Effects of Pyruvate and Taurine for *In Vitro* Preservation in Boar Semen and the Developmental Rates of Embryos Fertilized by Semen Treated with Antioxidant

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ABSTRACT

Oxidative stress is one of the major causes of failure in *in vitro* storage of boar semen. Reactive oxygen species (ROS) are known to be important mediators of such stress. The present study examined the effects of pyruvate and taurine on sperm motility and expression of BAD, Cytochrome c, Caspase-3 and Cox-2 protein in *in vitro* storage of boar semen, and tested the effect of semen treated with antioxidant with or without hydrogen peroxide on the development of IVM/IVF porcine embryos. Semen samples were transported to the laboratory at 17℃ within 2 hr after collection and were treated with different concentration of pyruvate (1~10mM) and taurine (25~100mM) with or without 250uM H_2O_2, respectively. The supplementation of pyruvate and taurine increased sperm motility in boar semen during *in vitro* incubation at 37℃. Expression of apoptosis protein (BAD, cytochrome c, caspase-3 and cox-2) were reduced in the group of boar semen treated with pyruvate and taurine when compared to the other groups. The developmental rates of IVM/IVF porcine embryos fertilized by semen treated with pyruvate and taurine were significantly increased when compared to control ($P<0.05$).

These results indicate that supplementation of pyruvate and taurine as antioxidants in boar semen extender can improve the semen quality and increase *in vitro* development of porcine IVM/IVF embryos when boar semen treated with antioxidants was used for *in vitro* fertilization.

(Key words: Cox-2, BAD, Cytochrome c, Caspase-3, Semen motility)

INTRODUCTION

The boar semen used for artificial insemination is stored at 17℃~19℃, following the dilution of an appropriate extender. The 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). There are many obstacle for *in vitro* preservation of boar semen because of the low resistance of low temperature. And also sperm function is altered rapidly *in vitro* storage by many factors, one of the greatest factor of failure is the reactive oxygen species (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 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 and the relative low antioxidant capacity of boar spermatozoa (Cerolini et al., 2000).

Therefore, in the present study, we investigated the effects of pyruvate and taurine in *in vitro* storage boar semen. Sperm motility and apoptosis protein(BAD, Cytochrome c, Caspase-3 adn Cox-2) as a reliable predictors of sperm fertilizing ability were determined. The developmental rates of porcine embryos fertilized by the semen treated with pyruvate and taurine with or without hydrogen peroxide were examined. Also the cell number of blastocysts were evaluated by differential fluorescence staining.

MATERIAL AND METHODS

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Semen Evaluation

Sperm-rich fraction were collected from 1~3 crossbred boar of known reproductive history in Won Ju A.I center. Just after collection, semen samples were diluted with Modena extender and transport to the laboratory at 17℃ within 2 hour. Semen was randomly aliquoted into 3 centrifuge tube and was treated with pyruvate(1~10mM) or taurine(25~100mM) with or without 250uM H_2O_2, respectively. The semen of each treated group was incubated for 3, 6, 9, and 12h at 37℃. Sperm motility were assessed by determining the percentage of spermatozoa showing any movement of the flagellum.

Procedure of *In Vitro* Maturation and *In Vitro* Fertilization

Culture Media

IVM-I medium were consisted of TC-199 supplemented with 10% porcine follicular fluid (pFF), 0.57mM cysteine, 10IU/ml eCG, 10IU/ml hCG, 75ug/ml potassium penicillin G and 50ug/ml streptomycin sulfate and the IVM-II was used as for IVM-I without hormone. The fertilization medium (IVF-medium) was used the modified Tris-buffered medium containing 2mM caffeine and 0.1% BSA(mTBM, Abeydeera and Day, 1997). Embryo culture medium was PZM 4 medium supplement with 4mg/ml BSA (Yoshioka et al., 2002).

Collection of Cumulus Oocyte Complexes

Porcine ovaries obtained from a local slaughterhouse were transported at 39℃ within 2hr in 0.9% NaCl solution to the laboratory. Immature oocytes were aspirated in ovarian follicles(3~5mm diameter) and cumulus oocyte complexes (COC) containing a compact cumulus mass were washed three times in IVM-I medium. Then 20~25 oocytes were cultured in 100ul of IVM-I medium for 22h at 38.5℃ and 5% CO_2 in air. After culture for 22hr, the COCs were cultured in IVM-II for another 20~22hr at above conditions.

Semen Preparation and *In Vitro* Fertilization

Boar semen treated with 250uM H_2O_2 +1mM pyruvte, 1mM pyruvte, 250uM H_2O_2 +50mM taurine, 50mM taurine were incubated at 37℃ for 6hr, they were used for *in vitro* fertilization. After maturation, 20 oocytes were transferred into a 50ul droplet of IVF medium. Sperm suspension(1×10^5 spermatozoa/ml) were introduced to 50ul of the fertilization drops containing oocytes. After 5~6hr of sperm-oocytes coincubation, oocytes were transferred in 100ul of culture medium. After 35~38hr of culture, cleaved embryos(2~8 cell) were alloted in each 100ul drop of culture medium and then cultured for 6~8 days in 5% CO_2 and 20% O_2 at 38.5℃.

Differential Staining of Blastocysts

Differential fluorescence staining of blastocysts was performed the technique described by Machaty et al. (1998). Embryos were exposed to a 1:5 dilution of TNBS (2, 4, 6 - trinitrobenzene sulfonic acid, Sigma) in PBS for 30min and treated TNBS embryos were rinsed in PBS and exposed to a 1:10 dilution anti-DNP-BSA (ICN, USA) in PBS. They were placed into a 1:10 dilution of guinea pig complement (Gibco, USA) containing 10ug/ml propidium iodide and 10ug/ml bisbenximide (Hoechst 33342, Sigma) for 30 min. The stained embryos were examined under fluorescence microscope (Excitation 280~365nm, Emission 425~480nm, Ziess, Germany).

Western Blot Analysis for Cytochrome c, Bad, Casapase-3 and Cox-2 in Boar Semen

Sperm pellet(1×10^7 spermatozoa/ml) were harvested, washed twice with ice cold PBS, and resuspended in 20mM Tris-HCL buffer containing a10 g/mL of apoprotinin, 1mM of phenylmethylsulfonyl fluoride, 50 g/mL of trypsin inhibitor and 5 mM benzamidine. Whole cell lysates were prepared for immunoblotting analysis of Cytochrome c, BAD, Caspase-3 and Cox-2(Transduction Laboratories, USA) by sonication and centrifugation at 13,000×g for 20 min at 4℃. The membrane was hybridized with Bad, Cytochrome c, Caspase-3 and Cox-2 by an ECL plus Western blotting detection system (Amersham, U.S.A). The amount of expressed protein was quantified by scanning the blots by image densitometer (Image quant 5.2; NIH, U.S.A). The relative protein expression was calculated in comparison to the signal intensity at control.

Statistical Analysis

Values are expressed as the mean±SEM. The percentage data were analyzed using the General Linear Model procedure and Duncan's Multiple Range test of SAS. Significance was determined at $P\leq 0.05$.

RESULTS

Effects of different concentration of pyruvate(1~10mM) against free radial (250uM H_2O_2) were evaluated on the sperm motility for 3, 6, 9 and 12hr incubation at 37℃ in boar semen(Table 1).

The motility were remained over 90% for 3hr in all treatment groups. Sperm motility decreased gradually in pyruvate treatment groups and pyruvate plus H_2O_2 groups according to incubation times (≥ 73.3±0.9), but sperm motility of hydrogen peroxide groups was significantly dropped for 9hr and 12hr (4.0±0.6 and 0.0±0.0). Sperm motility of pyruvate groups (1 and 2mM) for 6hr and 9hr were significantly higher results compared to control, and for 12hr, the 2mM pyruvate group was the highest score when compared to any other groups ($P<0.05$).

The sperm motility of boar semen treated with different

concentration of taurine (25~100mM) with or without 250uM H_2O_2 were assessed at 3, 6, 9 and 12hr during incubation at 37℃ in boar semen(Table 2). The motility for 3h were not statistically differ in results of all treatment groups. The taurine groups for 6hr and 9hr were significantly higher results(≥ 94.3% and 87.3%) compared to control (85.1±0.5 and 72.4±0.3, $P<0.05$). In taurine plus H_2O_2 for 6hr, the sperm motility were significantly higher results in 75mM taurine plus H_2O_2 and 100mM taurine plus H_2O_2 when compared to control but for 9hr and 12hr, the taurine plus H_2O_2 were not a positive effect on motility of sperm.

The overall means of sperm motility for antioxidants treatments(pyruvate and taurine with or without hydrogen peroxide groups) were summarized in Table 3.

No significant changes of motility were observed until 3hr of incubation in all treatment groups. At 6hr incubation, H+P and H+T groups (87.1±1.2 and 88.3±1.2) were not differ to control group(85.1±2.4), but for 9hr H+T group (3.2±2.0) was sharply decrease when compare to control (72.3±2.6) and H+P group (68.5±2.0)($P<0.05$).

Protective effect of antioxidant against hydrogen peroxide on motility in boar semen during incubation at 37℃ for 12hr was only shown the pyruvate plus hydrogen peroxide group (76.1±0.9).

The motility for 6hr and 9hr were higher result in taurine group (95.3±0.3 and 90.0±0.9) when compare to control (85.1±2.4 and 72.3±2.6) but for 12hr was the highest in pyruvate group (83.0±1.6) than those of any other groups (47.5±1.9 in control and 83.0±1.6 in taurine, Table 3).

To elucidate the protective mechanism of antioxidants in boar semen, boar semen were incubated for 6hr at 37℃ in Modena extender supplemented with antioxidants such as 1mM pyruvate, 50mM taurine and antioxidants plus 250uM H_2O_2, The detection of BAD, Cytochrome c, Caspase-3 and Cox-2 expression were performed by western blot analysis.

The treatments of pyruvate and taurine were reduced the expression of BAD protein when compared to the other groups. The lowest expression of Cytochrome c from mitochondria in boar sperm were detected in taurine group(Fig. 1).

Level of Caspase-3 were reduced in H_2O_2 plus pyruvate and taurine groups when compared to the other groups. The expression of Cox-2 were dramatically reduced in pyruvate, H_2O_2 plus taurine and taurine groups when compared to the control (Fig. 1).

Boar semen treated with pyruvate (1mM) and taurine (50mM) were significantly increased the developmental rates beyond morula stage (70.0% and 70.7%) when compared of any other groups (44.4% in control, 38.6% in 250uM H_2O_2, 54.5% in 250uM H_2O_2+1mM pyruvate and 67.4% in 250uM H_2O_2+50mM taurine, $P<0.05$).

Table 1. Effects of pyruvate with or without hydrogen peroxide on motility in boar semen during incubation at 37℃

Incubation time (hours)	Motility(%)									
	Control	H	H+P1	H+P2	H+P5	H+P10	P1	P2	P5	P10
3	97.3±0.3^{a}	94.3±2.2^{a}	94.3±0.7^{a}	95.7±0.7^{a}	94.3±0.9^{a}	96.3±0.3^{a}	94.7±0.9^{a}	94.0±0.6^{a}	94.7±0.9^{a}	95.7±0.7^{a}
6	85.1±0.5^{b}	16.3±2.0^{c}	86.3±0.9^{b}	87.3±0.9^{b}	85.7±1.2^{b}	89.0±0.6^{ab}	92.3±1.2^{a}	91.1±0.8^{a}	87.3±0.9^{b}	85.3±0.3^{c}
9	72.4±0.3^{b}	4.0±0.6^{d}	73.3±0.9^{c}	82.3±0.3^{b}	79.0±0.6^{bc}	84.0±0.6^{b}	89.6±1.5^{a}	89.7±3.8^{a}	83.3±0.9^{b}	85.0±1.5^{b}
12	47.5±1.4^{d}	0.0±0.0^{e}	71.3±0.3^{c}	73.3±0.9^{c}	75.7±1.3^{c}	78.9±0.6^{b}	81.0±0.3^{b}	86.7±3.4^{a}	76.7±0.8^{bc}	80.0±0.4^{b}

$^{a\sim e}$ Different superscripts within rows are significantly differ, $P<0.05$.

Control, Modena with 0.4% BSA; H, 250uM H_2O_2; H+P1, 250uM H_2O_2 +1mM pyruvate; H+P2, 250uM H_2O_2 +2mM pyruvate; H+P5, 250uM H_2O_2 +5mM pyruvate; H+P10, 250uM H_2O_2 +10mM pyruvate; P1, 1mM pyruvate; P2, 2mM pyruvate; P5, 5mM pyruvate; P10, 10mM pyruvate.

Table 2. Effects of taurine with or without hydrogen peroxide on motility in boar semen during incubation at 37℃

Incubation time (hours)	Motility(%)									
	0	H	H+T25	H+T50	H+T75	H+T100	T25	T50	T75	T100
3	97.3±0.3^{a}	94.3±2.2^{a}	97.0±0.6^{a}	95.3±0.3^{a}	96.0±0.6^{a}	97.0±0.6^{a}	96.3±0.3^{a}	97.3±0.5^{a}	95.3±0.3^{a}	96.3±0.3^{a}
6	85.1±0.5^{b}	16.3±2.0^{c}	89.5±0.3^{b}	87.3±0.3^{b}	88.0±0.6^{a}	88.3±0.9^{a}	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}

$^{a\sim e}$ Different superscripts within rows are significantly differ, $P<0.05$.

Control, Modena with 0.4% BSA; H, 250uM H_2O_2; H+T25, 250uM H_2O_2 +25mM taurine; H+T50, 250uM H_2O_2 +50mM taurine; H+T75, 250uM H_2O_2 +75mM taurine; H+T100, 250uM H_2O_2 +100mM taurine; T25, 25mM taurine; T50, 50mM taurine; T75, 75mM taurine; T100, 100mM taurine.

Table 3. Protective effect of antioxidants with or without hydrogen peroxide on motility in boar semen during incubation at 37℃

Incubation time (hours)	Treatments					
	Control	H_2O_2	H+P	H+T	Pyruvate	Taurine
3	97.3±0.8[a]	94.3±0.8[a]	95.2±0.4[a]	96.3±0.4[a]	94.6±0.4[a]	96.1±0.2[a]
6	85.1±2.4[bc]	46.3±2.4[c]	87.1±1.2[b]	88.3±1.2[ab]	87.3±1.0[b]	95.3±0.3[a]
9	72.3±2.6[b]	33.3±4.0[c]	68.5±2.0[b]	3.2±2.0[d]	86.7±1.4[a]	90.0±0.9[a]
12	47.5±1.9[d]	0.0±2.0[e]	76.1±0.9[b]	0.0±0.9[e]	83.0±1.6[a]	64.0±1.4[c]

[a~e] Different superscripts within rows are significantly differ, $P<0.05$.

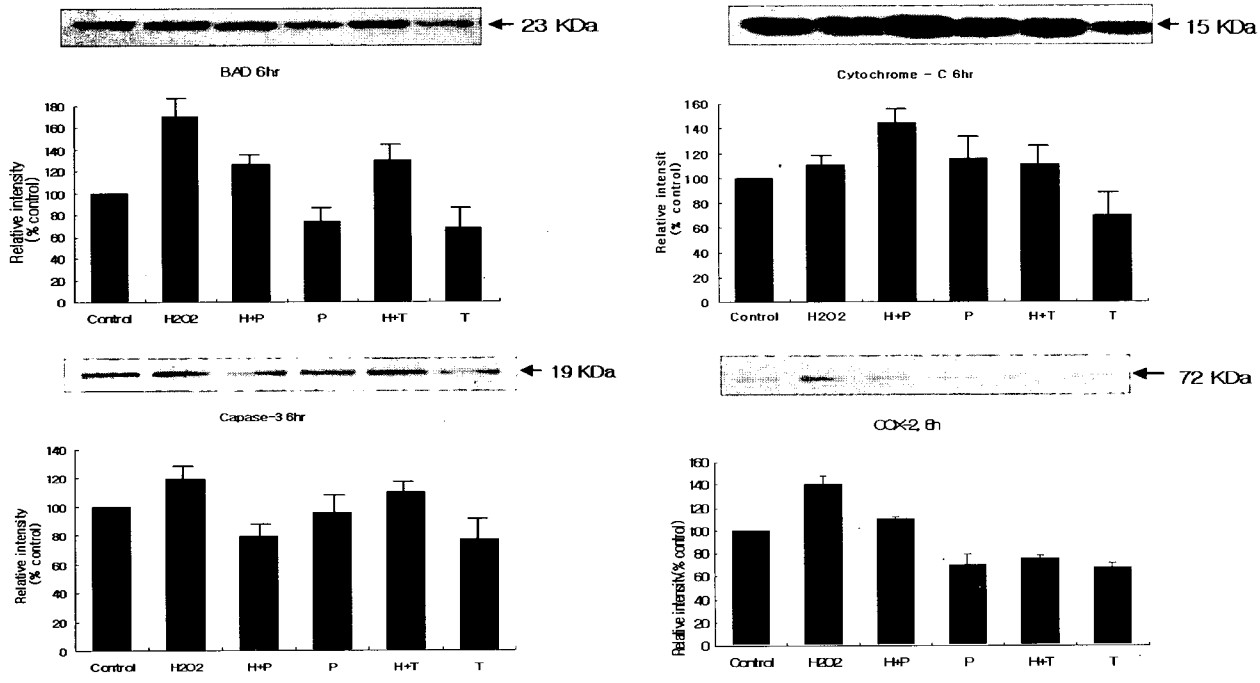

Fig. 1. Levels of BAD, cytochrome c, caspase-3 and cox-2 protein were measured during incubation at 37℃ by western blot analysis in boar semen. Upper panel shows a representative immunoblot and down panel shows quantitative values of immuoblots. Data represent three independent experiments and were expressed as mean±SD.

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)[c]	5(11.1)[b]	20(44.4)[c]
H	44	27(61.4)[a]	13(29.5)[c]	4(9.1)[b]	17(38.6)[c]
H+P	44	20(45.5)[b]	21(47.8)[b]	3(6.9)[c]	24(54.5)[b]
P	40	12(30.0)[c]	24(60.0)[a]	4(10.0)[b]	28(70.0)[a]
H+T	43	14(32.6)[c]	23(53.5)[b]	6(11.3)[b]	29(67.4)[a]
T	41	12(29.3)[c]	20(48.9)[b]	9(22.5)[a]	29(70.7)[a]

[a~c] Values with different superscripts within column are significantly differ, $P<0.05$.
H, H_2O_2 250uM; H+P, H_2O_2 250uM + pyruvate 1mM; P, pyruvate 1mM; H+T, H_2O_2 250uM+ taurine 50mM; T, taurine 50mM.

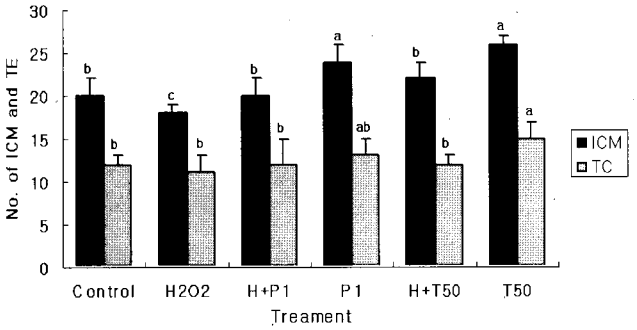

Fig. 2. Number of inner cell mass(ICM) and trophectoderm cell (TE) of blastocysts produced by IVM/IVF porcine oocytes. a~c Values with different superscripts within the number of ICM and TE are significantly differ, $P<0.05$.

The number of inner cell mass and tropectoderm cell of blastocyst produced by IVM/IVF in 1mM pyruvate (24.7± 1.7 and 15.5±1.5) and 50mM taurine (26.6±0.6 and 16.8± 1.3)groups were higher increased than those of control (21.0±1.2 and 13.9±0.8), 250uM H_2O_2 (18.5 ±0.4 and 14.3±0.5), and 250uM H_2O_2+1mM pyruvate (22.3±1.2 and 14.3±1.5) and 250uM H_2O_2+50mM taurine (22.5±1.4 and 13.9±0.6) groups ($P<0.05$, Fig. 2).

DISCUSSION

The generation of ROS is an essential prerequisite for the normal function of many cells, however, excessive formation of ROS can lead to cellular damage and pathology (Halliwer, 1991). Exposure of sperm to ROS is associated with decreased fertility, and the formation of lipid peroxidation and DNA damage (Aitken and Clarkson, 1989; Chen et al., 1997). The lipid peroxidation in sperm by the oxidative stress is associated with a loss of cell motility (Aitken and Clarkson, 1989; Alvarez and Storey, 1995). Mature mamalian 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). 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 spermatozoa that contain damaged DNA.

Pyruvate plays a pivotal role in both primary energy metabolism and the redox potential. Pyruvate may be secreted by some cells to function as an extracellular antioxidant(O'Dommell-Tormey et al., 1987). Pyruvate prevents peroxide-induced injury of *in vitro* cultured bovine embryos (O'Dommell-Tormey et al., 1987) and, when associated with lactate by being added to culture medium, prevents the effects of ROS on human spermatozoa(de Lamirande and Gagnon, 1992).

Taurine has indirect antioxidant effects and also taurine 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 (Van Winkle and Dickinson, 1995). Hypotaurine and taurine might have a sequential and complementary action on gametes(Van Winkle and Dickinson, 1995). However, whether or not supplement of taurine in boar semen storage prevent the deterious effect of ROS has not been attempted.

Our finding indicated that the supplementation of pyruvate and taurine were increased the sperm motility on boar semen during *in vitro* incubation but taurine plus H_2O_2 were not a positive affect on sperm characteristics from 9hr to 12hr during *in vitro* incubation. For that reason, taurine had a indirect antioxidant effects when compare to the pyruvate. Boar semen treated with pyruvate and taurine were increased on *in vitro* development of IVF porcine embryo, and cell number of blastocysts in pyruvate and taurine groups were significantly increased when compare to control ($P<0.05$). Therefore, supplementation of antioxidants in boar semen during *in vitro* storage could be improved the semen quality using the artificial insemination. 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 boar semen treated with pyruvate and taurine were increased the *in vitro* development of porcine IVM/IVF embryos.

Spermatozoa in mammals have rich mitochondria in tail piece. The integrity of mitochondria is established by the presence of BAD-mediated Cytochrome c in the inner membrane space. The presence of apoptotic proteins in the spermatozoa may be linked to defects in cytoplasmic remodeling during later stages of spermatogenesis (Sakkas et al., 1999; Sakkas et al., 2002). Release of BAD, Cytochrome c, Caspase-3 and Cox-2 proteins from mitochondrial inner space is likely to accelerate the process of apoptosis, possibly leading to DNA damage. Early apoptosis cannot be detected by conventional semen examinations, but spermatozoa undergoing apoptosis events by externalization of phosphatidylserine (Vermes and Haanen, 1995; Wang et al., 2001). Considerable evidence exists that disruption of mitochondrial functions (e.g., loss of transmembrane potential, permeability transition, and release of Cytochrome c leading to impaired electron transport) are important events in many apoptotic cell deaths (Wang et al., 2001; Yang et al., 1997). Conventional semen variables are not always sufficient in the assessment of sperm function and male infertility. Increased levels of Caspase 9 and 3 were reported in spermatozoa from infertile patients and low motility (Wang et al., 2001; Weng et al., 2002). A increase in

Cytochrome c levels in pyruvate, H_2O_2, H_2O_2 + pyruvate and H_2O_2+taurine groups were suggest the significant mitochondrial damage by high ROS for 6h during incubation at 37℃ in our study. Our findings, Caspases-3 were increased in boar semen treated with H_2O_2 and H_2O_2 plus taurine group and the increased expression of Cox-2 was shown in only H_2O_2 group but taurine group was reducing the expresstion of Caspase-3 and Cox-2. This results suggest that taurine had a protective effect for apoptosis events leading to the membrane and DNA damage by free radical.

The expression of BAD, Cytochrome c, Caspase-3 and Cox-2 proteins are important to identify the spermatozoa that are positive for apoptotic events and are continues to be related to both regulation of cell death and mitochondrial pathology. However, whether or not the expression of apoptosis protein play the critical roles in semen preservation has not yet elucidated.

Our results indicated that sperm membrane and mitochondria play a important role during long-term storage in boar semen, and supplementation of antioxidants into semen extender might be need to improve the semen quality in boar semen preservation and *in vitro* porcine embryos.

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