig. 3), respectively but the maturation rates of MII-stage oocytes for all these treatment groups did not significantly differ from those for the control (63.44, 64.29 and 67.5%, respectively; p>0.05).

IVF and developmental competence of ovine embryos after incubation of oocytes with BL-I for 8 h: Oocytes were allowed to recover for 16, 20 and 24 h after they were treated with BL-I for 8 h and then the blastocyst rate and cleavage rate of the *in vitro* fertilized oocytes were analyzed. The blastocyst rate of oocytes allowed to recover for 16 h was lower than that of the control group (2.99 vs. 24.39%, p<0.05). The blastocyst rate and cleavage rate did not significantly differ (p>0.05) between the control group and the experimental groups that were allowed to recover for 20 and 24 h (Table 1).

Effect of disinhibition of BL-I on blastocyst cell number: The number of blastocysts in the group allowed to recover for 20 h after BL-I treatment for 8 h did not significantly differ (p>0.05) from that for the control group allowed to recover for 20 h after 8 h of BL-I treatment. The number of blastocysts in the group allowed to recover for 24 h was higher than that in the control group but the results were not significantly different (p>0.05; Table 2).
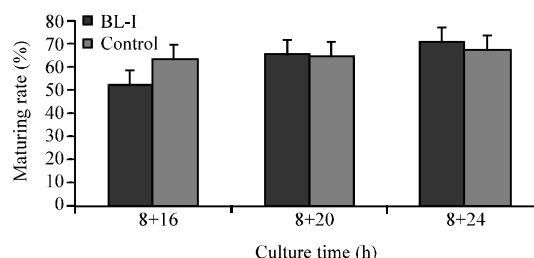

Fig. 3: Maturation rates of ovine oocytes treated with BL-I for different durations

Table 1: *In vitro* fertilization and developmental competence of ovine embryos after removal of BL-I

Treatments	Oocyte number	Cleavage rate (%)	8-16 cell rate (%)	Blastocyst rate (%)
8+16 h	134	56.72 (76/134)[a]	41.04 (55/134)[a]	2.99 (4/134)[a]
8+20 h	110	62.73 (69/110)[ab]	50.91 (56/110)[ab]	13.64 (15/110)[b]
8+24 h	123	69.92 (86/123)[b]	54.47 (67/123)[b]	20.33 (25/123)[bc]
24 h (control)	123	66.67 (82/123)[ab]	50.41 (62/123)[ab]	24.39 (30/123)[c]

[a-c]Means within the same row that do not share a common superscript are significantly different (p<0.05); n = 6

Effect of BL-I on the ultrastructure of ovine oocytes:
Before *in vitro* culture, the granular cells had many organelles. The oocyte had a polymorphous nucleus. The cytoplasm tuber traversed the pellucid zone and reached

Table 2: Effect of disinhibition of BL-I on the inner cell number of ovine blastocysts

Treatments	No. of blastocysts	Inner cell number of ovine blastocysts
8+20 h	11	55.09±22.08
8+24 h	15	71.26±32.68
(24 h) Control	15	66.73±21.64

the oocyte surface. The outer layer of the pellucid zone showed rarefaction and the inner layer was dense. The pellucid zone integrated tightly with the oocyte. Microvilli were rare and did not exit the pellucid zone. GVs were localized to 1 side of the oocytes. Lipids were distributed throughout the cortical area in two forms: One was grey and uniform in texture and the other was electron-lucent. Many mitochondria were distributed in the cortical area. After 8 h of IVM, in the normally cultured groups, some granular cells (Fig. 4(1)) were scattered and the microvilli

JEM-1230 80 kv
1200× 0002 20 μm

JEM-1230 80 kv
1200× 0002 20 μm

JEM-1230 80 kv
12000× 0002 20 μm

JEM-1230 80 kv
10000× 0002 20 μm

JEM-1230 80 kv
8000× 0002 5 μm

JEM-1230 80 kv
8000× 0002 2 μm

JEM-1230 80 kv
20000× 0002 1 μm

JEM-1230 80 kv
20000× 0002 1 μm

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15000× 0002 1 μm

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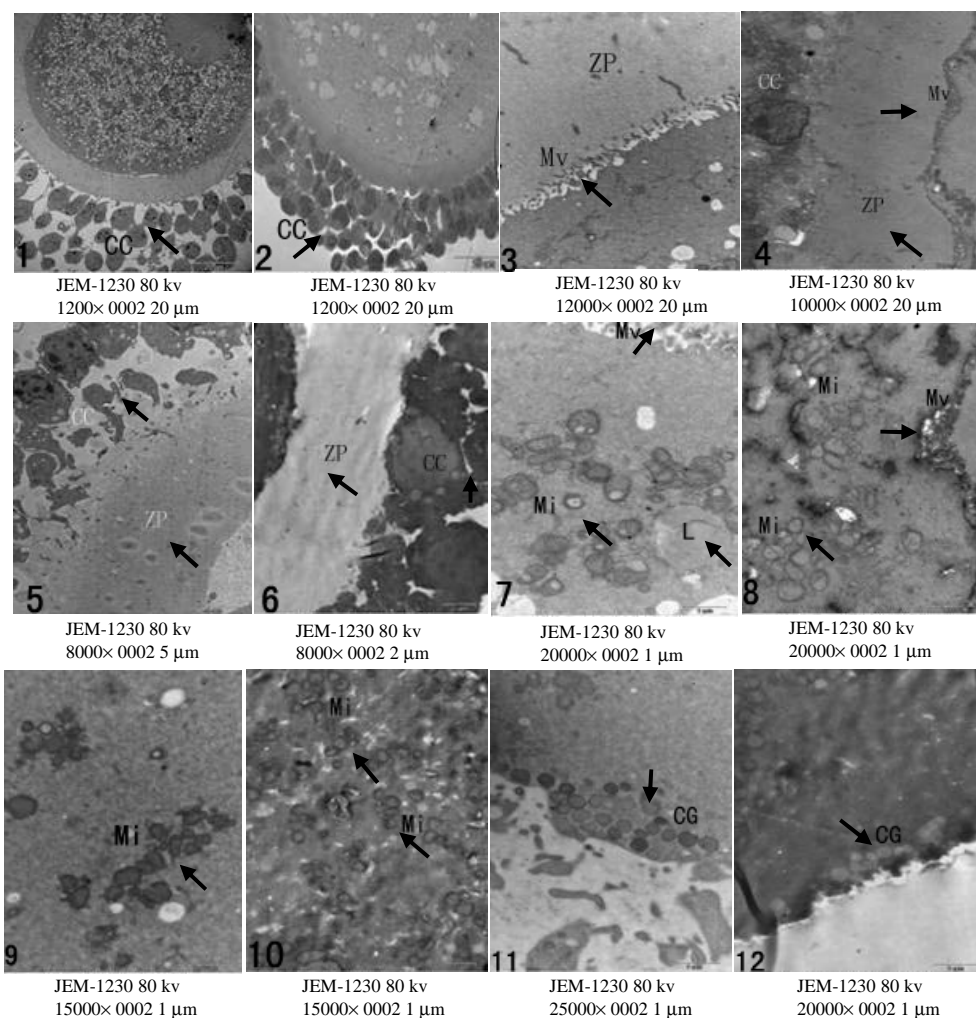25000× 0002 1 μm

JEM-1230 80 kv
20000× 0002 1 μm

Fig. 4: Electronic microscope photographs of ovine oocytes subcellular structure before or after BL-I treatment; 1) Granular cells incubated for 8 h (×1200); 2) Granular cells incubated with BL-I for 8 h (×1200); 3) Zona pellucida and microvilli of oocytes incubated for 8 h (×12,000); 4) Zona pellucida and microvilli of oocytes incubated with BL-I for 8 h (×10,000); 5) Zona pellucida and microvilli of oocytes incubated for 24 h (×8000); 6) Zona pellucida and microvilli of oocytes incubated with BL-I for 24 h (×8000); 7) Mitochondria of oocytes incubated for 8 h (×20,000); 8) Mitochondria of oocytes incubated with BL-I for 8 h (×20,000); 9) Mitochondria of oocytes incubated for 24 h (×15,000); 10) Mitochondria of oocytes allowed to recover for 24 h after treatment with BL-I for 8 h (×15,000); 11) Cortical granule of oocytes incubated with BL-I for 24 h (×25,000); 12) Cortical granule of oocytes allowed to recover for 24 h after treatment with BL-I for 8 h (×20,000)

were slender and did not exit the zona pellucida (Fig. 4(3)). The zona pellucida appeared as it did before culture. Some GVs were integrated and many mitochondria had gathered in the cortical area (Fig. 4(7)).

In the BL-I-treated groups, the granular cells (Fig. 4(2)) gathered together tightly. The microvilli (Fig. 4(4)) increased in number and coarseness. All GVs were integrated and many mitochondria and cortical granules gathered in the cortical area. However, the cristae of the mitochondria were few in number and appeared blurry (Fig. 4(8)).

Ovine oocytes cultured *in vitro* with normal medium for 24 h: The cell process of the granular cells was found to have retracted and the amount of cytoplasm had decreased. The outer layer of the zona pellucida had ramified but the inner layer had loosened. A perivitelline space had appeared between the zona pellucida and the oocyte. The microvilli had increased in number and become short and thick. The microvilli had exited the zona pellucida and had become lodged on the oocyte surface (Fig. 4(5)). The cortical granules were clustered or gathered in 2 or 3 layers under the plasma membrane (Fig. 4 (9)). Most of the mitochondria were scattered (Fig. 4(11)).

Recovery of ovine oocytes for 24 h after treatment with BL-I for 8 h: The connection between the granular cells and oocytes had decreased. Vacuoles had appeared in the cytoplasm of the granular cells. The zona pellucida was dense and the microvilli were rare and thick (Fig. 4(6)). Cortical granules were found to be distributed homogeneously in the monolayer under the plasma membrane (Fig. 4(12)). The mitochondria were distributed homogeneously in the cytoplasm (Fig. 4(10)).

DISCUSSION

It is well known that MPF which comprises the catalytic subunit p34^{cdc2} and the regulatory subunit cyclin B is an important regulatory factor of the meiotic cell cycle. MPF activity is necessary for oocyte GVBD (Zhang and Cao, 2005) and is regulated by binding to cyclin B as well as phosphorylation and dephosphorylation of p34^{cdc2}. BL-I is a specific inhibitor of Cyclin-Dependent Kinases (CDKs) that can competitively bind to cdc2 with ATP and then inhibit cdc2 dephosphorylation. When MPF cannot be activated, oocyte meiosis cannot be completed and oocytes are kept in the retention stage (Mermillod *et al.*, 2000; Hashimoto *et al.*, 2002). Many studies have shown that

the oocytes of cattle, pigs, horses and mice stay in the GV stage and maintain their developmental potential under solo or synergistic treatment with BL-I.

The present investigation indicated that BL-I could inhibit the GVBD of ovine oocytes at different doses and incubation durations. The inhibitory effect of BL-I on the IVM of ovine oocytes increased with increase in its concentration but there were no significant differences between the 2 inhibitor treatment groups and the inhibition did not vary upon supplementation with >200 µmol L^{-1} roscovitine. However, significant differences were noted among groups receiving different concentrations of BL-I. It has been reported that 80% of the GVBD oocytes from bovine were inhibited in medium supplemented with Polyvinyl Alcohol (PVA) or FBS and 100 µmol L^{-1} BL-I and that the inhibition effect in the group receiving PVA was much better than that in the group receiving FBS (Kubelka *et al.*, 2000; Hashimoto *et al.*, 2002). Previous studies have reported that the GVBD for immature swine oocytes in medium containing PVA and 12.5 µmol L^{-1} BL-I was inhibited by >90% (Wu *et al.*, 2002) and that 25 µM ROSC inhibited 83.7% of bovine oocytes at the GV stage (Mermillod *et al.*, 2000). In another study, approximately 86.2% of swine oocytes were found to be inhibited at their GV stage when incubated with 50 µM ROSC for 30 h (Krischek and Meinecke, 2001) and Adona *et al.* (2008) reported that 89% of cattle oocytes were arrested in the GV stage after 24 h of treatment with 6.25 µM BL-I and 12.5 µM ROS. Although, these differing results may have been the result of the use of different animal species, these results all show that inhibition of livestock oocyte maturation by BL-I can persist for a long duration. The results also indicate that the maturation of ovine oocytes was not affected by the removal of these inhibitors. The fertilization and blastocyst rates, as well as blastocyst cell numbers were not significantly different between the control group and the BL-I-treated groups after recovery in normal medium for 20 and 24 h. These results suggest that the developmental capacity of ovine oocytes matured *in vitro* was not damaged by BL-I treatment. Similar results have also been reported for swine and bovine oocytes (Kubelka *et al.*, 2000; Marchal *et al.*, 2001; Weng *et al.*, 2007).

For further analysis of the cytoplasmic maturity of ovine oocytes that were treated with BL-I for 8 h and then allowed to recover for 24 h, transmission electron microscopy was used for analyzing ultrastructural changes in connection with the IVF results. The degree of granular cell diffusion and the morphological features of microvilli in the BL-I-treated oocytes were inferior to those

in the control group. Fair *et al.* (2002) and Lonergan *et al.* (2003) have also reported similar results on bovine. Cumulus cells were mature and diffuse such that the tight junctions between cumulus cells or between cumulus cells and oocytes were interrupted and the nutrient and substance exchange channels were deoppilated. The gap junctions between oocytes and granular cells are tight and the microvilli are normally used to transmit signals among the gap junctions to regulate the growth and maturation of oocytes (Zhang *et al.*, 2005). *In vitro* development of oocytes may stop if the gap junctions are damaged at an early stage (Wang and Zeng, 2002). The morphological features of the microvilli and granular cells of the BL-I-treated oocytes suggested that the exchange of material between the cumulus oophorus and oocytes was interrupted such that the appropriate development of these oocytes was also negatively influenced.

The mitochondrial cristae of oocytes treated with BL-I for 8 h were parum and ambiguity but those of oocytes allowed to recover for 24 h after being treated with BL-I for 8 h seemed to be more well-proportioned than those of the control and the distribution of cortical granules in the treated oocytes was not different from that of the control group. Mitochondria are widely considered to play an important role in cell metabolism and their shape, quantity and distribution are closely related to cellular metabolism, proliferation and differentiation. Mitochondria scattered from the cortex to the central region of cytoplasm would provide favorable energy utilization during oocyte maturation. In addition, the distribution and metabolic activation of mitochondria are important factors for cytoplasmic maturation and recovery of meiosis during this process (Van Blerkom *et al.*, 1995). Stojkovic *et al.* (2001) evaluated the distribution of mitochondria of bovine oocytes before and after IVM and concluded that mitochondrial structure and distribution differed between mature and immature mitochondria. Tiny mitochondrial clusters were distributed to the perimeter of the cytoplasm before IVM but these clusters became larger and darker and were also distributed to the center of the cytoplasm. A previous human study showed that the developmental capacity of the *in vitro* fertilized oocytes was reflected by the mitochondrial structure and ATP levels (Van Blerkom *et al.*, 1995). Mitochondria lack cristae and become primitive after oocyte maturation and some become pyknotic and migrated to the center of the cytoplasm which suggests that the metabolic activity of oocytes decreases (Yang *et al.*, 2005). In the present study, the number of mitochondria in the BL-I-treated oocytes seemed to be slightly higher than that in the control group and the structural changes in the

mitochondria may affect the further development of the fertilized eggs. It possible that this ultrastructural change in ovine oocytes treated with BL-I may underlie their failure to develop further *in vitro*.

CONCLUSION

Collectively, the present results provide evidence that BL-I can reversibly inhibit IVM of ovine oocytes but does not improve their maturation and subsequent *in vitro* development.

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