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; Pavlo 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 O2 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, 2003).
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 chonic hormone (Mochida Pharmaceutical 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 Tesque, 100 μg/ml streptomycin sulfate, No.32237-72, Nacalai Tesque, 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% CO2, 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 benzoate
(No.C-4144, Sigma Aldrich, 50% w/w) plus 50 μg/ml heparin (No.411201010, derived from porcine intestinal mucosa anticogulant, Acros Organic, Geel, Belgium) to adjust the sperm concentrations of each bull to 600 to 660×10⁶ 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 300-330×10⁶/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) 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 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 CR1aa supplemented with 50 μM DMSO (Tsuzuki et al., 1998), 15% CS and antibiotics with 5 μl/COC of the culture volume of the IVF 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 taurine group was increased (p<0.05) compared with that of the control group (Table 2).

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<sup>b</sup> |
| 1                            | 134| 60.0±5.98                                              | 22.6±6.59<sup>ab</sup> |
| 10                           | 134| 67.0±4.79                                              | 21.3±4.28<sup>ab</sup> |
| 50                           | 134| 59.4±4.52                                              | 24.6±5.14<sup>a</sup> |
| 100                          | 134| 66.5±4.22                                              | 23.0±4.76<sup>ab</sup> |

<sup>a,b</sup> 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 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±3.48a                                               | 17.0±1.39b                                 |
| 1                            | 152| 66.1±3.15a                                               | 20.5±1.97ab                                |
| 10                           | 152| 73.6±3.91a                                               | 18.4±1.68ab                                |
| 50                           | 152| 64.9±3.89a                                               | 30.8±4.69a                                 |
| 100                          | 152| 37.1±10.42b                                              | 6.6±2.92c                                  |

*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±2.98b                                 |
| 1                            | 152| 61.4±4.85                                                 | 33.0±4.99a                                 |
| 10                           | 152| 65.4±6.02                                                 | 35.8±6.07a                                 |
| 50                           | 152| 66.9±4.60                                                 | 31.3±8.18a                                 |
| 100                          | 152| 51.9±6.98                                                 | 13.6±2.82b                                 |

*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 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| 61.6±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.
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 cysteine (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% O2. 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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