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Effect of Thiol Compounds on the Blastocyst Formation of *In Vitro* Matured and Fertilized Bovine Embryos

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체외에서 성숙되고 수정된 소 난자의 배반포 형성에 있어 항산화제의 역할

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요 약

본 연구는 체외에서 성숙되고 수정된 소 수정란의 체외 발달에 thiol compound인 β-mercaptoethanol(β-ME)과 cysteine(CYS)의 첨가 효과를 알아보기 위하여 실시하였다. 미성숙 난포란을 회수한 후 TCM-199 배양액내에서 24시간 동안 성숙을 유도하였다. 수정은 Fert-TALP 배양액에서 실시하였으며, 2세포기로 발달한 수정란만을 선별하여 CR1aa 배양액내에 β-ME 및 CYS를 첨가하여 체외배양하였다. 실험 1에서는 β-ME과 CYS의 최적농도를 알아보기 위해서 0, 5, 25, 125μM의 β-ME과 0, 0.01, 0.1, 1mM의 CYS이 첨가된 배양액에 2세포기 난자를 9일 동안 배양하면서 배반포까지 발달율을 조사하였다. 그 결과 25μM의 β-ME 그리고 0.1mM의 CYS이 첨가된 배양액에서 배반포까지의 발달율은 각각 30.7%와 31.0%로 대조군의 발달율(8.0%, 15%)에 비해 유의적으로 높은 결과를 보였다(P<0.05). 실험 2에서는 배반포 형성에 있어 최적농도인 25μM의 β-ME과 0.1mM의 CYS을 각각 초기배(2→8세포기)와 후기배 (8세포기 이후) 배양시 첨가하였을 때, 그 효과를 알아보았다. 그 결과 후기배 배양시 25μM의 β-ME과 0.1mM의 CYS이 첨가된 배양액내에서 배반포까지의 발달율은 각각 60.2%와 43.1%로 초기배 배양에서의 첨가군(18.2%, 23.7%)과 대조군(22.5%, 18.1%)에 비해 유의적으로 높게 나타났다(P<0.05). 따라서 배양액내 β-ME과 CYS의 첨가는 체외에서 생산된 소 난자의 배발달율을 향상시키며, 초기배 수정란의 genome 활성이 일어나는 시기 (8~16 세포) 이후에 첨가효과가 큼을 알 수 있었다.

(Key words : Thiol compound, β-Mercaptoethanol, Cysteine)

I. INTRODUCTION

In order to establish effective culture system for *in vitro* matured and *in vitro* fertilized bovine embryos, it is important to overcome the developmental arrest of embryos developing *in vitro*. Although the mechanism of the arrest during *in vitro* development is not exactly known at present, the block seems to coincide with the time of embryonic genome activation is initiated (Jarrell et al., 1991). Previously, the block has

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been overcome by changing slightly the composition of media (Davis and Day, 1978; Petters et al., 1990; Reed et al., 1992), adding growth factors (Saito and Niemann, 1991), supplementing amino acids (Meyen et al., 1989), coculturing with the somatic cells (Allen and Wright, 1984; Jin et al., 1991), using the secretions of somatic cells (Kane, 1992), and adding the body fluids such as oviductal fluid (Archibong et al., 1989; Collas et al., 1991).

One of the major differences between *in vivo* and *in vitro* environment for perimplantation embryonic development is high oxygen concentration (Bishop, 1956, Mastroianni and Jones, 1965, Mass et al., 1976). Some researchers have suggested a relationship between embryonic developmental arrest and an increase in free radical formation in embryos cultured *in vitro* (Nasr-esfahani, 1991, Nasr-esfahani, 1992, GoTo, 1993, Jonson et al., 1994). Moreover, Takahashi et al. (1993) reported that the increase in intracytoplasmic glutathione concentration caused by the addition of β-mercaptoethanol (β-ME) and cysteamine of the culture medium was beneficial for the development of 6- to 8-cell bovine embryos to the blastocyst stage. Low-molecular-weight thiol compounds such as β-ME and cysteamine appeared to promote cell viability and to enhance various cell reaction in lymphocytes (Fanger et al., 1970) and embryonal carcinoma cells (Rizzino and Oshima et al., 1978). In addition, these thiol compounds have been known to increase intracellular GSH (γ-glutamylcysteinylglycine) in mouse lymphoma cells (Ishii, 1981, Zmuda et al., 1983) and CHO (Chinese hamster ovary) cells (Issels et al., 1988) Therefore, the stimulation of glutathione synthesis by β-ME and cysteine may promote the development of bovine embryos at the time of genomic activation. In the present study we determined the effects of β-mercaptoethanol, glutathione and cysteine on the development of bovine embryos produced from *in vitro* matured and *in vitro* fertilized oocytes.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* maturation

Prepubertal bovine ovaries were collected from a local slaughterhouse and transported to the laboratory at 35℃ in Dulbecco's phosphate buffered saline supplemented with 75μg/L potassium penicillin G and 50μg/L streptomycin sulphate (mDPBS). Cumulus-Oocyte complexes (COCs) were aspirated with an 18-gauge needle into a disposable 10 ml syringe from follicles 2 to 6 mm in diameter. The follicular fluid was pooled in petri dish and the oocytes were collected from the fluid under stereomicroscope. The collected COCs were washed three times with TL-HEPES medium (Parrish et al., 1985) and washed three times in equilibrated tissue culture medium (TCM-199: 400-1100, GIBCO BRL Co., USA) supplemented with 2.2 g/L sodium bicarbonate ($NaHCO_3$), 10% heat-treated fetal bovine serum (FBS: 200-614, GIBCO), 0.22μg/ml Na pyruvate, 25μg/ml gentamycin sulfate, 1μg/ml FSH-p (Schering Co., UK) and 1μl/ml estradiol-17β (BCE-8875, Sigma, Missouri, USA). Then COCs were cultured in 50μl drops of TCM-199 under paraffin oil for 24 hrs at 39℃, 5% CO_2 in humidified atmosphere.

2. Sperm preparation and *in vitro* fertilization

In vitro fertilization was carried out by a method of sirard et al. (1988). Matured COCs were washed Sp-TALP and then with Fert-TALP (Rosenkrans et al., 1993). After washing, ten mature COCs were pulled into 44μl Fert-TALP droplets under paraffin oil. Bull spermatozoa recovered from frozen-thawed semen were separat-

ed on a discontinuous percoll gradient. Separated highly motile spermatozoa were added to a final concentration of 1×10^6 sperm/ml. Then 2μl of heparin stock solution (final concentration 10μg/ml) to induce sperm capacitation and 2μl of PHE stock solution (2mM phenicillamine, 20μM hypotaurine and 1μM epinephrine) to stimulate sperm motility were added into Fert-TALP droplets. Then COCs and sperm were incubated in 50μl drops of Fert-TALP under paraffin oil for 24 hrs at 39℃, 5% CO_2 in humidified atmosphere.

3. *In vitro* culture

The cumulus cells surrounding the inseminated oocytes were mechanically removed by gentle aspiration with a glass pipette, and the fertilized oocytes were transferred to drops of embryo culture medium, CR1aa (Rosenkrans and First, 1991) supplemented with 4 mg/ml fatty-acid-free BSA, 20μl/ml MEM essential amino acid, 10μl/ml MEM non-essential amino acid, 0.44μg/ml Na pyruvate, 1.46μg/ml glutamine, 25μg/ml gentamycin. Following culture for 72 hrs, the embryos were then transferred to the CR1aa medium supplemented with 10% FBS.

4. Treatments of β-ME and CYS on *in vitro* culture

The fertilized oocytes were transferred into CR1aa medium. The embryos developed to the 2-cell stage were transferred into the CR1aa medium containing 0.4% BSA, supplemented with β-ME or CYS. After 9 days of culture with or without the thiol compounds, the developmental stage of embryos was observed under a inverted microscope. The number of embryos developed to the blastocyst and hatched blastocyst stage were recorded.

5. Statistical analyses

All experiments were replicated four times. In each experiment, the embryos were allocated randomly and equally among the experimental groups. Statistical analysis of from four replicate trials was carried out by analysis of χ^2-test.

Ⅲ. RESULTS AND DISCUSSION

1. Effect of β-ME and CYS on the development of 2-cell bovine embryos

To determine the effect of thiol compounds on the early developmental potential of bovine embryos, we cultured 2-cell bovine embryos in the presence of 0, 5, 25 or 125 μM β-ME and 0, 0.1, 0.01 or 1 mM cysteine (Table 1, 2).

As shown in Table 1, addition of β-ME to the culture medium enhanced *in vitro* development of 2-cell stage embryos to the blastocyst or hatched blastocyst. The percentage of embryos developed to the blastocyst and hatched blasto-

Table 1. Effect of β-ME on *in vitro* development of 2-cell bovine embryos (Day 9)

Concentrations of β-ME	No. of 2-cell embryos(r)	No. (%) of embryos developed to	
		Blastocyst	Hatched blastocyst
None	198 (4)	14 (7.0)	2 (1.0)
5 μM	216 (4)	8 (3.7)	18 (8.3)
25 μM	202 (4)	22 (10.9)*	40 (19.8)*
125 μM	240 (4)	14 (5.8)	38 (15.8)

* Different superscripts within columns denote significant differences ($P<0.05$)

(r) : number of replication

Table 2. Effect of cysteine on *in vitro* development of 2-cell bovine embryos (Day 9)

Concentrations of cysteine	No. of 2-cell embryos(r)	No. (%) of embryos developed to	
		Blastocyst	Hatched blastocyst
None	172 (4)	22 (12.7)	4 (2.3)
0.01 mM	252 (4)	41 (16.6)	16 (6.3)
0.1 mM	203 (4)	28 (13.5)*	36 (17.5)*
1 mM	191 (4)	16 (8.4)	6 (3.1)

*Different superscripts within columns denote significant differences ($P<0.05$)
(r) : number of replication

cyst stage was significantly higher when embryos were cultured in CR1aa containing 25 μM (30.7%) β-ME than when they were cultured in CR1aa alone (Table 1). This result is similar to the previous results that β-ME has been shown to improve embryos development in mice (Oshima et al., 1978) and bovine (Caamano et al., 1996). β-ME was exerted on cystine, converting it to a compound other than CYS. It has also been reported that β-ME transports CYS into the cells resulting in the formation of β-ME-CYS compounds (Ishii et al., 1982). The mixed-disulfide compounds formed in the β-ME supplemented medium which was beneficial for growth of mouse lympoma cells (Ishii et al., 1981). Moreover, the increase of intracellular GSH levels caused by β-ME addition into the culture medium which was helpful for embryos development.

As shown in Table 2, when the embryos were cultured in the medium without of CYS and with of 1 mM CYS for 9 days, the percentages of the embryos developed to the blastocyst and hatched blastocyst stage was 15% and 11.5%. In the presence of 0.1 mM CYS in the culture medium the percentage of embryos developed to the blastocyst stage was 31.0%. Previous results showed that CYS present in medium is rapidly oxidized to form cystine (Toohey, 1975, Mohindru et al., 1985). Once the CYS is oxidized to cystine in the medium, intracellular GSH synthesis is significantly inhibited because cells can not utilize cystine for the synthesis GSH (Ishii et al., 1981). Therefore, the addition of proper CYS levels may be beneficial for embryo development, but excessive or defective addition of CYS may be resulted in the detrimental effect on the embryo development.

2. Effect of β-ME and CYS addition on the different stages development of bovine embryos

This experiment evaluated the effects of β-ME and CYS addition on the blastocyst formation when embryos at different stages were exposed to 25 μM β-ME and 0.1 mM of CYS. As shown in Table 3, the addition of 25 μM β-ME after 8-cell stage embryos enhanced *in vitro* development to the blastocyst or hatched blastocyst (Fig. 1). The percentage of embryos developed to the blastocyst and hatched blastocyst stage was significantly higher when embryos were cultured in the β-ME supplemented medium after 8-cell stage (18.2%, 42.0%) than embryos cultured with CR1aa containing β-ME from 2-cell to 8-cell stage (13.2%, 5.0%).

As shown in Table 4, we have used a cell-free culture system to assess the effects of 0.1 mM CYS on blastocyst and hatched blastocyst rate. The percentage of embryos developed to the blastocyst and hatched blastocyst stage was significantly higher when embryos were cultured in

Table 3. Effect of 25 μM β-ME on the early and late development of bovine embryos

Cell stage at treatment	No. of embryos (r)	No. (%) of embryos developed to			
		Blastocyst			Hatched blastocyst
		Day 7	Day 8	Day 9	Day 9
none	196 (4)	37 (19.2)	39 (19.9)	25 (12.8)	19 (9.7)
2 → 8 cell	242 (4)	103 (42.6)	61 (25.2)	32 (13.2)	12 (5.0)
<8 cell	214 (4)	97 (45.3)	117 (54.7)	39 (18.2)	90 (42.0)*

* Different superscripts within columns denote significant differences ($P<0.05$)

2 → 8 cell : 2-cell embryos were cultured with CR1aa containing 25 μM β-ME to 8-cell stage embryo and then transferred into the CR1aa culture medium without β-ME

< 8 cell : The embryos developed to 8-cell stage were transferred into the CR1aa medium containing 25 μM β-ME

Table 4. Effect of 0.1 mM cysteine on the early and late development of bovine embryos

Cell stage at treatment	No. of embryos (r)	No. (%) of embryos developed to			
		Blastocyst			Hatched blastocyst
		Day 7	Day 8	Day 9	Day 9
none	276 (4)	48 (17.4)	51 (18.4)	29 (10.5)	21 (7.6)
2 → 8 cell	228 (4)	56 (24.6)	41 (18.0)	34 (14.9)	20 (8.8)
<8 cell	204 (4)	58 (28.4)	81 (39.7)	30 (14.7)	58 (28.4)*

*Different superscripts within columns denote significant differences ($P<0.05$)

2 → 8 cell : 2-cell embryos were cultured with CR1aa containing 0.1 mM CYS to 8-cell stage embryo and then transferred into the CR1aa culture medium without CYS

<8 cell : The embryos developed to 8-cell stage were transferred into the CR1aa medium containing 0.1 mM CYS

Fig. 1. The blastocyst stage of *in vitro* derived bovine embryos after 9 days of culture in CR1aa medium supplemented with 25μ M β-ME and 10% FBS. Magnification is 200×.

the CYS supplemented medium after 8-cell stage (14.7%, 28.4%) than embryos cultured with CR1aa containing CYS from 2-cell to 8-cell stage (14.9%, 8.8%).

In an attempt to understand the mechanism of action of β-ME or CYS, data were gathered on the effect of this compound on the composition of the embryo culture medium. Culture media normally contain sulfhydryl compounds in an oxidized state at the initiation of the culture period. These medium constituents could have a dramatic effect on the ability of embryos to synthesize GSH, to perform normal functions of protein synthesis, or to accomplish a variety of other important cell signaling processes.

In conclusions, our study suggested that the

effects of thiol compounds (β-ME and CYS) on bovine embryos development may be more effective in the late stage embryos than early stage embryos.

Ⅳ. SUMMARY

The objective of this study was to determine effects of β-mercaptoethanol (β-ME) and cysteine (CYS) on the development of bovine embryos obtained from *in vitro* matured and fertilized oocytes. Cumulus-oocyte-complexes (COCs) were matured in micro-drop of TCM-199 medium containing 10% FBS, 17β-Estradiol and FSH-p under paraffin oil at 39 ℃ for 24 hrs. The fertilization of COC were induced in Fert-TALP medium supplemented with PHE, heparin, BSA and then the fertilized oocytes were cultured in CR1aa medium for 24 hrs. To investigate the effects of the agents on the development of the embryos, the embryos developed to the late 2-cell stage were cultured in the media with and without β-ME, CYS for 9 days. In experiment 1, to select appropriate concentration of β-ME and CYS during whole culture period (9 days), various concentrations of β-ME and CYS were added to the CR1aa medium. Addition of 25μM of β-ME and 0.1mM of CYS to the culture medium increase the incidence of embryos developed to the blastocyst. In experiment 2, we evaluated the effects of 25μM of β-ME and 0.1mM of CYS addition on the blastocyst formation when embryos at different stages were exposed to 25μM β-ME and 0.1mM of CYS. β-ME and CYS enhanced *in vitro* development of embryos to the blastocyst stage. The effect was greater in 8-cell to morula embryos than in embryos fewer than 2-cells at the initiation of treatment. These results suggested that the addition of 25μM B-ME and 0.1mM cysteine enhanced development to the blastocyst and hatching stage of *in vitro* derived bovine embryos, also addition of β-ME and cysteine were effective later stage embryo than early embryo development.

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