Improvement of *In Vitro* Development of Bovine Embryos in a Medium Containing Selenium

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ABSTRACT : The objective of this study was to investigate the effect of selenium (Se) on *in vitro* development of bovine embryos. In CR1BSA, FBS-free medium, the bovine embryos could not proceed past the developmental block more efficiently to morula stage than in chemically undefined media. Addition of glutathione precursor, cysteine, with β-mercaptoethanol did not improve the development in chemically defined medium and neither did glutathione alone. Exogenous selenium improved the embryonic development to the morula and blastocyst stages at 6 days post-insemination (dpi) significantly (67.1% vs 57.5%, $p<0.05$), and blastocyst stage at 8 dpi (30.1% vs 20.5%, $p>0.05$). These improvements might be induced by elevated glutathione peroxidase activity due to addition of Se, and a possible mechanism of selenium to elevate the activity of glutathione peroxidase is discussed. (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 2 : 170-173*)

Key Words : Bovine, Embryos, Cysteine, Glutathione, Selenium, *In Vitro* Development

INTRODUCTION

One of the major differences in embryo development conditions between *in vivo* and *in vitro* is the oxygen concentration (Maas et al., 1976). Three to four times higher oxygen concentration *in vitro* than *in vivo* resulted in the production of a variety of reactive oxygen species: superoxide radicals (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide ($H_2O_2^-$) (Luvoni et al., 1996). The biomolecules such as proteins, lipids, carbohydrates and DNA modified by these free radicals in murine embryos may be involved in two-cell developmental block (Noda et al., 1991).

Glutathione (GSH) redox cycle was one of the protective systems against free radicals (Yu, 1994). GSH is produced through γ-glutamyl cycle using cysteine (Cys), glutamate (Glu) and glycine (Gly). The reduced GSH is oxidized by glutathione peroxidase (GPx) which is a homotetramer selenoprotein, and therefore it requires selenium (Se) and catalyzes the degradation of peroxides and hydroperoxides to the corresponding alcohol by using reduced GSH as a specific hydrogen donor (Meister, 1983). The addition of cysteine, cystine and β-mercaptoethanol (β-ME) to IVM medium increased GSH synthesis in bovine oocytes during maturation (de Matos et al., 1996). Supplementation with GSH precursor and β-ME in culture medium may improve development of embryos *in vitro*.

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The addition of Se to culture media containing insulin and transferrin improved the development and viability of bovine embryo (Shamsuddin et al., 1994), while normal embryonic development and cell proliferation was inhibited in the Se deprived medium (Chada et al., 1989; Ozolin et al., 1996; Sun et al., 1997). Furthermore, Se has been known to elevate the transcription and translation of GPx in the cells (Spallholz, 1994; Sun et al., 1997). However, the specific effect of Se supplementation on embryo development during *in vitro* culture has not been studied.

The objective of this study was to investigate the effect of antioxidant and its precursor, such as GSH, Cys and β-ME as well as Se on the development during *in vitro* culture of bovine embryos.

MATERIALS AND METHODS

In vitro maturation

Immature bovine cumulus-oocyte complexes (COCs) were aspirated from antral follicles in ovaries of slaughtered cows and heifers. Aspirated follicular fluid was pooled into 15 ml conical tube and COCs were allowed to gravitate during a 10-15 min interval. Mature COCs were selected and then placed in TCM-199 supplemented with 10% (v/v) FBS, 1 μg /mL estradiol, 1 μg/mL FSH, 2 IU/mL LH, 1 μg /mL EGF and 1% (v/v) kanamycine and incubated for 24-25 hrs (Park et al., 2000).

In vitro fertilization

The sperms for IVF were prepared by the method of Niwa and Ohgoda (1988) as modified in our laboratory. Briefly, frozen-thawed semen was washed two times in 2 ml BO medium (Brackett and

Table 1. Comparison of 4 different media for embryonic development of *in vitro* fertilized bovine oocytes*

Medium	No. of embryos (%)				
	Inseminated (0 day)	Cleaved (1 day)	8-cells (3 day)	Morulae (6 day)	Blastocysts (8 day)
TLP	184	127 (69.0)	73 (39.7)^{a}	57 (31.0)^{a}	32 (17.4)^{ab}
TCM-199	189	141 (74.6)	95 (50.3)^{b}	87 (46.0)^{b}	44 (23.3)^{a}
CR1aa	200	154 (77.0)	102 (51.0)^{b}	82 (41.0)^{ab}	54 (27.0)^{a}
CR1BSA	165	112 (67.9)	81 (49.1)^{b}	39 (23.6)^{c}	21 (12.7)^{b}

* Pooled data from four replicates.
Numbers with same superscripts within column are not significantly different ($p<0.05$).

Oliphant, 1975) supplemented with 10 mM caffeine-benzoate by centrifuging at 1,200 rpm for a 5 min in a 15 mL conical tube and removing the supernatant. Motile sperms were collected by means of a modified percoll gradient preparation. On the surface of 1 ml 90% percoll solution, the washed sperms were loaded. After centrifugation at 1,200 rpm for 10 min, the supernatant was removed and the pellet was washed again with BO medium supplemented with 10 mM caffeine-benzoate.

Matured oocytes were transferred to 50 μL of BO medium (Brackett and Oliphant, 1975), supplemented with 20 mg/mL fatty acid free-bovine serum albumin and 20 μg/mL heparin, and coincubated with approximately $3\text{-}4\times10^{7}$/mL motile spermatozoa for 8-12 hrs (Park et al., 2000).

In vitro culture

The cumulus cells still surrounding the inseminated oocytes were mechanically removed by vortexing for 1 min. Denuded oocytes were transferred to culture media. Medium was replaced with fresh medium every two days. In undefined condition, TLP, TCM-199 and CR1aa (Rosenkrans et al., 1993) supplemented with FBS (10% v/v) were used as culture media and culture was performed with cumulus monolayer. In FBS-free condition, CR1 containing 3 μg/mL fatty acid free-BSA and 1% (v/v) MEM non-essential amino acid was used as culture medium and culture was performed without a somatic cell monolayer.

Experimental design and analysis

In experiment I, each undefined and defined medium for supporting growth past the developmental block was compared. Differences were reflected by data on embryonic development to the morula and blastocyst stages recorded at 6 and 8 days post-insemination (dpi), respectively.

The effects of addition of 0.1 mM Cys, 50 μM β-ME (Caamano et al., 1998) and 5 ng/ml Se (Shamsuddin et al., 1994), and 1 mM GSH (Luvoni et al., 1996) and 5 ng/mL Se to defined culture medium for supporting growth to proceed past the developmental block were compared to the control group using 8 cell stage embryos obtained at 3 dpi. Effects were reflected by embryonic development to the morula or blastocyst stage recorded at 6 and 8 dpi, respectively.

Statistical differences between proportions reflecting various treatments were ascertained by ANOVA test (SAS, 1990). P values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

There was no significant difference in embryonic development between defined and undefined medium before 3 dpi. However at morula stage, the developmental rate of embryos in CR1BSA was significantly lower (23.6%, $p<0.05$) than in undefined media (31.0%, 46.0% and 41.0% in TLP, TCM-199 and CR1aa, respectively). Also, the development of embryos to blastocyst stage in CR1BSA was significantly lower ($p<0.05$) than in TCM-199 and CR1aa (23.3% and 27.0%, respectively). These results indicate that the undefined medium used in this experiment contained some unknown factors which were absent in FBS-free medium but essential for embryo development.

As shown in table 2, addition of 0.1 mM Cys and 50 μM β-ME together had no significant effect on the development of 8-cell embryos to morulae and

Table 2. Effects of added cysteine (0.1 mM), β-mercaptoethanol (50 μM) and selenium (5.0 ng/mL) on the development of *in vitro* fertilized bovine embryos*

Treatment	No. of embryos (%)		
	8-cells (3 day)	M+BL (6 day)	BL (8 day)
Control	81	41 (50.6)	16 (19.8)
Cys+ β-ME	83	43 (51.8)	15 (18.1)
Cys+ β-ME+Se	83	39 (47.0)	14 (16.9)

M: Morulae, BL: Blastocysts.
* Pooled data from three replicates.
Data are not significantly different at $p=0.05$.

blastocysts compared to control group at 6 and 8 dpi (51.8% vs 50.6% and 19.8% vs 18.1% respectively). This conflicts with the results of Caamano et al. (1998) which showed that the addition of Cys and β-ME promoted the development of bovine embryos. However, our results were consistent with those of Kishi et al. (1994) that reported the failure of rat embryos to proceed past the developmental block by the addition of Cys. Cys is oxidized to cystine which can not be transported into the cell membrane, or the mercapuric acid derived from the Cys-conjugate is exported to the outer cell membrane so that it can not participate in γ-glutamyl cycle (Meister, 1983). Although the transport system of Cys or β-ME-Cys conjugate into the cell membrane is not clarified in the developing bovine embryos yet, these results could be explained by the oxidation of Cys or excretion out of the cell. Addition of 5.0 ng/mL Se along with Cys and β-ME also did not improve embryonic development to morulae and blastocysts at 6 and 8 dpi. It has been speculated that the competition of Se with sulfur in cysteine might result in selenocysteine, which had no chance to play a role as a antioxidant GSH (Yan, 1993).

In table 3, the development of 8-cell stage embryos to the morulae and blastocysts was improved by addition of 1 mM GSH+5.0 ng/mL Se compared with the control group at 6 dpi (66.7% vs 57.5%, respectively, $p<0.05$). Furthermore, addition of Se alone in defined medium improved the development to morula and blastocyst at 6 dpi (67.1% vs 57.5%, respectively, $p<0.05$). But addition of GSH alone did not show a significant difference compared to the control group (61.6% vs 57.5%, respectively). Thus, it is considered that GSH did not affect the embryonic development. Although the difference was not significant, addition of Se alone or with GSH tended to improve the embryonic development to blastocyst stage compared to that of the control group at 8 dpi (30.1% and 33.3% vs 20.5%).

While antioxidant enzymes such as glucose-6-phosphate dehydrogenase, glutathione peroxidase and glutathione reductase are known to start to be transcribed from the early embryonic stage to blastocyst stage, GPx mRNA was shown to be expressed highly from blastocyst stage (Harvey et al., 1995; Lequarre et al., 1997). Exogenous Se was incorporated into GPx through seryl-tRNA, which oxidized GSH to remove free radicals (Ganther, 1986; Spallholz, 1994; Sun et al., 1997). Chada et al. (1989) reported that GPx was not translated sufficiently without Se in medium. In selenium-deplete culture condition, the GPx activity was decreased significantly but this enzymatic activity was sharply increased in selenium-replete medium (Chada et al., 1989). Our results showed that exogenous GSH could not improve the development of embryos under the present experimental conditions. On the other hand, the addition of Se alone might play a role as an effective antioxidant enough to proceed the developmental block. Considering the study of Chada et al. (1989), these results suggested that exogeneous Se promotes GPx activity, which removes free radicals sufficiently to allow growth to proceed past the developmental block.

Table 3. Effect of added glutathione (1 mM) and selenium (5.0 ng/mL) on the development of *in vitro* fertilized bovine embryos*

Treatment	No. of embryos (%)		
	8-cells (3 day)	M+BL (6 day)	BL (8 day)
Control	73	42 (57.5)^{a}	15 (20.5)
Se	73	49 (67.1)^{b}	22 (30.1)
GSH	73	45 (61.6)^{ab}	15 (20.5)
Se+GSH	75	50 (66.7)^{b}	25 (33.3)

M: Morulae, BL: Blastocysts.
* Pooled data from five replicates.
Numbers with same superscripts within column are not significantly different ($p<0.05$).

In conclusion, addition of GSH or Cys as a GSH precursor to the defined culture medium did not efficiently pass the developmental block in bovine embryos. But Se, the major component of GPx, was shown to be effective for growth past the developmental block under the conditions used in this study. These improvements might be induced by elevated GPx activity due to addition of Se, but the exact mechanism of Se to elevate the transcriptional or translational levels of GPx remains to be clarified.

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