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The Effect of Various Concentrations of Taurine during *In vitro* Fertilization on the Development of Bovine Embryos Fertilized with Spermatozoa from Three Different Bulls

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ABSTRACT : We investigated the effect of various concentrations of taurine during *in vitro* fertilization (IVF) on the embryonic development up to the blastocyst stage of bovine oocytes fertilized with three different Japanese Black bulls (Bull A, B and C). *In vitro* matured oocytes were fertilized with various concentrations of taurine (0, 1, 10, 50 and 100 mM) in the presence of 2.5 or 5.0 mM caffeine plus 25 μg/ml heparin (CH) for 6 hr or 100 μg/ml heparin (H) for 24±2 h. After IVF, the cleavage rates from the 2 to 16 cell stage determined at 3 days and the development rates up to the blastocyst stage determined at 7-8 days from the onset of IVF were assessed. Although the cleavage rates for the taurine concentration groups were not significantly increased in any of the three bulls in the CH groups, the development rates up to the blastocyst stage of the 50 mM taurine group of Bulls A and B, and of the 1 to 50 mM groups of Bull C were increased ($p<0.05$) compared to those of the control (0 mM taurine) groups. On the other hand, none of the bulls in the H groups showed any significant increase either in the cleavage rates or blastocyst formation rates in any taurine concentrations groups compared with those of the control groups. These results indicate that the addition of 50 mM taurine to a fertilization medium containing caffeine and heparin may stimulate embryonic development up to the blastocyst stage when fertilized with different bull semen. (**Key Words :** Taurine, *In vitro* Fertilization, Embryonic Development, Bull Variation)

INTRODUCTION

Bovine oocytes can be developed to the blastocyst stage, which is a possible implantation stage. However, the success of IVF is markedly dependent on individual bulls, as well as on ejaculates from the same bull (Kreysing et al., 1997; Zhang et al., 1997; Zhang et al., 2003; Alomar et al., 2008; Xu et al., 2009). To decrease the variation in the embryonic development from different bulls, some reagents such as caffeine, heparin, caffeine plus heparin, caseine phosphopeptides and pentoxyfylline have been used during IVF (Niwa and Ohgoda, 1988; Fukui et al., 1990; Saeki et al., 1995; Kreysing et al., 1997; Numabe et al., 2001; Pavlok et al., 2001; Tartaglione and Ritta, 2004). However, bull variation when using the IVF technique is still one of the problems affecting the *in vitro* production of bovine embryos (Almoar et al., 2008; Xu et al., 2009).

The O_2 tension in the oviducts and uteri of rabbits, hamsters and rhesus monkeys were only 7.5-40% of that in air (20% oxygen tension in air, Maas et al., 1976; Fischer and Bavister, 1993). The air culture conditions for *in vitro* production of embryos is in a range from 5% to 20% oxygen tension, and this may produce reactive oxygen species (ROS, Fowler and Callingham, 1978; Guérin et al., 2001). Mammalian sperm are highly susceptible to oxygen damage (Aitken and Fisher, 1994) and spontaneously produce ROS (Aitken and Clarkson, 1987). ROS increase DNA fragmentation, modify the cytoskeleton and cause a loss of fluidity, integrity and competence of the sperm membrane to participate in the membrane events associated with fertilization (Gil et al., 2008). To avoid damage due to these radicals during IVF, the addition of antioxidants such as α-tocopherol, ascorbic acid or β-mercaptoethanol to the IVF medium were considered to be effective for the subsequent development of the embryos up to the blastocyst stage in cattle (Dalvit et al., 1998) and pigs (Funahashi,

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2005). However, Gonçalves et al. (2010) reported that addition of antioxidants such as β-mercaptoethanol, cysteamine and buthione sulfoximine to the IVF medium inhibited the bovine sperm quality, normal pronuclear formation and resultant embryonic development up to the blastocyst stage. Ali et al. (2003) also reported no positive effects with the addition of antioxidants (N-acetyl-L-cysteine, superoxide dismutase and catalase) to the IVF medium for the embryonic development up to the blastocyst stage in cattle. This discrepancy about the efficacy of antioxidants added to the IVF medium on bovine embryonic development may be caused by the types of antioxidant and/or composition of the culture medium or oxygen tension in the incubator etc, and further study will be needed.

Taurine (2-aminoethane sulfonic acid) is the main end-product of cysteine metabolism in mammals, and it is one of the most abundant of the low-molecular-weight organic constituents (Huxtable, 1992; Ekremoğlu et al., 2007). In mammalian tissues, taurine is ubiquitous and is the most abundant free amino acid in the heart, retina, skeletal muscle, brain and lukocytes. It is well known that taurine is a non-enzymic antioxidant in human, pig and bull semen (Johnson et al., 1972; Ibrahim and Boldizsár, 1981; Zini et al., 2009). Taurine was also detected at a higher concentration than the other amino acids in the mouse (approximately 0.33 mM) and cattle (approximately 0.05-0.5 mM) oviductal fluid (Dumoulin et al., 1992; Elhassan et al., 2001; Harris et al., 2005; Hugentobler et al., 2007; Leese et al., 2008). It is thought that taurine in the oviducts is a compound necessary for sperm capacitation and fertilization in mammals, playing an important role in the protection of spermatozoa against ROS (Guérin et al., 1995; Sariözkan et al., 2009). Boatman (1997) reported that the addition of 10 μM taurine to the IVF medium increased frozen-thawed hamster sperm motility and penetration to oocytes *in vitro*. However, mM level taurine added to the IVF medium did not stimulate the fertilization rate of mouse oocytes (Dumoulin et al., 1992). In the light of these reports, the effect of taurine during IVF appears to differ between species. For cattle, there have been no reports about the relationship among taurine added to the IVF medium, bull variations and subsequent embryonic development.

Therefore, we investigated the influence of taurine combined with caffeine added to the IVF medium using spermatozoa derived from three different bulls on the embryonic development of bovine oocytes *in vitro*.

MATERIALS AND METHODS

Oocyte aspiration and *in vitro* maturation (IVM)

Bovine ovaries of Japanese Black and Holstein cattle were obtained from a local slaughterhouse, immersed in physiological saline (27-32°C) and brought within 4 h to our laboratory. Oocytes were aspirated from superficial follicles (3-6 mm in diameter) with a 20 G needle attached to a 5 ml disposable syringe. Then, oocytes enclosed with cumulus cells (COCs) were selected, washed with Hank's salted TCM-199 (No.21200, Gibco BRL products, Grand Island, NY, USA) and loaded into maturation medium. The maturation medium we used consisted with 25 mM Hepes buffered TCM-199 (No.12340, Gibco BRL Products), 0.12 U/ml follicle stimulating hormone (No.F-2293, Sigma Aldrich, St Louise, MO, USA), 50 iu/ml human chrionic hormone (Mochida Phaumaceutical Co., LTD, Tokyo Japan), 50 μM dimethyl sulfoxide (DMSO, No. 13407-45, Nacalai Tesque, Kyoto, Japan, Tsuzuki et al., 1998), 5% (v/v) heat inactivated (56°C, 30 min) calf serum (CS, No.16170-086, Gibco BRL Products) and antibiotics (100 U/mL penicillin G potassium, No. 26239-42, Nacalai Tesuque, 100 μg/ml streptomycin sulfate, No.32237-72, Nacalai Tesuque, and 100 μg/ml dibekacin sulfate, No. DBK, Meiji Seika Co., Tokyo, Japan) in a plastic dish (35 mm in diameter, No.153066, Nunc Brand Products, Roskilde, Denmark) (Tsuzuki et al., 2005). The COCs were covered with paraffin oil (No.26137-85, Nacalai Tesque) matured for 22-26 h under 5% CO_2, 95% air and 100% humidity at 39°C. During IVM, the culture volume was adjusted to 8.33 μl/COC.

After maturation, some COCs were randomly selected and washed with physiological saline supplemented with 2% CS twice and denuded cumulus cells by a vortex mixer, and fixed in acetic alcohol (ethanol:acetic acid = 3:1, V/V) for two days at room temperature. The remaining ones were assigned into 5 taurine groups (20-30 COCs/group) for IVF. Before maturation, some COCs were also selected, denuded and fixed using the same method used for the matured ones mentioned above.

In vitro fertilization (IVF) and *in vitro* culture (IVC) of the embryos

We used two types of IVF methods in this study. One was the caffeine plus heparin (CH) method reported by Niwa and Ohgoda, (1988) with some modification of the concentrations of caffeine, heparin and BSA. Frozen semen samples in a 0.5 ml plastic straw from each bull were thawed for 1 min in 39°C warm water, and washed and diluted 2, 4, 8, 16 and 32 times at 2 min intervals with BO solution (Brackett and Oliphant, 1975) in a 39°C water bath to remove glycerol. Thereafter, the semen was centrifuged at 750 xg for 8 min at room temperature and added to an equal volume of BO solution drops containing 20-30 matured COC supplemented with 15 mg/ml bovine serum albumin (No.01-2030, Fraction V, Katayama Chemical Co., Osaka, Japan), 5 or 10 mM caffeine sodium benzoade

(No.C-4144, Sigma Aldrich, 50% w/w) plus 50 μg/ml heparin (No.411210010, derived from porcine intestinal mucosa anticogulant, Acros Organic, Geel, Belgium) to adjust the sperm concentrations of each bull to 600 to 660× 10^4 spermatozoa/ml. With these treatments, the final concentrations of caffeine and heparin were 2.5 or 5 mM and 25 μg/ml, respectively. The final concentrations of spermatozoa from each bull and BSA were 300-330× 10^4/ml and 7.5 mg/ml. In our preliminary study, matured oocytes fertilized with various concentrations of caffeine (0, 2.5, 5, 10 and 25 mM) combined with various concentrations of heparin (0, 10, 25, 50 and 100 μg/ml) with the same sperm concentration (300-330×10^4/ml), showed the highest rate of development up to the blastocyst stage when fertilized with 2.5 mM caffeine combined with 25 μg/ml heparin for Bulls A and C, and 5 mM caffeine plus 25 μg/ml heparin for Bull B. Therefore, we used these concentrations of caffeine and heparin for IVF with semen from each bull. During IVF, 0, 1, 10, 50 and 100 mM taurine (No.T-8691, Sigma-Aldrich) were added to the IVF medium (the culture volume of the IVF medium was adjusted to 5 μl/COC in all groups). After 6 hrs' incubation, the COCs were washed with TCM-199 and transferred to CR1aa (Rosenkrans and First, 1991) supplemented with 50 μM DMSO (Tsuzuki et al., 1998), 1% CS and antibiotics, as mentioned above. For the other IVF method, we used 100 μg/ml heparin sodium (H) only for 24±2 h (Tsuzuki et al., 2005) to induce sperm capacitation with modification from previous papers (Fukui et al., 1990; Gordon, 1994; Saeki et al., 1995; Guyader-Joly et al., 1998; Chohan and Hunter, 2004) because in our preliminary study, when the oocytes were fertilized with the same bull's semen in the presence of 1, 10 and 100 μg/ml H for the same period (24±2 h), the development rates up to the blastocyst stage in the 100 μg/ml H group in all bulls were higher than those of the other H concentration groups. This concentration (100 μg/ml) of H has been used for IVF in cattle (Fukui et al., 1990; Tsuzuki et al., 2005) and buffalo (Mehmood et al., 2007). During fertilization, the same concentrations of taurine in the CH groups mentioned above were added to the medium. The cultural volume in both the CH and H groups was adjusted to 5 μl/COC during IVF. On 3 day from the onset of IVF, the culture medium was changed to CRlaa supplemented with 50 μM DMSO (Tsuzuki et al., 1998), 15% CS and antibiotics with 5 μl/COC of the culture medium, and the cleavage rates from 2 to 16-cells stage were observed by an inverted phase contrast microscope. At 7-8 days from the onset of IVF, the blastocyst formation rates in each group were also observed. All fertilization treatments were replicated five or six times (20-30 COCs/group per replicate).

Statistical analysis

All data collected from the 5 or 6 replicates were analyzed with ANOVA followed by Duncan's Multiple Range Test (Duncan, 1955).

RESULTS

Before IVM, all 133 oocytes (100%) collected from 5 replicates were at the GV stage, and 94 of 100 oocytes (94%) collected from the 5 replicates had reached the metaphase stage of the second meiotic division after IVM.

The cleavage rates from the 2 to 16 cell stage and the development rates up to the blastocyst stage after fertilization by each bull capacitated with various concentrations of taurine under the presence of CH are shown in Table 1, 2 and 3.

For Bull-A, the cleavage rates from the 2 to 16 cell stage determined on the third day from the onset of IVF were the same rates in all taurine concentration groups. However, the development up to the blastocyst stage in the 50 mM taurine group were increased ($p<0.05$) compared with that of the control (0 mM) group (Table 1).

For Bull-B, the cleavage rates in the 1 to 50 mM groups were the same as those of the control group. However, the development rate up to the blastocyst stage in the 50 mM group was increased ($p<0.05$) compared with that of the control group (Table 2). The 100 mM taurine group reduced the cleavage and blastocyst formation rates ($p<0.05$) compared with the other concentration groups.

For Bull C, the cleavage rates in the 1 to 100 mM groups were almost the same as those of the control group.

Table 1. Effect of various concentrations of taurine on the oocytes fertilized with Bull A's semen in the presence of CH

Concentration of taurine (mM)	N	Cleavage rate of the oocytes from the 2 to 16 cell stage %	Development rate up to the blastocyst stage %
0	134	66.5±5.04	9.5±0.44[b]
1	134	60.0±5.98	22.6±6.59[ab]
10	134	67.0±4.79	21.3±4.28[ab]
50	134	59.4±4.52	24.6±5.14[a]
100	134	66.5±4.22	23.0±4.76[ab]

[a, b] Values with different superscripts in a column are significantly different ($p<0.05$).

Values are mean±SE of 5 replicates. CH: 2.5 mM caffeine plus 25 μg/ml heparin.

Table 2. Effect of various concentrations of taurine on the oocytes fertilized with Bull B's semen in the presence of CH

Concentration of taurine (mM)	N	Cleavage rate of the oocytes from the 2 to 16 cell stage %	Development rate up to the blastocyst stage %
0	152	65.6±3.48[a]	17.0±1.39[b]
1	152	66.1±3.15[a]	20.5±1.97[ab]
10	152	73.6±3.91[a]	18.4±1.68[ab]
50	152	64.9±3.89[a]	30.8±4.69[a]
100	152	37.1±10.42[b]	6.6±2.92[c]

[a, b, c] Values with different superscripts in a column are significantly different ($p<0.05$).
Values are mean±SE of 6 replicates. CH: 5 mM caffeine plus 25 μg/ml heparin.

Table 3. Effect of various concentrations of taurine on the oocytes fertilized with Bull C's semen in the presence of CH

Concentration of taurine (mM)	N	Cleavage rate of the oocytes from the 2 to 16 cell stage %	Development rate up to the blastocyst stage %
0	152	58.4±3.89	15.5±2.98[b]
1	152	61.4±4.85	33.0±4.99[a]
10	152	65.4±6.02	35.8±6.07[a]
50	152	66.9±4.60	31.3±8.18[a]
100	152	51.9±6.98	13.6±2.82[b]

[a, b] Values with different superscripts in a column are significantly different ($p<0.05$).
Values are mean±SE of 5 replicates. CH: 2.5 mM caffeine plus 25 μg/ml heparin.

In the blastocyst stage, the 1 to 50 mM groups showed the higher rates ($p<0.05$) compared with that of the control group (Table 3).

In H experiments for each bull, there were no significant increases in the cleavage rates or the development rates up to the blastocyst stage in any bulls treated with various concentrations of taurine compared with those of the control (0 mM) groups (Tables 4-6). For bull C, the development rate of the embryos up to the blastocyst stage in the 100 mM taurine group was lower ($p<0.05$) than those of the other taurine concentration groups.

DISCUSSION

In the present study, all of the oocytes were at the GV stage, and almost all oocytes after IVM were at the M-II stage. This suggests that our selection of the COCs for IVM and our IVM system is valid.

Fifty mM taurine added to the IVF medium for Bulls A and B, 1 to 50 mM for Bull C in the presence of CH increased ($p<0.05$) the development rates up to the blastocyst stage compared to those of the control (0 mM) groups for each bull in the present study. This suggests that the addition of taurine to the IVF medium may stimulate the development of bovine oocytes up to the blastocyst stage. Sariözkan et al. (2009) reported that taurine at 2 mM added to the medium increased the catalase activity, an antioxidant enzyme, of frozen-thawed bovine spermatozoa. This may have induced a positive effect for spermatozoa in the 50 mM taurine groups in this study.

In hamsters, 10 μM taurine added to the IVF medium increased the sperm motility and resultant penetration rate to the oocytes (Boatman, 1997). However, Dumoulin et al. (1992) reported that taurine added to the IVF medium at a maximum concentration of 20 mM did not stimulate the fertilization rate of mouse oocytes. This suggests that the effects of taurine may be different in different species.

In the present study, the CH group showed positive effects of taurine for the embryonic development, but H groups did not. 10 mM caffeine inhibited superoxide

Table 4. Effect of various concentrations of taurine on the oocytes fertilized with Bull A's semen in the presence of H

Concentration of taurine (mM)	N	Cleavage rate of the oocytes from 2 to 16 cell stage %	Development rate up to the blastocyst stage %
0	110	53.7±4.00	18.2±3.05
1	110	56.2±8.70	20.4±5.20
10	110	51.3±10.22	19.0±4.85
50	110	61.9±7.51	16.3±8.10
100	110	55.8±4.53	10.0±4.29

Values are mean±SE of 5 replicates. H: 100 μg/ml heparin.

Table 5. Effect of various concentration of taurine of the oocytes fertilized with Bull B's semen in the presence of H

Concentration of taurine (mM)	N	Cleavage rate of the oocytes from the 2 to 16 cell stage %	Development rate up to the blastocyst stage %
0	124	63.4±2.78[ab]	24.0±2.34[ab]
1	124	55.5±3.89[b]	22.6±3.78[ab]
10	124	58.1±4.16[ab]	30.9±7.43[a]
50	124	57.9±4.96[ab]	23.3±4.54[ab]
100	124	70.0±5.89[a]	14.0±2.63[b]

[a, b] Values with different superscripts in a column are significantly different ($p<0.05$).
Values are mean±SE of 5 replicates. H: 100 μg/ml heparin.

Table 6. Effect of various concentrations of taurine on the oocytes fertilized with Bull C's semen in the presence of H

Concentration of taurine (mM)	N	Cleavage rate of the oocytes from the 2 to 16 cell stage %	Development rate up to the blastocyst stage %
0	108	58.6±3.76	19.8±1.89[a]
1	108	56.7±6.77	24.1±3.04[a]
10	108	61.8±3.81	25.5±4.48[a]
50	108	60.0±4.50	24.8±5.28[a]
100	108	54.5±4.93	5.0±2.50[b]

[a, b] Values with different superscripts in a column are significantly different ($p<0.05$).
Values are mean±SE of 5 replicates. H: 100 μg/ml heparin.

dismutase and augmented superoxide anion radical generation in human spermatozoa (Sinha et al., 1993). Caffeine induced cell death via apoptotic signal activation and survival signal inactivation in human osteoblasts, and these negative effects can be prevented with antioxidants such as α-tocopherol or N-acetyl cyteine (Lu et al., 2008). On the other hand, Córdoba et al. (2008) reported that 60 μg/ml heparin did not increase ROS production of frozen-thawed bovine spermatozoa. Therefore, ROS production may be increased by only CH treatment, but not H treatment, resulting in the increase in embryonic development in the CH groups.

The highest development rates up to the blastocyst stage in each group varied between bulls in our results. This indicates that taurine under the presence of caffeine and heparin cannot improve the bull variation IVF results and further study will be needed to decrease the bull variation in embryonic development.

For Bull B of the CH group and Bull C of the H group, the development rates up to the blastocyst stage with 100 mM taurine were significantly lower than those of the control (0 mM) groups. O'Flaherty et al. (1999) reported that superoxide anion and hydrogen peroxide, two ROS, are required for sperm capacitation and acrosome reaction, respectively. From this we infer that 100 mM taurine may inhibit these ROS required for sperm capacitation during IVF, resulting in a decrease in the development up to the blastocyst stage.

In the present study, 50 mM taurine had a much larger effect on the embryonic development compared with that in the bovine oviductal (around 10 to 100 fold) and uterine fluids (around 15 to 125 fold). In our IVF system, the sperm-oocyte coculture was done under 20% O_2. Mammalian oviducts contained approximately 7.5% to 40% oxygen of that in air (Maas, 1976; Fischer and Bavister, 1993). From these reports, a higher concentration of taurine may be required to inhibit the toxic effects of ROS.

In conclusion, the addition of 50 mM taurine to a fertilization medium containing caffeine and heparin may stimulate embryonic development up to the blastocyst stage when fertilized by different bulls.

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