



Effects of Taurine on Sperm Characteristics during *In vitro* Storage of Boar Semen

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ABSTRACT : The objective of this study was to investigate the anti-oxidative effects of taurine on sperm characteristics for *in vitro* storage of boar semen. Semen was randomly divided into 10 groups in conical tubes and treated with different concentrations of taurine (25-100 mM) with or without 250 μ M H₂O₂. The percentage of motile spermatozoa in taurine groups after 6 and 9 h were significantly higher at >94% and 87%, respectively, compared to the control group (85.1 \pm 0.5 and 72.4 \pm 0.3, p <0.05). The sperm motility in taurine with H₂O₂ after 6 h incubation was slightly decreased compared to the taurine alone treatment, but after 9 and 12 h incubation % sperm motility dropped sharply in taurine with H₂O₂ (75.3 \pm 0.3 and 69.6 \pm 2.9, p <0.05). For 3, 9 and 12 h incubation, sperm viability in the control was lower than in taurine groups, irrespective of taurine concentration. In eosin Y and nigrosin staining (ENS), the sperm survival rates (%) for 6 h incubation were significantly higher in 25 mM (76.0 \pm 0.6) and 50 mM taurine groups (78.0 \pm 0.7), respectively. Sperm survival rates for 9 and 12 h incubation were higher in taurine groups (\geq 48% in 9 h and \geq 42% in 12 h) compared to controls (43.0 \pm 2.1 and 31.0 \pm 0.6, respectively). In the hypoosmotic swelling test (HOST), sperm membrane integrity was similar to the results of sperm survival. These experiments indicate that supplementation of taurine to the semen extender can increase the sperm characteristics (motility, viability, survival and membrane integrity). (**Key Words :** Boar Semen, Sperm Characteristic, Hydrogen Peroxide (H₂O₂), Taurine, Hypoosmotic Swelling Test (HOST))

INTRODUCTION

The boar semen used for artificial insemination (AI) is stored at 17°C-19°C following the addition of an appropriate extender. The research of a selection of boar semen extenders has been focused on storage periods over 5 to 7 days by many researcher (Alexopoulos et al., 1996; Korniewicz et al., 1996; Laforest and Allard, 1996).

There are many problems for the exactly assessment of sperm function. The sperm function is altered rapidly for *in vitro* storage at 17-19°C in pig, one of the greatest factor of failure is reactive oxygen species (ROS, Alexopoulos et al., 1996; Jang et al., 2004). Boar sperm seems to be especially sensitive to ROS damage due to the relative high content of unsaturated fatty acids in the phospholipids of the boar

sperm membrane. Fluidity is linked to the integrity of the membrane lipids (Stubbs and Smith, 1984) and changes in the lipid composition of the plasma membrane may therefore be associated with the cooling and storage effects. As spermatozoon cells are characterised by a high level of polyunsaturated fatty acids, lipid peroxidation may be one of the mechanisms responsible for the negative biochemical and physiological changes during sperm storage (Cerolini et al., 2000) and the relative low antioxidant capacity of boar seminal plasma (Brezekinska-Slevbodzinska et al., 1995).

Taurine (2-aminoethanesulfonic acid) is the major intracellular free-amino acid, which is normally present in most mammalian tissues (Chesney, 1985). Although taurine is not a constituent of any structural mammalian protein, it plays various important physiological roles including osmoregulation, cell proliferation, viability and prevention of oxidant-induced injury in many tissues (Chesney, 1985; Huxtable, 1992; Redmond et al., 1996). The beneficial effects of taurine as an antioxidant in biological systems have been attributed to its ability to stabilize biomembranes

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(Wright et al., 1986), scavenge reactive oxygen species (Wright et al., 1985) and reduce the production of lipid peroxidation end products (Huxtable, 1992).

Therefore, in the present study, we investigated the effects of taurine as antioxidant in boar semen and the analysis of spermatozoa were assessed the reliable predictors of sperm fertilizing ability (motility, viability, survival and membrane integrity).

MATERIAL AND METHODS

Semen preparation

Sperm-rich fractions (30 to 50 ml) were collected from 1-3 purebred (Duruc, Yorkshire and Landrace) with 85% motile sperm by the gloved hand method at the local A. I center (Wonju) and were diluted three times with Modena to give a concentration to 2×10^7 spermatozoa/ml. The diluted semen were transported to the laboratory at 17°C within 2 h of collection. Modena solution extender (138.75 mM glucose, 6.99 mM EDTA, 11.9 mM NaHCO_3 , 46.66 mM Tris, 23.46 mM Na-citrate, 15.10 mM citric acid, 0.306 mM cysteine, 3.0 g/L BSA and 10 mg/L gentamycin sulfate) was used as a semen extender. Semen was randomly aliquoted into 3 centrifuge tube and was treated with taurine (25-100 mM) with or without H_2O_2 (250 μM). All of the treatments were repeated at least 3 times with the semen samples from the different boars. The semen of each treated group was incubated for 3, 6, 9, and 12 h at 37°C in 5% CO_2 incubator.

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich.

Sperm analysis

The analysis of semen quality were evaluated the motility, viability, survival and membrane integrity.

Sperm motility : To assess motility, 50 μl of the semen from each groups were placed on warm slide glass at 37°C and examined the percentage of motile spermatozoa under 400 magnification with an inverted phase contrast microscope (Nikon, Japan). Sperm movement was classified the five following categories, vigorous-forward movement, an active forward movement, a weak-forward movement, a circular or pendulum movement and no movement.

Sperm viability by makler counting chamber : Semen treated with taurine with or without H_2O_2 was measured the viability using Makler counting chamber. Briefly, a 20 μl drop of treated semen was placed in the center of the lower platform of the counting chamber and was covered with lid. The number of dead or motile spermatozoa in the whole grid was counted. After counting, counting chamber was transferred into refrigerator at -20°C for 5 min and then take out the chamber. The total sperm in chamber were

immediately counted under an inverted microscope at 400 magnification. Sperm viability was assessed by determining the percentage of motile spermatozoa out of total spermatozoa.

Analysis of sperm viability by Eosin-Y and nigrosin staining (ENS) : To prepare the eosin-nigrosin staining solution, eosin Y (11.1 mg/ml) and nigrosin (66.7 mg/ml) was dissolved in distilled water on warm stirring plate at 40°C. Trypan blue (TB) staining solution (0.4% TB) was used. Semen sample were made by placing of 50 μl ENS staining solution in the center of warm slide glass and then add 20 μl semen to the drop of staining solution, and mixed and smeared the staining solution containing semen with another clean slide glass. Specimen was dried at room temperature. Nigrosin was used a background stain to facilitate the reading of eosin preparation. TB staining was prepared by adding 50 μl TB solution on the ENS stained slide glass. Semen sample stained were evaluated under phase contrast microscope (Olympus, Japan) at 400 magnification. Stained spermatozoa (dead) appeared partial and complete purple or red against a blue back ground while those (live) that were not stained appeared white. Spermatozoa that stain only in the posterior portion of the head were classified as stained. A total 200 spermatozoa was evaluated by counting in at least 3 times under a phase contrast microscope at 400 magnification.

Hypoosmotic swellig test (HOST) : The HOS test was preformed by employing the technique developed by Maxwell and Johnson (1997). Briefly, a 50 μl semen sample was added and mixed with 1 ml of 150 mOsm/kg HOS diluent (Na-citrate, 7.35 g and fructose, 13.51 g in 1 L of distilled water) and then incubated for 30 min at 37°C in 5% CO_2 incubator. The assessment of total sperm swelling and individual swelling patterns was decided as sperm tail coiled and swelon. A total of 200 spermatozoa were evaluated for coiled tails by counting in at least 3 times under a phase contrast microscope at 400 magnification.

Statistical analysis

Statistical analysis of replicated experiment results were used for treatment comparisons and were carried out one-way analysis of variance (ANOVA) using SAS program. The p value was <0.05 in ANOVA, treated means were compared for differences through use of Duncan's modified multifl range test. All data were expressed as mean \pm SEM. $p \leq 0.05$ was considered to be significant.

RESULTS

The sperm characteristics (motility, vability, survival and membrane integrity) of boar semen treated with different concentration of taurine (25-100 mM) with or without 250 μM H_2O_2 were assessed at 3, 6, 9, and 12 h

Table 1. Effects of taurine with or without hydrogen peroxide on motility in boar semen during incubation at 37°C

Incubation time (h)	Treatments									
	0	H	H+T25	H+T50	H+T75	H+T100	T25	T50	T75	T100
3	97.3±0.3 ^{NS}	94.3±2.2 ^{NS}	97.0±0.6 ^{NS}	95.3±0.3 ^{NS}	96.0±0.6 ^{NS}	97.0±0.6 ^{NS}	96.3±0.3 ^{NS}	97.3±0.5 ^{NS}	95.3±0.3 ^{NS}	96.3±0.3 ^{NS}
6	85.1±0.5 ^b	16.3±2.0 ^c	89.5±0.3 ^{ab}	87.3±0.3 ^{ab}	88.0±0.6 ^{ab}	88.3±0.9 ^{ab}	94.5±0.3 ^a	96.3±0.3 ^a	94.3±0.3 ^a	95.3±0.4 ^a
9	72.4±0.3 ^c	4.0±0.6 ^d	4.0±0.6 ^d	5.0±0.0 ^d	5.0±0.0 ^d	5.0±0.0 ^d	87.5±0.3 ^b	92.3±1.5 ^a	94.3±0.3 ^a	87.3±0.3 ^b
12	47.5±1.4 ^d	0.0±0.0 ^e	0.0±0.0 ^e	0.0±0.0 ^e	0.0±0.0 ^e	0.0±0.0 ^e	65.0±0.6 ^{ab}	67.0±0.5 ^a	63.3±0.0 ^b	57.3±1.0 ^c

NS: Non-significant.

^{a-c} Different superscripts within rows are significantly differ, $p < 0.05$.Control, Modena with 0.4% BSA; H, 250 μ M H₂O₂; H+T25, 250 μ M H₂O₂+25 mM taurine; H+T50, 250 μ M H₂O₂+50 mM taurine; H+T75, 250 μ M H₂O₂+75 mM taurine; H+T100, 250 μ M H₂O₂+100 mM taurine; T25, 25 mM taurine; T50, 50 mM taurine; T75, 75 mM taurine; T100, 100 mM taurine.**Table 2.** Effects of taurine with or without hydrogen peroxide on viability in boar semen during incubation at 37°C

Incubation time (h)	Treatments									
	0	H	H+T25	H+T50	H+T75	H+T100	T25	T50	T75	T100
3	75.3±0.3 ^c	69.6±2.9 ^d	88.0±1.5 ^b	88.5±0.6 ^b	87.0±0.6 ^b	85.7±0.3 ^b	93.6±1.6 ^a	88.4±0.7 ^b	91.4±1.1 ^{ab}	89.9±0.1 ^b
6	72.0±0.9 ^b	44.3±1.5 ^d	52.7±5.6 ^c	56.4±1.0 ^c	54.4±0.1 ^c	47.1±1.1 ^d	82.8±2.1 ^a	86.0±1.1 ^a	82.8±2.0 ^a	84.7±0.7 ^a
9	60.7±2.0 ^c	33.3±6.2 ^d	6.4±0.9 ^e	3.4±0.4 ^e	2.8±0.2 ^e	0.0±0.0 ^e	69.4±1.3 ^b	75.6±2.4 ^a	75.5±1.1 ^a	60.9±0.6 ^c
12	35.1±0.6 ^d	14.1±2.0 ^e	0.0±0.0 ^f	0.0±0.0 ^f	0.0±0.0 ^f	0.0±0.0 ^f	59.2±0.5 ^b	65.4±1.1 ^a	66.6±2.1 ^a	43.4±0.4 ^c

^{a-f} Different superscripts within rows are significantly differ, $p < 0.05$.**Table 3.** Effects of taurine with or without hydrogen peroxide on survival in boar semen during incubation at 37°C

Incubation time (h)	Treatments									
	0	H	H+T25	H+T50	H+T75	H+T100	T25	T50	T75	T100
3	77.6±1.9 ^{NS}	76.3±0.9 ^{NS}	79.0±1.0 ^{NS}	78.0±1.5 ^{NS}	76.3±1.7 ^{NS}	77.6±0.9 ^{NS}	80.3±1.2 ^{NS}	82.3±1.2 ^{NS}	80.6±1.2 ^{NS}	78.3±0.9 ^{NS}
6	72.0±0.6 ^b	46.3±2.3 ^d	72.0±4.2 ^b	71.0±1.5 ^b	72.7±1.2 ^b	68.7±1.5 ^b	76.0±0.6 ^a	78.0±0.7 ^a	70.3±1.5 ^b	61.0±3.8 ^c
9	43.0±2.1 ^b	22.3±1.5 ^c	6.8±1.5 ^d	8.3±0.3 ^d	6.7±0.9 ^d	7.3±1.2 ^c	48.7±1.3 ^a	50.7±1.5 ^a	48.7±1.2 ^a	50.7±4.6 ^a
12	31.0±0.6 ^c	5.0±2.5 ^d	2.7±0.3 ^d	2.0±0.0 ^d	3.0±0.6 ^d	2.0±0.6 ^d	43.7±2.4 ^b	46.7±2.6 ^a	45.0±2.5 ^a	41.0±2.1 ^b

NS: Non-significant ^{a-d} Different superscripts within rows are significantly differ, $p < 0.05$.**Table 4.** Effects of taurine with or without hydrogen peroxide on membrane integrity in boar semen during incubation at 37°C

Incubation time (h)	Treatments									
	0	H	H+T25	H+T50	H+T75	H+T100	T25	T50	T75	T100
3	41.0±1.2 ^{NS}	41.7±0.3 ^{NS}	43.3±0.9 ^{NS}	40.3±0.9 ^{NS}	43.0±2.2 ^{NS}	43.7±0.6 ^{NS}	42.0±0.6 ^{NS}	43.3±0.6 ^{NS}	41.7±1.2 ^{NS}	41.3±0.3 ^{NS}
6	33.7±0.9 ^a	11.0±0.6 ^d	29.8±0.9 ^c	29.0±0.9 ^c	30.0±0.3 ^b	31.0±0.6 ^b	34.0±0.6 ^a	36.3±3.7 ^a	32.7±1.2 ^{ab}	30.7±0.9 ^b
9	21.3±0.9 ^c	3.7±0.3 ^d	3.5±0.9 ^d	5.7±0.3 ^d	7.0±0.3 ^d	5.3±0.3 ^d	25.7±0.7 ^b	28.3±0.3 ^a	25.0±0.6 ^b	22.3±1.5 ^c
12	10.0±0.6 ^b	2.7±0.3 ^c	2.5±0.7 ^c	4.7±0.3 ^c	3.0±0.0 ^c	5.0±0.0 ^c	16.3±1.2 ^{ab}	18.7±1.9 ^a	16.3±0.3 ^{ab}	14.3±2.3 ^b

NS: Non-significant. ^{a, b, c, d} Different superscripts within rows are significantly differ, $p < 0.05$.

during incubation at 37°C in boar semen. The results were shown in Table 1 to 4.

The motility for 3 h was not statistically differ in results of all treatment groups. The taurine groups for 6 and 9 h were significantly higher results over 94 and 87% compared to control (85.1±0.5 and 72.4±0.3, $p < 0.05$) respectively. For 12 h, sperm motility was remained over 57% in results of taurine groups. In taurine with H₂O₂ for 6 h, the sperm motility were significantly higher results in 75 mM taurine plus H₂O₂ when compared to control, but for 9 and 12 h, the taurine plus H₂O₂ were not a positive effect on sperm motility (Table 1).

The viability were higher result over 85% of taurine and taurine with H₂O₂ groups for 3 h than in control (75.3±0.3, $p < 0.05$). For 6, 9 and 12 h, sperm viability were

significantly higher result in taurine groups than in control, but taurine plus H₂O₂ groups for 6, 9 and 12 h were not sharply dropped in sperm viability (Table 2).

The survival for 3 h was not statistically differs in results of all treatment groups. In the 25 and 50 mM taurine groups for 6 h, the survival were significantly higher results (76.0±0.6 and 78.0±0.7, respectively) compared to control (72.0±0.6, $p < 0.05$). Sperm survival for 9 and 12 h were higher result ($\geq 48\%$ in 9 h and $\geq 41\%$ in 12 h, respectively) in taurine groups compare to controls (43.0±2.1 and 31.0±0.6), but the taurine plus H₂O₂ for 6 to 12 h were not change the sperm survival rates (Table 3).

The membrane integrity 3 and 6 h were not statistically differ in results of taurine groups but 9 h, the membrane integrity were significantly higher result of taurine groups

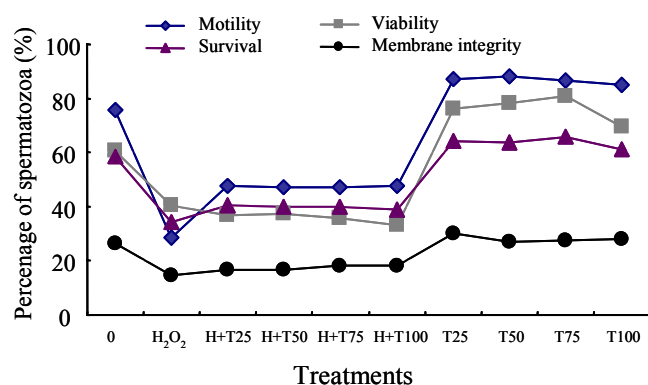


Figure 1. Overall means of sperm characteristic of boar semen diluted in Modena extender supplemented with taurine with or without hydrogen peroxide.

(25.7±0.7, 28.3±0.3, 25.0±0.6 and 22.3±1.5) respectively, than in control (21.3±0.9) and for 12 h, the membrane integrity was higher in 50 mM taurine group (18.7±1.9) when compare to control (10.0±0.6, $p<0.05$). In a taurine with H₂O₂ groups, the results of the membrane integrity were similar to the result of survival (Table 4).

The overall means of sperm characteristic of boar semen treated with different concentration of taurine with or without hydrogen peroxide are shown in Figure 1. Sperm characteristic of taurine treated groups regardless of concentration were higher than that of control group, and taurine with H₂O₂ groups were not positive effects on sperm characteristics when compared to control.

DISCUSSION

The generation of ROS is an essential prerequisite for the normal function of many cells however excessive formation can lead to cellular damage and pathology (Halliwell and Arunoma, 1991). Exposure of sperm to ROS is associated with decreased fertility, and the formation of lipid peroxidation and DNA damage (Aitken et al., 1989; Chen et al., 1997). In addition, the production of lipid peroxidation in sperm due to oxidative stress has been associated with a loss of cell motility (Aitken et al., 1989; Alvarez and Storey, 1994). Spermatozoa are particularly susceptible to ROS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PSFA; Alvarez and Storey, 1994) and their cytoplasm contains low concentrations of scavenging enzymes (de Lamirande and Gagnon, 1995). Oxidative stress-mediated damage to the sperm plasma membrane may account for defective sperm function observed in a high proportion of infertility patients (Aikten, 1997). Oxidative stress attacks not only the fluidity of the sperm plasma membrane but also the integrity of DNA in the sperm nucleus (Sun et al., 1997). Oxidative stress-induced DNA damage may accelerate the process of germ cell apoptosis leading to the

decline in sperm counts associated with male infertility and the apparent deterioration of semen quality observed over the past 4 to 5 decades. The antioxidant capacity of sperm and the extracellular environment within which they live, therefore, would seem to be significant factors in determining the etiology of male infertility and potentially the incidence of germ line mutations through spermatozoal DNA damage. Mature human spermatozoa do not possess significant DNA repair mechanism (Chen et al., 1997) and contain negligible levels of antioxidants (Aitken et al., 1995; Alvarez and Storey, 1995). Oxidative DNA damage, therefore, has the potential to accumulate, increasing the possibility of mutagenesis during fertilization. In addition, Fraga et al. (1991) demonstrated that oxidative damage to sperm DNA was not necessarily associated with decreased cell motility or viability, indicating fertilization may still be possible by a cell that contain damaged DNA.

Taurine has indirect antioxidant effect: it contributes to limit on the deleterious effect of ROS by neutralizing cytotoxic aldehydes, the end-products of the peroxidation cascade reaction (Ogasawara et al., 1993). Mammalian embryos are capable of taurine uptake. The protective effect of taurine on cells is effective at a low concentration (0.3 μM). Hypotaurine and taurine might have a sequential and complementary action on gametes and embryos (Van Winkle and Dickinson, 1995). For taurine, the results are contradictory: it has been observed that supplementing taurine may have beneficial effects on embryo development (Dumoulin et al., 1992; Li and Foote, 1993), though others have not found to be the this case (Van Winkle and Dickinson, 1995). However, whether or not supplement of taurine in boar semen storage exerts the ROS has not been attempted. These study were examined the effects of taurine and in boar semen during incubation at 37°C.

Our finding indicated that the taurine were increased the sperm characteristics (motility, viability, survival and membrane integrity) on boar semen during *in vitro* incubation but taurine with H₂O₂ were not a positive affect on sperm characteristics from 6 to 12 h during *in vitro* incubation. Therefore, we conclude that supplementation of taurine as antioxidant in boar semen during *in vitro* storage could be improved the semen quality using the artificial insemination.

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