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Maintenance of Sperm Characteristics and *In vitro* Developmental Rate of Embryos against Oxidative Stress through Antioxidants in Pig

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ABSTRACT : Oxidative stress is one of the major causes of failure of *in vitro* storage of boar semen. Reactive oxygen species (ROS) are one of the important mediators of oxidative stress during *in vitro* storage of boar semen. Our study examined the effects of taurine on sperm characteristic and on *in vitro* developmental embryos during *in vitro* storage of boar semen for 7 days. Semen was randomly aliquoted into 3 centrifuge tubes and treated with different concentrations of taurine (25-100 mM). The characteristics of boar sperm were analyzed for motility by light microscopy, viability by using a Makler counting chamber and membrane integrity by a hypoosmotic swelling test (HOST). The percentages of motile spermatozoa in taurine groups after 5 days were significantly higher compared to the control. Sperm viability in the control was lower than in taurine groups after 7 days irrespective of different taurine concentration. In the hyoosmotic swelling test (HOST), significantly higher results were obtained in taurine groups after 3 days. Also, the developmental rates of *IVM/IVF* porcine embryos from semen treated with pyruvate and taurine were significantly increased when compared with the control ($p<0.05$). These results indicate that supplementation of taurine as an antioxidant in boar semen extender can improve the semen quality.
(**Key Words :** Reactive Oxygen Species (ROS), Boar Semen, Taurine, Embryo Antioxidant, Sperm Characteristic)

INTRODUCTION

The boar semen used for artificial insemination (AI) is stored at 17-19°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 researchs (Korniewicz et al., 1992; Alexopoulos 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 (Alexopoulos et al., 1996). 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 (Brezezinska-Slevbodzinska et al., 1995).

Antioxidants overcomes the cell damage by ROS production, limited portion of cells begin the process of programed cell death called apoptosis. ROS are regulated by superoxide dismutase (SOD) that changes superoxide into H_2O_2 or by catalase and glutathione peroxidase (GPx) which decompose H_2O_2 into H_2O. Because of their ability to degenerate ROS into nontoxic compounds, these antioxidative enzymes play important roles in protecting cells from oxidative stress-induced cell death (Majima et al., 1998; Chandra et al., 2000; Kim et al., 2007).

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 osmo-regulation, cell proliferation, viability and prevention of oxidant-induced injury in many tissues (Chesney, 1985; Huxtable, 1992; Redmond et al., 1996). The beneficial

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effects of taurine as an antioxidant in biological systems have been attributed to its ability to stabilize biomembranes (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) and the effect of semen treated with taurine with or without hydrogen peroxide on the development of *IVM/IVF* porcine embryos.

MATERIAL AND METHODS

Semen preparation

Sperm-rich fractions (30 to 50 ml) were collected from 1-3 purebreed (Duruc, Yorkshire and Landrace) with 85% motile sperm by the gloved hand method at the local A. I center (Wonju) and was diluted three times with Modena to give a concentration to 2×10^7 spermatozoa/ml. The diluted semen was 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). 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 storaged for 1-7 days at 17°C.

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

Sperm analyisis

The analysis of semen quality were evaluated for 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℃ 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 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 takes 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.

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 swellon. 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.

Procedure of *in vitro* maturation and *in vitro* fertilization in porcine oocytes

Culture media : Cumulus-oocyte complexes (COCs) were washed in IVM-I medium consisting of TC-199 supplemented with 10% porcine follicular fluid (pFF), 0.57 mM cysteine, 10 IU/ml eCG, 10 IU/ml hCG, 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulfate. The maturation medium for the first 22 h of *in vitro* maturation designated IVM-I. For the second 20-22 h of maturation (IVM-II), the IVM-II was used as for IVM-I without hormone. The fertilization medium (IVF-medium) was modified Tris-buffered medium (mTBM, 113.1 mM NaCl, 3.0 mM KCl, 20.0 mM Tris, 11.0 mM D-glucose, 7.5 mM $CaCl_2$-$2H_2O$ and 5.0 mM Na-Pyruvate; Abeydeera and Day, 1997) containing 2 mM caffeine and 0.1% BSA (Sigma, USA). Embryo culture medium was PZM 4 medium supplmented with 4 mg/ml BAS (Fraction V, Yoshioka et al., 2002).

Collection of cumulus oocyte complexes : Porcine ovaries obtained from a local slaughterhouse were transported at 39°C within 2 h in 0.9% NaCl solution to the laboratory and they were washed four times in sterile 0.9% NaCl solution. Ovarian follicles 3-5 mm in diameter were aspirated using an 18 gauge needle fixed to a 10 ml disposable syringe and cumulus oocyte complexes (COC) containing a compact cumulus mass and even cytoplasmic pigmentation were washed three times in IVM-wash medium (IVM-I medium). Then 20-25 oocytes were cultured in 100 μl of IVM-I medium which had peviously been covered with mineral oil and equilibrated for 22 h at 38.5°C and 5% CO_2 in air prior to use. After culture for 22 h, the COCs were washed three times in IVM-II medium and then cultured in IVM-II for another 20-22 h at above conditions.

In vitro fertilization : After maturation, oocytes surrounded by expanded cumulus cell were washed twice, each in maturation medium and in IVF medium, and 20 oocytes were transferred into a 50 μl droplet of IVF medium covered with warm mineral oil. Frozen semen was thawed in 37°C water bath for 30-40 sec. After thawing, spermatozoa were washed twice by centrifugation at 1,500 rpm for 10 min and resuspended with IVF medium to give a concentration to 2×10^6 spermatozoa/ml, and 50 μl of the sperm suspension was introduced to 50 μl of the fertilization drops containing oocytes.

After 5-6 h of sperm-oocytes coincubation, oocytes were washed 3 times in culture medium and transferred in 100 μl of culture medium.

After 35-38 h of culture, oocytes were stripped of cumulus and corona cell in maturation medium containing 0.1% hyaluroniase by repeated passage using fine pipette and 2-8 cell embryos were pick up and alloted in each 100 μl drop of culture medium and then cultured for 6-8 days in 5% CO_2 and 20% O_2 at 38.5°C.

Semen preparation and embryo culture : Boar semen in treated with 250 μM H_2O_2, 250 μM H_2O_2+100 mM taurine, 100 mM taurine were incubated at 17°C for 4 days, they were used for *in vitro* fertilization and 2- to 8-cell embryos were alloted in each 100 μl drop of culture medium (PZM 4) in 5% CO_2 and 5% O_2 at 38.5°C. Embryos were cultured 6-7 day after fertilization. The culture medium was changed every 2 days and embryos were checked briefly at that time.

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 valus was <0.05 in ANOVA, treated means were compared for differences through use of Duncan's modified multiful range test. All data were expressed as mean±SEM. $p\leq0.05$ was considered to be significant.

RESULTS

The sperm characteristics (motility, viability and membrane integrity) of boar semen treated with different concentration of taurine (0 mM-100 mM) were assessed at 1 day to 7 day in boar semen. The results were shown in Table 1 to 3.

The motility for 3 day was not statistically differs in results of all treatment groups. The taurine groups (for 5 days) were significantly higher results over 63% compared to control (53.7±2.9, $p<0.05$). For 7 days, sperm motility was remained over 54% in results of taurine groups (Table 1).

For 3 day to 6 days, the viability was not statistically differs in all treatment groups. Sperm viability for 7 day was significantly higher result in 75 mM and 100 mM taurine groups compared with any other groups (Table 2).

The membrane integrity for 3 days were not statistically differ in results of taurine groups but over 4 days, the membrane integrity were significantly higher result of 75 mM and 100 mM taurine groups than in control, the results of the membrane integrity were similar to the result of motility. The overall means of sperm membrane integrity of boar semen treated with different concentration of taurine were higher when compared to control (Table 3).

When boar semen treated with 0, 250 μM H_2O_2, 250 μM H_2O_2 +100 mM taurine and 100 mM taurine were used for *in vitro* fertilization, the developmental rates of porcine embryos in PZM 4 medium were summarized in Table 4. Boar semen treated with taurine (100 mM) was significantly increased the developmental rates of morula and blastocyst (70.7%) when compared of any other groups (44.4% in control, 38.6% in 250 μM H_2O_2, and 67.4% in 250

Table 1. Effects of taurine on motility in boar semen during *in vitro* storage

Storage periods (days)	Taurine (mM)				
	0	25	50	75	100
1	97.3±0.7^a	96.6±0.7^a	98.0±0.0^a	98.0±0.0^a	96.0±1.0^a
2	76.6±7.3^a	83.3±7.3^a	81.6±1.9^a	92.6±3.9^a	95.3±0.3^a
3	75.0±5.0^a	80.0±1.6^a	85.3±1.8^a	86.6±8.3^a	85.0±2.9^a
4	66.0±2.9^b	68.2±1.9^b	72.4±1.81^a	80.0±1.5^a	80.9±4.3^a
5	53.7±2.9^c	63.3±7.2^b	65.0±1.1^b	76.6±1.8^a	78.3±1.7^a
6	42.3±1.7^c	56.6±7.3^b	65.0±1.7^b	78.6±1.6^a	66.6±2.2^b
7	30.6±1.2^c	54.0±8.3^b	55.2±1.6^{ab}	65.0±2.3^a	63.3±1.9^a
Overall means					
Ta-0 mM	63.1±3.1^B				
Ta-25 mM	65.1±7.7^B				
Ta-50 mM	65.3±5.5^B				
Ta-75 mM	82.5±3.2^A				
Ta-100 mM	80.8±4.5^A				

$^{a, b, c, d}$ Values with different superscripts within and $^{A, B}$ overall mean with columns are significantly differ, $p<0.05$.

Table 2. Effects of taurine on viability in boar semen during *in vitro* storage

Storage periods (days)	Taurine (mM)				
	0	25	50	75	100
1	89.7±2.3[a]	92.4±0.4[a]	92.6±0.4[a]	91.8±1.6[a]	90.2±1.6[a]
2	84.8±1.6[ab]	87.4±0.9[ab]	88.3±1.3[a]	84.1±3.6[a]	81.0±1.4[b]
3	81.9±0.4[a]	81.9±0.8[a]	82.2±2.7[a]	79.6±3.4[a]	75.1±2.0[a]
4	76.1±0.2[b]	78.5±3.3[a]	77.4±0.8[a]	75.1±2.3[a]	72.8±3.6[a]
5	71.3±1.3[a]	71±3.3[a]	70.3±3.5[a]	73.9±2.4[a]	68.8±2.4[a]
6	61.7±3.9[a]	61.1±5.2[a]	67.3±3.3[a]	69.6±1.8[a]	61.9±4.6[b]
7	46.7±6.1[b]	64±3.0[a]	65.4±0.8[a]	66.2±0.2[a]	63.4±1.9[a]
Overall means					
Ta-0 mM	73.2±3.2				
Ta-25 mM	69.9±5.0				
Ta-50 mM	77.7±2.3				
Ta-75 mM	73.9±3.8				
Ta-100 mM	73.3±2.2				

[a, b] Values with different superscripts within rows are significantly differ, $p<0.05$.

Table 3. Effects of taurine on membrane integrity in boar semen during *in vitro* storage

Storage periods (days)	Taurine (mM)				
	0	25	50	75	100
1	89.7±2.3[a]	90.3±0.4[a]	92.6±0.4[a]	91.8±1.6[a]	90.2±1.6[a]
2	84.8±1.6[ab]	87.4±1.0[ab]	88.3±1.2[a]	84.1±3.6[ab]	81.0±1.4[b]
3	61.8±0.4[c]	67.8±0.8[c]	72.3±2.7[b]	81.1±3.4[a]	81.3±2.0[a]
4	55.1±0.2[c]	66.9±3.3[b]	68.5±0.8[b]	74.3±2.3[a]	76.5±3.6[a]
5	28.8±1.2[c]	54.2±3.3[b]	54.2±3.5[b]	61.7±2.4[ab]	70.1±2.4[a]
6	28.7±3.9[c]	53.5±5.2[b]	53.7±3.3[b]	59.1±1.8[ab]	67.8±4.6[a]
7	24.0±6.1[c]	52.2±3.0[b]	51.7±0.8[b]	55.9±0.2[ab]	61.4±1.9[a]
Overall means					
Ta-0 mM	44.8±2.9[C]				
Ta-25 mM	58.1±1.4[B]				
Ta-50 mM	61.0±1.5[B]				
Ta-75 mM	67.2±1.7[A]				
Ta-100 mM	71.1±2.0[A]				

[a, b, c] Values with different superscripts within rows and [A, B, C] overall mean with columns are significantly differ, $p<0.05$.

Table 4. Effect of semen treated with antioxidants with or without hydrogen peroxide on the development of porcine IVM/IVF embryos

Semen treatments	No. of IVM/IVF embryos	No. of embryos developed to (%)			Morulae plus Blastocysts (%)
		Pre-morulae	Morulae	Blastocysts	
0	45	25 (55.6)[b]	15 (33.3)[b]	5 (11.1)[b]	20 (44.4)[b]
250 μM H_2O_2	44	27 (61.4)[a]	13 (29.5)[b]	4 (9.1)[b]	17 (38.6)[b]
250 μM H_2O_2 +100 mM taurine	43	14 (32.6)[c]	23 (53.5)[a]	6 (11.3)[b]	29 (67.4)[a]
100 mM taurine	41	12 (29.3)[c]	20 (48.9)[a]	9 (22.5)[a]	29 (70.7)[a]

[a, b, c] Values with different superscripts within column are significantly differ, $p<0.05$.

μM H_2O_2+100 mM taurine, $p<0.05$).

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 enviroment 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 this case (Van Winkle and Dickinson, 1995). However, whether or not supplement of taurine in boar semen storage exerts the ROS has a little attempted. These study were examined the effects of taurine and in boar semen during storage at 17°C.

Our finding indicated that the supplementation of taurine were increased the sperm characteristics on boar semen during *in vitro* storage. Boar semen treated with taurine was significantly increased on *in vitro* development of *IVF* porcine embryo when compare to control ($p<0.05$). This finding, is consistent with that reported by Lamirande and Gagnon (1992) who added to pyruvate into semen extender to prevent the damage by ROS in human spermatozoa. Also Van Winkle and Dickinson (1995) reported that taurine had a positive effect as antioxidant on human embryos development in *in vitro*, this results were agreement with our finding that taurine was increased the *in vitro* development of porcine *IVM/IVF* embryos.

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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