Effect of β -Mercaptoethanol on the Preimplantation Development of Bovine Embryos Fertilized *In Vitro*

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Abstract. The present study investigated effects of β -mercaptoethanol (β -ME) on the development of bovine embryos derived from *in-vitro* matured and fertilized (IVM-IVF) oocytes. IVM oocytes were inseminated with frozen-thawed bovine sperm, and at 24, 48 and 72 h after insemination, 2-, 8- and 16-cell embryos, respectively, were transferred to TCM-199 medium containing 5% fetal bovine serum and 0, 5, 10, 20, 50 or 100 μ M β -ME without a cumulus cell monolayer or with a cumulus cell monolayer but without addition of β -ME as a control. All embryos were subsequently incubated such that the total duration of culture was 8 days. The addition of low concentrations (5 or 10 μ M) of β -ME significantly enhanced the development of all the embryos to the blastocyst stage. High concentrations (20, 50 or 100 μ M) of β -ME were effective only for the development of 8- and 16-cell embryos, but not of 2-cell embryos, to the blastocyst stage. Compared with embryos in the co-culture system (control), 2-cell embryos that were cultured with β -ME but without a cumulus cell monolayer showed significantly lower rates of development to the blastocyst stage. However, even though without a cumulus cell monolayer, 8-cell embryos showed the same rates of development to the blastocyst stage as controls when low concentrations of β -ME (5 or 10 μ M) were added to the medium. Furthermore, significantly higher rates of development of embryos to the blastocyst stage were obtained when 16-cell embryos were cultured in medium supplemented with 20 μ M β -ME than that of controls. Twenty blastocysts developed from 16 cell IVM-IVF embryos in the medium containing 20 μ M β -ME were transferred to 20 recipients, and 11 of them became pregnant, resulting in 10 calves. These results indicate that β -ME is effective for blastocyst development of embryos that are cultured without a cumulus cell monolayer, and the effect is remarkable for embryos of advanced stages.

Key words: *In-vitro* fertilization, Bovine embryo, *In-vitro* culture, β -Mercaptoethanol.

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It is well established that live calves can be obtained by *in vitro* maturation and fertilization of

follicular oocytes collected from the ovaries of slaughtered cows [1-3]. Successful *in-vitro* maturation and fertilization (IVM-IVF) of bovine embryos was achieved in co-culture systems with

fibroblast monolayers [4], trophoblastic vesicles [5], oviductal cell monolayers [6] or cumulus cell monolayers [7, 8]. However, these co-culture systems are time consuming, labor intensive, and the results are quite variable. Furthermore, analysis of the cellular physiology of embryos requires a reliable, simple embryo culture system that eliminates the potential interference of co-cultured cells. Recently, Takahashi *et al.* [9] reported that β -mercaptoethanol (β -ME) enhances development of IVM-IVF bovine oocytes from the 6- to 8-cell stages to blastocysts without co-culture. However, the effect of β -ME on the development of embryos at earlier stages has not been investigated. The present study evaluates the effects of β -ME on development of bovine IVM-IVF embryos from different developmental stages to the blastocyst stage without a cumulus cell monolayer.

Materials and Methods

Oocyte collection and in vitro maturation

Ovaries of Japanese Black cows were obtained from a slaughter house and transported to the laboratory within 1 h of slaughter in 0.9% saline containing 0.1 mg/ml Kanamycin at 37 C. All visible follicles (2 to 5 mm in diameter) on the ovarian surface were aspirated by syringe with an 18-gauge needle.

Cumulus-oocyte complexes were washed several times with maturation medium and cultured in a CO₂ incubator (2% CO₂ in air at 38.5 C) for 20 to 21 h in 1.0 ml of maturation medium under mineral oil (Squibb, Wako Pure Chemical, Osaka, Japan) in a culture dish (35 × 10 mm, Nunclon 153066, Inter-med., Roskilde, Denmark). The maturation medium was TCM-199 (Earle's salts with 25 mM HEPES, GIBCO, Grand Island, NY, USA) supplemented with 5% heat treated fetal bovine serum (FBS, Filtron, PTY, Ltd., Victoria, Australia) and antibiotics.

Sperm capacitation

Sperm capacitation was induced by the method described by Parrish *et al.* [10]. Frozen semen from a Japanese Black bull in a 0.5 ml straw was thawed at 37 C. Semen was suspended in 10 ml of BO medium [11] containing 10 mM caffeine (Sigma Chemical, St. Louis, MO, USA), and sperm were washed twice by centrifugation for 5 min at 800 ×

g. After washing, the sperm concentration was adjusted to 2×10^7 cells/ml. Then the sperm suspension was diluted 2-fold with BO medium containing 10 mg/ml bovine serum albumin (Fraction V, Wako Pure Chemical, Japan) and 10 μ g/ml heparin (NOVO-Heparin, Kodama Industries, Tokyo, Japan).

In vitro fertilization

At the end of maturation culture, cumulus-oocytes complexes were washed twice by pipetting 2 or 3 times in BO medium and then introduced into 0.5 ml drops of sperm suspension. Oocytes and spermatozoa were co-cultured for 5 h. The maturation medium that contained cumulus cells from which the oocytes had been removed was kept in a CO₂ incubator. All inseminated oocytes, still surrounded by cumulus cells with attached motile spermatozoa, were then introduced into this maturation medium with cumulus cells.

In vitro coculture of fertilized oocytes

At 24, 48 or 72 h after insemination, oocytes were removed from cumulus cell masses by pipetting and examined for their stages of cleavage. The 2-, 8- and 16-cell embryos, respectively, were further cultured in 1.0 ml of TCM-99 with 5% FBS and antibiotics under mineral oil. For embryos cultured without a cumulus cell monolayer, 0, 5, 10, 20, 50 or 100 μ M β -ME was added to the medium. Controls were cultured with a cumulus cell monolayer and no β -ME. Six to 8 days after insemination, embryo development to the blastocyst stage was examined under a microscope.

Embryo transfer

Twenty blastocysts obtained by *in vitro* culture of 16-cell IVM-IVF embryos in medium containing 20 μ M β -ME without a cumulus cell monolayer were transferred to 20 recipients.

Statistical analysis

Statistical significance of the results was evaluated by Student's *t*-test and Chi-square test.

Results

As shown in Table 1, when low concentrations (5 or 10 μ M) of β -ME were added to culture medium without cumulus cells, significantly higher rates

of development to the blastocyst stage were observed than when embryos were cultured without β -ME irrespective of the initial developmental stage. High concentrations (20, 50 or 100 μ M) of β -ME were effective for blastocyst development from 8- and 16-cell embryos but not from 2-cell embryos. Two-cell embryos that were cultured in medium containing β -ME without cumulus cells showed a significantly lower rate of the development to the blastocyst stage than controls. However, 8-cell embryos showed the same rates of the development to the blastocyst stage as controls when low concentrations (5 or 10 μ M) of β -ME were added to the medium. Furthermore, significantly higher rates of development to the blastocyst stage were obtained when 16-cell embryos were cultured in the medium containing 20 μ M β -ME than when embryos were co-cultured with a cumulus cell monolayer without addition of β -ME (control).

Twenty blastocysts were developed from 16-cell embryos in medium containing 20 μ M β -ME without a cumulus cell monolayer. After transfer into

20 recipients, 11 of these became pregnant and 10 calves were born.

Discussion

Recently, Takahashi *et al.* [9] reported that including β -ME and cysteamine in culture media facilitates development of bovine 6- to 8-cell embryos from IVM-IVF to the blastocyst stage without any feeder cells. Our results indicate that β -ME is also effective for development of bovine 2- and 16-cell embryos, and the effect is remarkable for embryos of advanced stages.

Even without a co-cultured cumulus cell monolayer, 16-cell embryos cultured in medium containing β -ME (5 to 100 μ M; all concentrations used in the present study) showed blastocyst development at rates comparable to co-cultured embryos. A significantly higher rate of development to the blastocyst stage was obtained when they were cultured with 20 μ M β -ME than when co-cultured with a cumulus cell monolayer. How-

Table 1. Effect of various concentrations of β -mercaptoethanol(β -ME) on development of bovine 2-, 8- and 16-cell¹⁾ embryos obtained by *in-vitro* maturation and fertilization of oocytes

Source of embryos	Concentrations of β -ME (μ M)	No. of trials	No. of embryos cultured	No. of embryos developed to blastocysts on			Total No. of blastocysts
				Day 6 ²⁾	Day 7 ²⁾	Day 8 ²⁾	
2-cell	0	5	100	2	3	0	5 ^a
	5	5	100	7	12	2	21 ^b
	10	5	100	5	13	1	19 ^b
	20	5	100	2	5	2	9 ^a
	50	5	100	2	8	0	10 ^a
	100	5	100	2	5	0	7 ^a
	control ³⁾	5	100	14	20	5	39 ^c
8-cell	0	5	100	1	3	0	4 ^a
	5	5	100	8	24	7	39 ^b
	10	5	100	20	27	3	50 ^b
	20	5	100	6	15	4	25 ^c
	50	5	100	6	9	2	17 ^{cd}
	100	5	100	2	7	4	13 ^d
	control ³⁾	5	100	13	24	15	52 ^b
16-cell	0	5	100	8	15	0	23 ^a
	5	5	100	27	19	14	60 ^b
	10	5	100	28	20	11	59 ^b
	20	5	100	37	30	7	74 ^c
	50	5	100	34	25	13	72 ^{bc}
	100	5	100	29	20	7	56 ^b
	control ³⁾	5	100	24	30	9	63 ^b

1)Two, 8- and 16-cell embryos were collected 24, 48 and 72 h, respectively, after insemination. 2)Day 0=Day of insemination. 3)Embryos were cultured with a cumulus cell monolayer without β -ME. Different superscripts within columns for the same source of embryos denote significant differences.

ever, the beneficial effect of β -ME on the development of embryos was not so evident for 2-cell embryos, and the addition of β -ME at high concentrations (20 to 100 μ M) had no effect on their development. Although the addition of β -ME to the culture medium significantly enhanced the development of 8-cell embryos, only low (5 and 10 μ M) concentrations of β -ME were found to be effective for their development to the blastocyst stage when compared with those of co-cultured embryos.

Therefore, it was found that high concentrations (20 to 100 μ M) of β -ME are not effective for development of 2- and 8-cell embryos to the blastocyst stage but are effective for 16-cell embryos. In contrast, Takahashi *et al.* [9] reported that the percentage of 6- to 8-cell embryos developing to the blastocyst stage was significantly higher when embryos were cultured in medium supplemented with a high concentration of β -ME (50 μ M) than when they were cultured in the medium supplemented with a low concentration of β -ME (10 μ M). The reason for this discrepancy with respect to effective concentrations of β -ME on the development of embryos to the blastocyst stage is not clear. Differences in the source of oocytes (Japanese black *vs* Holstein) and/or culture conditions (2% CO₂ in air *vs* 5% CO₂ in air) might be reasons for this difference. Because 10 calves were born after the transfer of blastocysts obtained by *in vitro* culture with β -ME to recipients, addition of β -ME was determined not to be toxic to the embryos.

At present, the mechanism by which different concentrations of β -ME enhance development of embryos at different stages (2-, 8- and 16-cell) to

the blastocyst stage is not entirely understood. However, it is of interest that bovine IVM-IVF embryos at 2- and 4-cell stages can metabolize glutamine at a high rate, and then the rate declines before rising at a later preimplantation stage [12]. It has been postulated that the beneficial effects of glutamine on the development of embryos may arise from its ability to counteract lipid peroxidation under circumstances when other substrates are relatively ineffective [12]. If the promoting effect of β -ME on the development of IVM-IVF embryos is to increase intracellular concentration of glutathione, known to scavenge free radicals in the cells [9], embryos at 4- and 8-cell stages may not need a high concentration of β -ME in the culture medium to increase intracellular glutathione levels because the embryos may utilize glutamine to scavenge free radicals. Higher concentrations of glutathione in the cells and/or β -ME in the medium than needed might be detrimental for development of embryos.

Further investigations are required to explain the relationships between the optimal concentration of β -ME for development of embryos and their intracellular glutathione levels. Nevertheless, the usefulness of such a thiol compound in creating improved conditions for *in vitro* culture of IVM-IVF embryos at early stages without co-culture is quite evident.

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