Effects of β-mercaptoethanol on ATP Contents in Cumulus Cell-enclosed Bovine Oocytes Matured In Vitro and Sequential Development of Resultant Embryos from In Vitro Fertilization

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Abstract: *In the present study, we assessed the effects of β-mercaptoethanol (β-ME) added to in vitro maturation (IVM) medium on the development of resultant embryos derived from in vitro fertilization (IVF) of oocytes, the number of cumulus cells, and ATP content of bovine oocytes. Furthermore, we counted the number of cells of the blastocysts derived from IVF of oocytes matured in media with or without 100 μM β-ME. When β-ME was added into the medium of IVM at concentrations of 0, 10, 50, 100 and 500 μM, the rate of embryonic development to the blastocyst stage of resultant embryos increased significantly (P<0.05) in the 100 μM group compared to the 0 μM (control) group. Although β-ME was added to media with the same concentrations (0, 10, 50, 100 and 500 μM) for the embryonic culture following in vitro fertilization of oocytes matured without β-ME, the rates of embryonic development to the blastocyst stage decreased in a concentration-dependent manner. The number of cumulus cells attached to the oocytes matured in media with 100 μM β-ME was significantly greater (P<0.05) than that in the 0 μM group. There was no difference between ATP contents of cumulus cell-enclosed oocytes (CO) matured with or without 100 μM β-ME. In contrast, the ATP content of cumulus cell-denuded oocytes after maturation in media supplemented with 100 μM β-ME was significantly lower (P<0.05) than that of the 0 μM-treated group. In addition, the average cell number of blastocysts in the 100 μM β-ME group was significantly greater (P<0.05) than that of the 0 μM group. These results suggest that β-ME may increase both the numbers of cumulus cells attached to oocytes and the cell numbers of blastocysts derived from in vitro fertilization by influencing the ATP metabolism of bovine oocytes during IVM.*

Key words: *β-mercaptoethanol, Bovine oocytes, ATP*

It has been recently reported that bovine oocytes can be fertilized and developed to the blastocyst stage *in vitro* with success rates of 20 to 40% depending on the culture conditions [1, 2].

Low molecular weight thiol compounds such as cysteamine and β-mercaptoethanol (β-ME) can act as a defense against oxidative stress [3, 4]. It has been shown that adding β-ME to media for *in vitro* maturation (IVM) of oocytes and *in vitro* culture (IVC) of embryos improves the rate of embryonic development to the blastocyst stage in cattle in *in vitro* fertilization (IVF) [4, 5]. This improvement may be the result of production of glutathione (GSH) in bovine oocytes and embryos with β-ME [4, 5]. In addition, β-ME can act to improve the development of cloned bovine embryos as an inhibitor of apoptosis [6, 7].

Adenosine triphosphate (ATP) is produced by glycolysis in the cytoplasm and the Krebs cycle and oxidative phosphorylation in mitochondria in cells [8]. ATP is important for providing the energy substrate necessary for protein synthesis and other cellular functions such as GSH production and apoptosis. While ATP is needed for resumption of meiosis of the secondary oocytes, activation, fertilization and embryonic development, ATP contents of oocytes or embryos are changed by their physiological condition

Received: August 31, 2004
Accepted: January 28, 2005
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[8–10]. In humans and cattle, ATP contents of oocytes may be crucial criteria for the embryonic development after IVF [10–12].

In the light of these reports, we postulated that β-ME may influence the ATP contents of oocytes during IVM for embryo production in an IVF system. However, no studies have documented ATP content levels associated with a cattle IVF system using β-ME. In addition, it is widely accepted that cumulus cells during IVM support the nuclear maturation of mammalian oocytes and are involved in the cytoplasmic maturation needed for developmental competence of embryos after IVF [13]. It has been reported that the presence of adequate numbers of cumulus cells supports the IVM of bovine oocytes, thereby increasing the rate of embryonic development of the resultant embryos [14]. β-ME can increase the cell number of bovine embryos produced *in vitro* [15]. These results suggest that the number of cumulus cells may influence oocyte maturation and the subsequent development after IVF; however, there is no report on the effect of β-ME on the number of cumulus cells attached to bovine oocytes.

The present study was undertaken to assess the relationships among ATP contents of bovine oocytes, the number of cumulus cells during IVM and the development of embryos derived from IVF by adding β-ME to media for IVM and IVC.

Materials and Methods

IVM of the oocytes

Bovine ovaries were obtained from Miyazaki Prefectural Meat Inspection Center and brought to our laboratory immersed in physiological saline containing 400 U/ml penicillin G potassium salt (No.24-0783, Katayama Chemical Co., Osaka, Japan) and 500 μg/ml streptomycin sulfate (No.28-5158, Katayama Chemical Co.). Oocytes attached with one or more layers of cumulus cells were aspirated from superficial follicles (1–6 mm in diameter) using a 20-G needle (No.NN-2038R, Terumo, Tokyo, Japan) attached to a 5-ml syringe (Terumo) containing a small volume of Hank's salts balanced TCM-199 (No.21200-027, Gibco BRL Products, Grand Island, NY, USA) plus 2% (v/v) heat-inactivated (56°C, 30 min) calf serum (CS, No.16170-086, Gibco BRL Products). After washing with TCM-199, the oocytes were introduced to 25 mM Hepes-buffered TCM-199 (No.12340-030, Gibco BRL Products, Earl's salts) supplemented with 5% (v/v) CS, 50 μM dimethyl-sulfoxide (DMSO, No.134-07, Nacalai Tesque Co., Kyoto, Japan), 0.12 U/ml follicle stimulating hormone from pig pituitary (No.F-2293, Sigma, St Louis, MO, USA), 50 iu/ml human chorionic gonadotropin (No.B233, Mochida Pharmaceutical Co., Tokyo, Japan), and antibiotics (98 U/ml penicillin G potassium salt and 98 μg/ml streptomycin sulfate, Katayama Chemical Co., plus 100 μg/ml dibekacin sulfate, No.DKB, Meiji Seika Co., Tokyo, Japan). DMSO was included for the stimulation of nuclear maturation and the subsequent development of bovine oocytes *in vitro* [16, 17]. The IVM incubation of the oocytes was performed for 22–26 h at 39°C under 5% CO_2, 95% air and 100% humidity.

IVF and IVC of the embryos

Frozen-thawed semen from one Japanese Black bull were diluted with Brackett & Oliphant's solution (BO solution) [18] 2, 4, 8, 16 and 32 times, for 2 min for each dilution in a stepwise manner, to remove glycerol. After dilution, the semen was centrifuged for 8 min (750 × g, RT), and the concentration was adjusted to 600–660 × 10^4 spermatozoa/ml BO solution. Oocytes in 50 μl BO solution supplemented with 200 μg/ml heparin (No.411210010, Acros Organics, Geel, Belgium) and 15 mg/ml bovine serum albumin (BSA, No.01-2030, Fraction V, Katayama Chemical Co.) were transferred to a 50-μl semen drop using diluted semen as mentioned above. This adjusted the sperm concentration, heparin level and BSA level to 300–330 × 10^4 spermatozoa/ml, 100 μg/ml heparin and 7.5 mg/ml BSA. Co-culture of spermatozoa and oocytes was performed for 22–26 hr. After insemination, oocytes were transferred to CR1aa medium [19] supplemented with 1% CS, 50 μM DMSO and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml dibekacin sulfate), and incubated for an additional 8 days under 5% CO_2, 95% air at 100% humidity and 39°C.

Nuclear maturation assay of the oocytes

Oocytes were matured with various concentrations of β-ME (No.M-7522, Sigma, 0, 10, 50, 100 and 500 μM) by the method mentioned above. Some matured oocytes had one or more layers of cumulus cells removed by vortex mixing and were fixed with methanol-acetic acid (methanol:acetic acid=3:1, v/v) to observe the nuclear maturation of oocytes up to the metaphase stage of the second meiotic division (M-II stage). The remaining oocytes with cumulus cells were fertilized *in vitro* and cultured for 8 days to assess their potency of embryonic development.

Table 1. Effects of β-ME on the nuclear maturation of bovine oocytes

Conc. of β-ME (mM)	N	Nuclear maturation rate up to the metaphase of the second meiotic division
0	26	80.0
10	24	79.2
50	35	88.6
100	35	82.9
500	25	88.0

Values are totals of 5 replicates.

Assay for IVC of embryos after fertilization

After oocytes matured in the absence of β-ME were fertilized *in vitro*, they were cultured in medium supplemented with 0, 10, 50, 100 and 500 μM β-ME for an additional 8 days.

Cell counts of cumulus cells and embryos

The number of cumulus cells of oocytes matured with or without 100 μM β-ME was counted by a hemocytometer (Thoma type). The cell numbers of blastocysts at the middle stage, derived from IVM with or without 100 μM β-ME, were determined by fluorescence microscopy (fluorescence microscope: Y-FL type, Nikon, Tokyo, Japan) using 10 or 100 μM bisbenzimide (No.382065, Calbiochem, La Jolla, CA, USA) as a stain reagent.

Assay for ATP contents

To assess the ATP contents of the oocytes, approximately 25–30 of the cumulus-cells enclosed oocytes (COs) matured with or without 100 μM β-ME were transferred to 1.5-ml Eppendorf tubes (No.A-150, Assist Co., Tokyo, Japan) with 50 μl of physiological saline containing 20% (v/v) fetal bovine serum. Then 400 μL of pure water from a pure water apparatus (Milli-Q Lab., Bedford, MA, USA) was added to the tube, and the contents were boiled at 100°C for 4 min in a hot dry bath (No.HDB-1, As One Co., Osaka, Japan). After this treatment, the COs were frozen in a deep freezer (–70°C) until ATP assay a few weeks later. To measure the ATP contents, thawed COs were loaded into a lumicounter (No.A-237, Advantec Co., Tokyo, Japan), and their chemiluminesence was counted while crude luciferase (No.FTT, Lot No.69H7430, Sigma) was added.

To clarify the effect of β-ME on the cumulus cells and oocytes, cumulus cells were removed from COs after maturation with or without 100 μM β-ME by vortex mixing to yield cumulus cell-denuded oocytes (DOs). Then, the DOs were treated with the same method as the COs, described above, for measuring the ATP contents.

Statistical analysis

The embryonic developmental rates after IVF and the proportion of nuclear maturation up to the M-II stage of the oocytes were analyzed by the chi-square test [20]. The numbers of cumulus cells and embryonic cells and the level of ATP content were analyzed by student's t-test.

Results

The nuclear maturation rates of oocytes cultured with various concentrations of β-ME are shown in Table 1. No significant difference was seen among the groups in terms of the proportion of oocytes that reached to the M-II stage.

The developmental rates of embryos, which were derived from the oocytes matured in the presence of the various concentrations of β-ME and cultured without β-ME, are shown in Table 2. The embryonic developmental rates from the 2- to 16-cell stages in the 10, 50 and 100 μM groups showed no significant difference from that of the untreated (control, 0 μM) group. However, the rate of embryonic development up to the blastocyst stage in the 100 μM group was significantly higher ($P<0.05$) than that of the control group. The 500 μM group showed significantly lower rates ($P<0.05$) in both the 2- to 16-cell and blastocyst stages than those in the control group.

The development rates of the embryos up to the blastocyst stage, derived from oocytes matured without β-ME and cultured with various concentrations of β-ME after fertilization, are shown in Table 3. No groups treated with β-ME, except for the 500 μM group, showed a significant decrease of embryonic development at the 2- to 16-cell stage. However, the developmental rate up to the blastocyst stage of embryos cultured with 50 μM β-ME was significantly lower ($p<0.05$) than that of the

Table 2. Effects of β-ME added to the maturation medium on development of bovine embryos up to the blastocyst stage

Conc. of β-ME (μM)	N	2- to 16-cell stage %	Blastocyst stage %
0	129	56.6[a]	13.2[b]
10	125	50.4[a]	14.4[b]
50	130	59.2[a]	22.3[ab]
100	132	55.3[a]	28.0[a]
500	121	0.0[b]	0.0[c]

Values are totals of 5 replicates. a, b, c: Values with different superscripts in a column are significantly different (P<0.05).

Table 3. Effects of addition of β-ME to the development medium on embryonic development after IVF of bovine oocytes

Conc. of β-ME (μM)	N	2- to 16-cell stage %	Blastocyst stage %
0	151	42.4[a]	21.2[a]
10	151	43.7[a]	17.8[a]
50	151	43.7[a]	2.6[b]
100	151	46.6[a]	0.7[b]
500	151	0.7[b]	0.0[b]

Values are totals of 6 replicates. a, b: Values with different superscripts in a column are significantly different (P<0.05).

Table 4. Effect of β-ME on the number of cumulus cells attached to oocytes

	The number of cumulus cells / oocyte
Before IVM	4133.9 ± 714.33[c]
Conc. of b-ME during IVM (μM)	
0	6833.3 ± 68.03[b]
100	12750.0 ± 259.08[a]

a, b, c: Values with different superscripts in a column are significantly different (P<0.05). Values are mean ± S.E.M. of 4 replicates.

control group. No blastocyst was seen in the 100 and 500 μM groups.

The numbers of the cumulus cells attached to the oocytes are shown in Table 4. The numbers of the cumulus cells attached to oocytes matured with or without 100 μM were significantly greater (p<0.05) than that of oocytes before IVM. When the comparison was done using the numbers of the cumulus cells attached to oocytes matured with or without 100 μM β-ME, the cell number in the 100 μM group was significantly greater (P<0.05) than that of the control (0 μM) group.

The ATP contents of oocytes matured with or without β-ME are shown in Table 5. The ATP content from the COs matured with 100 μM β-ME was similar to that of the 0 μM group. However, the ATP content of the DOs matured with 100 μM β-ME was significantly lower (P<0.05) than that of the 0 μM group.

The cell number of the embryos derived from IVF of oocytes matured with 100 μM β-ME was significantly greater (P<0.05) than that of the 0 μM group (Table 6).

Discussion

In the present study, nuclear maturation rates up to the M-II stage did not increase in any group treated with β-ME (P>0.05). However, the developmental rate up to the blastocyst stage of IVF embryos derived from oocytes matured with 100 μM β-ME was significantly higher (P<0.05) than that of the control (0 μM) group. In addition, the cell number of blastocysts derived from

Table 5. ATP contents of the cumulus cells-enclosed and -denuded oocytes treated with or without β-ME during IVM

Conc. of β-ME during IVM (μM)	ATP content (p mol)	
	cumulus cells-enclosed oocyte	cumulus cells-denuded oocyte
0	7.05 ± 1.153	0.83 ± 0.133[a]
100	11.56 ± 2.048	0.33 ± 0.120[b]

a, b: Values with different superscripts in a column are significantly different (P<0.05). Values are mean ± S.E.M. of 4 replicates.

Table 6. The cell number of blastocysts produced from oocytes matured with or without β-ME

Conc. of β-ME during IVM (μM)	N	No. of blastomeres
0	10	62.5 ± 3.50[b]
100	10	100.7 ± 9.32[a]

a, b: Values with different superscripts in a column are significantly different (P<0.01). Values are mean ± S.E.M.

oocytes matured with 100 μM β-ME was significantly greater (P<0.05) than that of the 0 μM group. Leibfried-Rutledge [21] suggested that the development rate up to the blastocyst stage is determined by the conditions of IVM of bovine oocytes. In mammals, promotion of cytoplasmic maturation can result in higher embryonic quality such as that reflected by the cell number [22]. β-ME stimulates the intracellular GSH level of bovine oocytes during IVM [5]. A high level of GSH production is one of the criteria for improving the cytoplasmic maturation of oocytes, which may result in a higher success rate of embryonic development [5, 23, 24]. It has been reported that β-ME can reduce cystine to cysteine and promote the uptake of cysteine and synthesis of GSH in mammalian cells; also that β-ME may promote cystine uptake by bovine embryos in the presence of cystine and cysteine in the medium [24, 25]. We used TCM-199, which contains 83 μM cystine and 57 μM cysteine, for IVM [26]. The results of our study, taken together with these other reports, suggest that β-ME promotes the cytoplasmic maturation of oocytes by increasing some cytoplasmic factors such as GSH, induced by the uptake by cumulus-cells and/or oocytes of cystine or cysteine in the IVM medium, thereby inducing higher embryonic development with greater cell numbers.

The embryo culture with β-ME did not increase the developmental rate up to the blastocyst stage in the present study; however, the blastocyst formation rate was significantly increased (P<0.05) when the oocytes were matured in medium with 100 μM β-ME. Cornell and Crivano [27] indicated that β-ME can chelate divalent cations. For bovine embryos produced *in vitro*, addition of ethylene diamine tetraacetic acid (EDTA), a membrane-impermeable divalent cation chelator, to the culture medium before the first cleavage of the embryos inhibited embryonic development to the blastocyst stage [28]. In the light of these reports, we suggest that β-ME may remove some divalent cations that are needed for embryonic development before the 2-cell stage, resulting in the present finding of a decrease in embryonic development during IVC of embryos treated with β-ME.

The 500 μM β-ME group showed a decrease in embryonic development during IVM and IVC. It was shown that cysteamine, a similar thiol compound, at levels over 250 μM, decreased the development of bovine embryos *in vitro* by its toxicity [29]. It has been suggested that an excess of thiol or reductor compounds may be deleterious to subsequent embryonic development and that the redox equilibrium is important for oocyte maturation [3]. Altering the thiol-redox status in mouse embryos induced cell cycle arrest or cell death [30]. In the present study, it is possible that these negative effects occurring at the higher levels of β-ME, such as 500 μM, resulted in the decrease of the development rate up to the blastocyst stage during IVM and IVC.

In the present study, the ATP contents of the DOs were from 0.47 to 0.76 p mol/oocyte on average. These values were similar to the findings of previous papers [9, 31]. Thus, our method of measuring ATP contents seems to be an accurate one.

It has been reported that ATP content may not have any relationship with the maturation of pig oocytes [32]. In contrast, van Blerkom *et al.* [10] suggested that a higher ATP content of oocytes might reflect better embryonic development in human IVF. In the present study, DOs, derived from COs matured with 100 μM β-ME and showing lower levels of ATP content, produced more blastocysts with higher quality than DOs derived

from COs matured without β-ME, although the ATP contents of COs matured with or without 100 μM β-ME were almost the same. This led us to infer that β-ME may influence the ATP production or consumption of bovine oocytes rather than that of cumulus cells during IVM. The process of ATP production is a key regulator for embryonic development in cattle and sheep [33], and many related cellular events such as oxidation. If excess oxidative stress occurs, it would result in impaired mitochondrial function, which would further contribute to oxygen radical formation, reduced ATP content, and decreased intracellular GSH, all of which have been associated with decreased developmental competence [8]. In addition, there may be some relationship between ATP contents and apoptosis induced by oxidization [8]. In contrast, β-ME can act against oxidative stress in mammalian oocytes and embryos by stimulating GSH production [3, 4]. Oxidative stress induces consumption of reducing equivalents such as GSH. Glutathione reductase is dependent on NADPH, which has been shown to be related to ATP, and a main source of NADPH in the monophosphate shunt [3]. Otherwise, mammalian oocytes require the polymerization of actin for cytoplasmic maturation events such as the relocation of the oocytes' cortical granules [34]. Actin polymerization requires the presence of ATP *in vitro* [35].

In the light of these reports, we suspect that β-ME might have increased the consumption of ATP accelerating cellular events including GSH production and/or the polymerization of actin, thereby decreasing the oocytes' ATP contents. The number of cumulus cells after culture with 100 μM β-ME was significantly increased ($P<0.05$) compared to that of the untreated group, but the ATP contents of the cumulus cells attached to the oocytes were not increased regardless of the treatment of 100 μM β-ME during IVM. These results indicate that 100 μM β-ME affects the proliferation of cumulus cells without increasing their ATP content.

In conclusion, the maturation of oocytes in medium with 100 μM β-ME may affect the ATP contents thereby promoting the embryonic development of post-fertilization bovine oocytes.

Acknowledgement

We thank the Livestock Improvement Association of Miyazaki Prefecture and Miyazaki Prefectural Meat Inspection Center of Miyakonojou-Divison for providing the frozen semen and ovaries, respectively.

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