Effects of Reactive Oxygen Species (ROS) on Sperm Function and Plasminogen Activator Activity in Porcine Spermatozoa

Soo-Jin Sa[1,†], Chun-Keun Park[2], In-Cheul Kim[1], Seung-Hoon Lee[1], Oh-Sub Kwon[1], Myung-Jick Kim[1], Kyu-Ho Cho[1], Du-Wan Kim[1], Kyoung-Min So[1] and Hee-Tae Cheong[3]

[1]*Swine Science Division, National Institute of Animal Science, RDA, Cheonan City 330-801, Korea*

[2]*College of Animal Life Science and* [3]*Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea*

ABSTRACT

Plasminogen activators (PAs) are serine protease that cleave plasminogen to form the active protease plasmin and may participate in mammalian fertilization. Although correlations have been reported between reactive oxygen species (ROS) and sperm function, the relationship between PA activity and ROS is unknown. We determined the effects of ROS on sperm function and PA activities in boar spermatozoa preincubated under the X-XO system. When spermatozoa were treated with the X+XO group, a significant increase ($p<0.05$) was observed in the percentage of acrosome reacted spermatozoa compared with that of the control group. However, when antioxidants were added to the medium with X+XO, the rate of acrosome reaction tended to decrease. Also, a significantly lower percentage of acrosome reacted spermatozoa was observed in the X+XO+catalase group at 6 hr of incubation compared with that of X+XO group. The density of malondialdehyde (MDA) was higher in the X+XO group than in different treatment groups. In another experiment, incubation of spermatozoa in medium with X+XO was associated with a significant ($p<0.05$) increase in activity of tPA-PAI and tPA compared with the control group. Antioxidants decreased the increased activity of tPA-PAI and tPA by preincubation in the X-XO system. Also, a significantly lower ($p<0.05$) activities of tPA-PAI and tPA were observed in the X+XO+catalase group compared with the X+XO group. No significant differences, however, were observed in the activity of uPA. These results suggest that the increase of acrosome reaction by the X-XO system resulted in increase of PAs activity in the sperm incubation medium.

(Key words : Plasminogen activators (PAs), Reactive oxygen species (ROS), Spermatozoa, Pig)

INTRODUCTION

The role of reactive oxygen species (ROS) in the pathophysiology of human sperm function has been emphasized in many studies (de Lamirande and Gagnon, 1992; Aitken *et al.*, 1993). Toxic metabolites of oxygen, including the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\cdot$), are important mediators of inflammatory tissue injury (Weiss, 1986). Previous studies have demonstrated that peroxidative damage to the sperm membrane by ROS may impair sperm function, leading to the onset of male infertility (Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992). Human spermatozoa are able to generate superoxide anion radical ($O_2^{\cdot-}$) (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987) which spontaneously or enzymatically dismutates to hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1989). The spermatozoa are very sensitive to oxidative stress, leading to the initiation to peroxidative damage. Spermatozoa from several mammalian species, including mice and humans, are highly susceptible to oxygen-induced damage mediated by lipid peroxidation because of their high content in polyunsaturated fatty acid and relatively low levels of antioxidants (Jones *et al.*, 1979; Alvarez and Storey, 1989). However, the evidence has been presented not only for the detrimental effects of ROS on sperm function (Aitken and Clarkson, 1987; Aitken *et al.*, 1989, 1991; Iwasaki and Gagnon, 1992). There is increasing evidence suggesting that in many cell types (Fialkow *et al.*, 1994) or organelles (Bauskin *et al.*, 1991), ROS participate in signal transduction processes. Superoxide anion ($O_2^{\cdot-}$) and/or hydrogen peroxide (H_2O_2) may participate in the induction of hyperactivation and capacitating, two cellular phenomena that spermatozoa must go through before they can fertilize oocytes (Griveau *et al.*, 1994; de Lamirande and Gagnon, 1995a). Also, ROS are involved in the process of acrosome reaction (Aitken *et al.*, 1995; Griveau *et al.*, 1995). On the other hand, previous

† Corresponding author : Phone: +82-41-580-3450, E-mail: soojinsa@korea.kr

studies have shown that ROS inhibit the motility, capacitating and acrosome reaction in sperm and that the inhibitory effects are mediated mainly by lipid peroxidation in human (Aitken, 1994; Aitken and Fisher, 1994).

The mammalian sperm acrosome reaction is an exocytotic event that occurs prior to or during sperm penetration of the zona pellucida around the oocyte (Barros *et al.*, 1967). In addition to several enzymes located in the head of the mammalian spermatozoon (Zaneveld and De Jonge, 1991) the presence of plasminogen activators (PAs) as well as plasminogen activator inhibitors (PAIs) and plasmin inhibitors (PIs) has been shown in ejaculated spermatozoa of man and various animal species (Smokovitis *et al.*, 1987). PAs are specific proteolytic enzymes which convert the inactive proenzyme plasmiogen to plasmin. There are two types of PA on the basis of molecular mass: tissue-type (tPA) and urokinase-type PA (uPA). PAs and their inhibitors are localized to the outer acrosomal membrane and plasma membrane of human and boar spermatozoa (Smokovitis *et al.*, 1992a). In ram spermatozoa, PA activity shows breed and seasonal variation (Rekkas *et al.*, 1993) and is influenced by chromosomal anomalies (Smokovitis *et al.*, 1992b) and hormones (Rekkas *et al.*, 1991). Several studies provide evidence for a role of the plasminogen activator/plasmin system in mammalian fertilization. In mouse, ovulated oocytes contain and secrete tPA and ejaculated spermatozoa exhibit uPA (Huarte *et al.*, 1985, 1987). PAs was released during acrosome reaction in man, boar, bull and ram spermatozoa (Taitzoglou *et al.*, 1996) and the addition of plasminogen, the major substrate for PAs, to fertilization medium increases the yield of fertilized mouse eggs (Huarte *et al.*, 1993). Although correlations have been reported between effectiveness of ROS and sperm function, the relationship between ROS and PA activity in boar spermatozoa during preincubation *in vitro* has not been elucidated.

Therefore, this study was conducted to evaluate effects of ROS on sperm function and PA activity during boar spermatozoa preincubation in fertilization medium.

MATERIALS AND METHODS

Culture Media

All chemicals used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA) unless otherwise stated. The basic medium used for sperm preincubation was modified Tris-buffered medium (mTBM), consists of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and no antibiotics (Abeydeera and Day, 1997).

Generation of Reactive Oxygen Species (ROS)

Reactive oxygen species were generated by the xanthine-xanthine oxidase (X-XO) system described by McCord and Fridovich (1968). Xanthine oxidase catalyzes the univalent and divalent reduction of ground-state oxygen to generate both $O_2^{\cdot -}$ and H_2O_2 with the oxidation of xanthine to uric acid. Treatments in present experiment were done as follows: 1) sperm alone, 2) sperm + X (0.5 mM) + XO (0.05 U/ml), 3) sperm + X-XO + catalase (CAT, from bovine liver; 150 U/ml), 4) sperm + X-XO + superoxide dismutase (SOD, from bovine erythrocyte; 150 U/ml), 5) sperm + X-XO + β-mercaptoethanol (β-ME; 0.1 mM).

Preparation of Spermatozoa

Ejaculated semen was collected from three Duroc boars by artificial vagina. The same three boars were used for all experiments. Fresh spermatozoa were washed three times by centrifugation (1,900×g for 4 min) in Dulbecco's PBS (DPBS; Gibco, Grand Island, NY, USA) supplemented with 1% BSA, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate. After washing of fresh boar spermatozoa with PBS, washed spermatozoa were resuspended in mTBM containing X and XO.

Experimental Design

In experimental 1, to evaluate effects of ROS on fertilization ability *in vitro* and lipid peroxidation, spermatozoa were preincubated in mTBM containing X and XO. After preincubation with X-XO system, sperm viability and acrosomal status were determined by staining with eosin-nigrosin (E-N), assay of chlortetracycline (CTC) fluorescence, respectively. Also, the levels of lipid peroxidation were assessed by the determination of malondialdehyde (MDA).

In experimental 2, to determine effect of ROS on change of PA activities in boar spermatozoa during preincubation, spermatozoa were preincubated in mTBM containing X and XO. After preincubation, the changes of PA activity were determined in spermatozoa incubation medium by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography.

Assessment of Sperm Viability

The percentage of viability in porcine spermatozoa was measured by the E-N stain. The E-N stain was prepared by dissolving 0.67 g eosin Y in 100 ml tap water. This solution was heated gently, and 10.0 g nigrosin was added and dissolved before being brought to the boil. The stain was allowed to cool, filtered using Reeve grade 802 paper, and stored in a stopp-

ered glass bottle at 4℃. The stain was warmed to ambient temperature before use.

To study the effect of ROS on the viability of spermatozoa, washed spermatozoa (10^7) were resuspended in 100 μl caffeine-free mTBM containing X and XO and preincubated for 2, 4 or 6 hr at 39℃ under an atmosphere of 5% CO_2 in air. Subsequently, spermatozoa preincubated and E-N stain were mixed with equal volumes on a spotting plate and left for 30 sec. After air-dry, the ratio of live spermatozoa and dead spermatozoa are counted under a phase-contrast microscope at magnification of × 400. Spermatozoa that were white (unstained) were counted as live and those showed any pink or red coloration were counted as dead. At least 200 spermatozoa were counted for each sample. Experiment was replicated three times.

Assay of Chlortetracycline (CTC) Fluorescence of Spermatozoa

The functional state of the spermatozoa was assessed using the chlortetracycline (CTC) fluorescence assay method described by DasGupta *et al.* (1993). A CTC solution was prepared on the day of use and contained 750 μM CTC (Sigma) in a buffer of 130 mM NaCl, 5 mM cysteine, 20 mM Tris-HCl; the pH was adjusted to 7.8 with 1N HCl. This solution was kept wrapped in aluminum foil at 4℃ until just before use. Hoechst-treated sperm suspension (45 μl) was added to 45 μl of CTC solution at room temperature in a aluminum foil-wrapped centrifuge tube and mixed thoroughly. Spermatozoa were then fixed by adding 8 μl 12.5% (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.4). Slides were prepared by placing 10 μl of the stained, fixed suspension on a slide for observation. One drop of anti-fade solution [0.22 M 1,4-diazabicyclo(2.2.2)octane (DABCO; Sigma) was dissolved in glycerol:PBS (9:1)] was mixed with the drop of semen to retard fading of the fluorescence. After adding a clean coverslip, the slide was compressed firmly between absorbent tissues (Kimwipes) to remove any excess fluid and to maximize the number of spermatozoa lying flat on the slide. The coverslip was then sealed with colorless nail varnish, wrapped in aluminum foil and stored at 4℃ until used for microscopic observation.

An assessment was carried out on either the same or the following day using an Olympus BHS microscope equipped with phase-contrast and epifluorescent optics. Cells were assessed for CTC staining using ultraviolet light. The excitation beam (halogen lamp) was passed through a 405 nm band pass filter, and CTC emission was observed through a 455 nm diachronic mirror with an additional 375 nm barrier filter. Three kinds of CTC staining patterns were identified: an F pattern, spermatozoa with uniform fluorescence over the entire head (uncapacitated spermatozoa); a B pattern, spermatozoa with a fluorescence-diminished band in the post-acrosomal region and a relatively bright fluorescence in the acrosomal region (capacitated spermatozoa); and an AR pattern, spermatozoa with almost no fluorescence over the whole sperm head except for a thin band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa). At all three stages bright fluorescence on the midpiece could be seen. At least 200 spermatozoa were counted for each sample. Experiment was replicated three times.

Determination of Lipid Peroxidation

The levels of lipid peroxidation induced in porcine spermatozoa were assessed by the determination of malondialdehyde (MDA) using the thiobarbituric acid (TBA) reaction (Aitken *et al.*, 1993). For this assay, approximately 1×10^8 sperm/ml were treated with X-XO system in mTBM for 60 min at 37℃. Subsequently, 0.6 ml of reaction solution was mixed with 0.2 ml of 15.2% trichloroacetic acid and centrifuged at 8,000×g for 10 min. Then, 0.6 ml of the supernatant was mixed with 2 ml of 0.6% 2-thiobarbituric acid, and incubation was conducted at 95℃ for 40 min. The absorbance of the mixture, measured with a JASCO-V550 UV-visible spectrophotometer at 534 nm, was used to determine the MDA concentration.

SDS-PAGE and Zymography

SDS-PAGE and zymography were performed using procedures described by Dyk and Menino (1991) with a slight modification. After preincubation of boar spermatozoa with X-XO system, sperm incubation medium were diluted with equal volumes of sample buffer (5.0% [w/v] SDS, 20% [v/v] glycerol, and 0.0025% [w/v] bromophenol blue in 0.125 M Tris-HCl buffer) and frozen at −80℃ until used for zymographic analysis. As a standard of tPA, stock solution of 0.5 ng/ml tPA from human melanoma cell culture (Sigma) was prepared in sample buffer. Each sample (20 μl) being compared in each experiment, a stock solution of human tPA (7 μl), and molecular mass markers (10 μl; Bio-Rad Lab., Hercules, CA) were placed in a castellated well in a 4.5% stacking gel with a 12.5% separating gel. Electrophoresis was conducted at 20 mA for 2 hr.

After electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 for 45 min, rinsed with distilled water three times, and incubated for 30 min at 39℃ in phosphate-buffered saline (PBS). Each gel was carefully laid on a casein-agar gel (zymogram) containing purified human plasminogen (Sigma) supported in a plastic chamber. For preparing zymograms, 0.4 g of nonfat dry milk was dissolved in 10 ml of buffer containing 0.0013 M $CaCl_2\cdot2H_2O$, 0.1 M glycine, 0.038 M Tris, and 0.005 M sodium azide. The nonfat dry milk mixture were heated to 55℃ and combined with

9.9 ml of 1% (v/v) melted agarose maintained at 55℃. Purified human plasminogen stock solution (100 μl) was added to 19.9 ml of the warmed mixture to yield a final plasminogen concentration of 50 μg/ml and 10 ml of this mixture were cast into a warmed dish (100× 15 mm; Falcon 1012; Becton and Dickinson) and allowed to cool. Zymograms containing 0 μg/ml plasminogen were used for detection of any nonspecific proteolytic activity. Polyacrylamide gels and zymograms were incubated at 39℃ for 24~48 hr to allow the development of lytic bands. After the distance from the edge of the separating gel to the center of the clear lytic bands in each lane was measured, incubation of zymograms was terminated by separating the gels. Then the zymograms were fixed with 3% (v/v) acetic acid for 10 min, rinsed under tap water.

All experiments measuring PA activity were repeated at least three times. Protease activity was quantified by densitometric scanning of the zymograph using NIH Image 1.62 (Center for information Technology National Institutes of Health, Maryland, USA). PA activities were expressed relative to the activity in a fixed sample, which was different in each experiment.

Statistics

Data were analyzed by ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). When *F*-test results were significant in ANOVA, individual data were further tested by Duncan's multiple-range test. Differences with values of $p<0.05$ were considered to be statistically significant. Data are presented as mean±standard error of the mean (SEM).

RESULTS

Experiment 1

Spermatozoa were preincubated form 2 to 6 hr in the mTBM containing X and XO; or antioxidant catalase, SOD, β-ME. When boar spermatozoa were preincubated for different periods, although the percentage of viability in boar spermatozoa decreased as the preincubation time increased from 2 to 6 hr, no significant differences of viability were observed under the all conditions (Fig. 1).

When spermatozoa were treated with the X+XO group, a significant increase ($p<0.05$) was observed in the percentage of acrosome reacted spermatozoa compared with that of the control group. However, the percentage of acrosome-reacted spermatozoa tended to decrease when antioxidants were added to the medium containing X and XO. Also, a significantly lower percentage ($p<0.05$) of acrosome reacted spermatozoa was observed in the X+XO+catalase group at 6 h of incubation compared with that of X+XO group (Fig. 2).

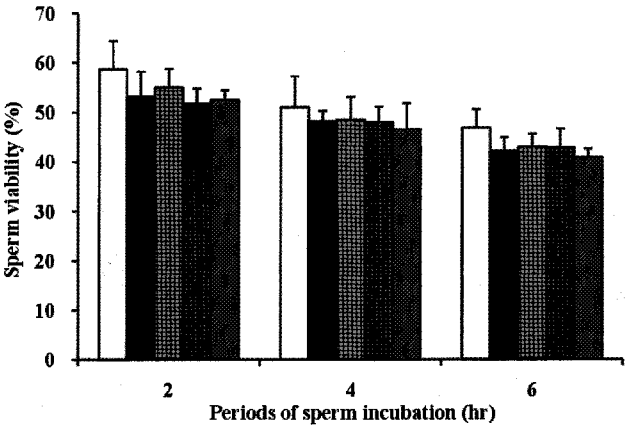

Fig. 1. Effect of antioxidants on viability of boar sperm treated with X-XO system. Sperm were preincubated (2~6 hr) in the fertilization medium (mTBM) containing xanthine (X, 0.5 mM) and xanthine oxidase (XO, 0.05 U/ml); or the antioxidant catalase (CAT, 150 U/ml), superoxide dismutase (SOD, 150 U/ml) and β-mercaptoethanol (β-ME, 0.1 mM). Values obtained from three replicates are expressed as mean±SEM.

The density of MDA was higher in the X+XO group than in different treatment groups. No significant differences, however, were observed under the all conditions (Table 1).

Experiment 2

Incubation of spermatozoa in the X+XO group was associated with a significant ($p<0.05$) increase in activity of tPA-PAI and tPA compared with the case of control

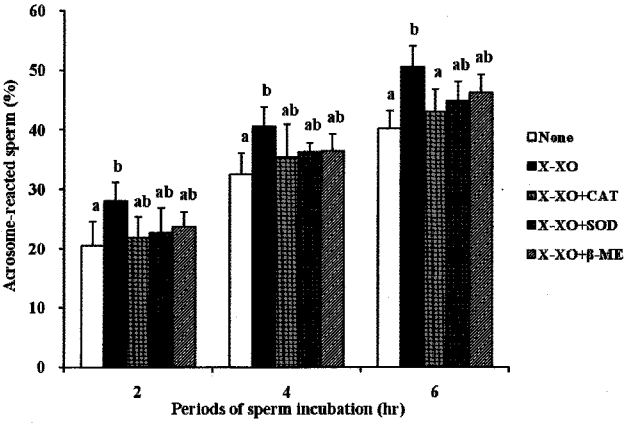

Fig. 2. Effect of antioxidants on CTC patterns of boar sperm treated with X-XO system. Sperm were preincubated (2~6 hr) in the fertilization medium (mTBM) containing xanthine (X, 0.5 mM) and xanthine oxidase (XO, 0.05 U/ml); or the antioxidant catalase (CAT, 150 U/ml), superoxide dismutase (SOD, 150 U/ml) and β-mercaptoethanol (β-ME, 0.1 mM). Values obtained from three replicates are expressed as mean±SEM. ^{ab} Values with different letters within each period of sperm incubation differ significantly ($p<0.05$).

Table 1. Effect of antioxidants on lipid peroxidation of boar sperm treated with X-XO system*

Treatments	Density of MDA (nM/10^8 spermatozoa)
None	28.41±0.55
X+XO	29.11±0.72
X+XO+CAT	28.58±0.26
X+XO+SOD	28.82±0.57
X+XO+β-ME	28.85±0.13

Spermatozoa were preincubated (1 hr) in the fertilization medium (mTBM) containing X and XO; or the antioxidant catalase (CAT, 150 U/ml), superoxide dismutase (SOD, 150 U/ml) and β-mercaptoethanol (β-ME, 0.1 mM). Values obtained from three replicates are expressed as mean±SEM.

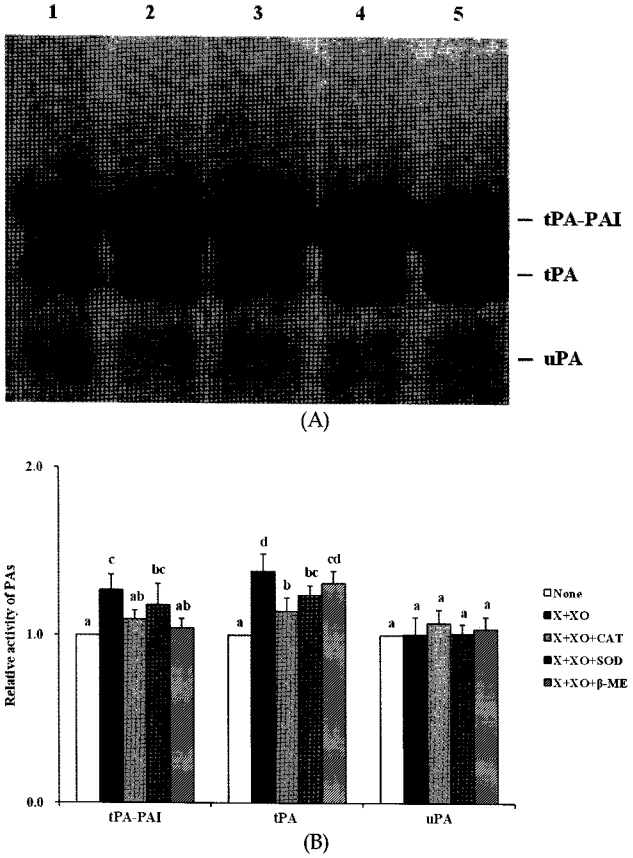

Fig. 3. Changes of PAs activities of sperm incubation medium at 6 hr of culture in fertilization medium (mTBM) containing containing none (lane 1), X+XO (lane 2), X+XO+CAT (lane 3), X+XO +SOD (lane 4) and X+XO+β-ME (lane 5). (A) Zymographic analysis of sperm incubation medium of culture in fertilization medium (mTBM). (B) Intensity of PAs activity was quantified by densitometric scanning of zymography and expressed relative to the activity for tPA-PAI, tPA and uPA in the control group (None). Results are shown as mean±SEM of three replicates using 40×10^6 spermatozoa were used for the analysis. $^{a\sim d}$ Value with different letters are significantly different ($p<0.05$).

group (Fig. 3). The addition of antioxidants to the preincubation medium decreased the increased activity of tPA-PAI and tPA by preincubation in the X-XO system. Also, a significantly lower activities ($p<0.05$) of tPA-PAI and tPA were observed in the X+XO+ catalase group compared with the X+XO group. No significant differences, however, were observed in the activity of uPA.

DISCUSSION

The present study was undertaken to examine effects of ROS generated by X-XO system on sperm viability, acrosome reaction, lipid peroxidation and plasminogen activators (PAs) activity. A combination of X and XO, an enzymatic free radical-generating system, primarily generates the superoxide anion (O_2^-) which is immediately dismuted into hydrogen peroxide (H_2O_2) (Ikeda *et al.*, 1999). ROS have beneficial or detrimental effects on sperm functions depending on the nature and the concentration of ROS involved, as well as the moment and the location of exposure.

The results of this experiment demonstrated that X-XO system is not influence on viability of boar spermatozoa (Fig. 1). This finding is consistent with that result in viability of equine spermatozoa incubated with X-XO at higher concentration than was used in our study (Baumber *et al.*, 2000) but is not consistent with that studies in mouse (Baiardi *et al.*, 1997), human (de Lamirande and Gagnon, 1992). These discords may be due to differences in the experimental methods used in our study or may be species differences in the susceptibility of spermatozoa to oxidative stress.

Spermatozoa are particularly susceptible to lipid peroxidation because of the high concentration of polyunsaturated fatty acids (PUFAs) in their plasma membrane. There was a close correlation between loss of motility and lipid peroxidation. Fluidity of the plasma membrane has important roles in sperm function, including motility. Block (1991) showed that H_2O_2 caused an increase in membrane rigidity through lipid peroxidation in endothelial cell. In sperm, increased membrane rigidity may also cause loss of motility. However, there was no influence in lipid peroxidation of boar spermatozoa incubated with the concentration of the X-XO system used in this study.

In the present study, spermatozoa treated with X-XO system had a stimulatory effect on acrosome reaction all periods of spermatozoa incubation examined. However, this stimulatory effect was repressed by antioxidants. Sperm acrosome reaction is a membrane event which occurs at the surface of the egg's zona pellucida and is characterized by the fusion of the plasma membrane and the underlying outer acrosomal membrane.

Recent evidence demonstrates that ROS are involved in the process of acrosome reaction. In human spermatozoa capacitated in BWW medium containing bovine serum albumin, the presence of catalase prevents the acrosome reaction incuced the calcium ionophore A23187, whereas H_2O_2 stimulates this process (Aitken *et al.*, 1995). Protein Kinase C (PKC), a key regulatory enzyme in signal transduction mechanisms, is involved in the acrosome reaction of human (Rotem *et al.*, 1992) and bovine (Breitbarth *et al.*, 1992) spermatozoa. In cell of vascular smooth muscle, the phospholipase A_2 (PLA_2) can be activated by H_2O_2 induced phosphorylation (Rao *et al.*, 1995). The PLA_2 enzyme is involved in the induction of acrosome reaction in many mammals, including human (Bennet er al., 1987), ram (Roldan *et al.*, 1993) and cattle (Manjunath *et al.*, 1994).

Plasminogen activators (tPA and uPA) are present in spermatozoa of various animal sepcies (Smokovitis *et al.*, 1987), they are both located in the plasma and the outer acrosomal membrane of spermatozoa (Smokovitis *et al.*, 1992a) and their release has been shown during the acrosome reaction (Taitzoglou *et al.*, 1996). Also, plasminogen activator inhibitors (PAIs) have been localized to plasma and outer membrane; PAI-I was identifyed in human sperm membranes (Smokovitis *et al.*, 1992a). The membrane localization of PAs in spermatozoa indicates that these proteolytic enzymes could participate in the whole process of ovum fertilization. The addition of plasminogen or antibodies to plasmin during fertilization could increase or decrease the fertilization rate, respectively. Trypsin-like proteases can stimulate capacitating (Talbot and Chacon, 1981) and plasmin induces the acrosome reaction in capacitated bovine spermatozoa (Taitzoglou *et al.*, 2003). Results of this study showed that boar spermatozoa release tPA-PAI, tPA and uPA during preincubation in mTBM. Also, the increase of acrosome reaction by the X-XO system resulted in increase of PAs activity in the sperm incubation medium. Previous reports suggested that the presence of PAs and PAIs in sperm membranes and their release during acrosome reaction might participate in the whole process of ovum fertilization and regulation of proteolytic effect (Smokovitis *et al.*, 1988).

In summary, hydrogen peroxide has a primary role in mediating spermatozoa damage due to ROS generation by the X-XO system. Although peroxidative damage to the sperm membrane by ROS cause impairment of sperm function, the low concentration of the X-XO system used in this study was not significant influence in either sperm viability or lipid peroxidation. However, ROS generated by the X-XO system increases the percentage of acrosome reacted spermatozoa and the activities of PA in the sperm incubation medium. These results suggest that the increase of acrosome reaction by the X-XO system resulted in increase of PAs activity in the sperm incubation medium.

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