韓國家畜繁殖學會誌 26(3) : 263~273 (2002)
Korean J. Animal Reprod.

Effects of β-Mercaptoethanol on Lipid Peroxidation and Fertilization Ability *In Vitro* by Xanthine-Xanthine Oxidase System in Pig

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Xanthine-Xanthine Oxidase System하에서 돼지 동결-융해정자의 Lipid Peroxidation과 체외수정능력에 대한 β-Mercaptoethanol의 영향

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ABSTRACT

This study was undertaken to evaluate the effects of β-mercaptoethanol (β-ME) on lipid peroxidation and fertilization ability *in vitro* by xanthine (X) - xanthine oxidase (XO) system in boar spermatozoa frozen-thawed. The boar spermatozoa were treated with X and/or XO, and the spermatozoa viability were measured by the eosin-nigrosin stain method. In control group, level of vitality in boar spermatozoa were higher than in medium with X, XO and X+XO groups. No significant differences, however, were observed under the all conditions. The percentage of spermatozoa that reached acrosome reaction were significantly ($P<0.05$) higher in sperm treated without that than with β-ME under the all conditions. On the other hand, when spermatozoa were inseminated in medium with X and/or XO, the penetration rates in all conditions were higher in medium with that than without β-ME. However, significant differences were not observed between medium with and without β-ME. The lipid peroxidation of sperm was evaluated on the basis of malondialdehyde (MDA) production. The MDA were higher in sperm treated without that than with β-ME under the above all conditions. However, significant differences were not observed between medium with and without β-ME. Sperm-SH group were higher detected in medium with that than without β-ME under the all conditions. The activity of sperm binding to zona pellucida was also evaluated through binding to salt-stored porcine oocytes. In control group, sperm binding to zona pellucida were significantly ($P<0.05$) higher than in medium with X+XO groups. The sperm binding in all conditions were higher in medium with that than without β-ME. However, significant differences were not observed between medium with and without β-ME. These results

This work was supported by grant No. R01-2000-00208 from the Basic Research Program of the Korea Science & Engineering Foundation.

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suggest that addition of β-ME in X-XO system may play a positive role in improving of fertilization ability *in vitro*.

(Key words: β-Mercaptoethanol, Lipid peroxidation, Fertilization ability, Xanthine-xanthine oxidase system, Pig)

Ⅰ. INTRODUCTION

Reactive oxygen species (ROS) have been shown to influence gamete function and embryo development. The evidence has been presented not only for the detrimental effects of ROS on sperm function (Aitken and Clarkson, 1987; Aitken et al., 1989; Aitken et al., 1991; Iwasaki and Gagnon, 1992), but also for their beneficial effects. Whereas H_2O_2 is the ROS responsible for toxicity in human spermatozoa (Aitken et al., 1993; de Lamirande and Gagnon, 1992a,b), the superoxide anion ($O_2^{\cdot -}$) can induce hyperactivation and capacitation, two cellular phenomena that spermatozoa must go through before they can fertilize oocytes (de Lamirande and Gagnon, 1995).

Sperm-oocytes fusion is mediated by fusion -related proteins in the plasma membranes of both the sperm and oocytes (Myles, 1993). The characteristics of reported that proteins, however, have been poorly defined. Mammoto et al. (1996) reported that proteins in the sperm plasma membrane that are sensitive to sulfhydryl(SH)-depleting reagents may be involved in sperm-oocyte fusion in mice. Sulfhydryl residues in proteins are highly sensitive to ROS (Di-Simplicio et al., 1991; Snyder et al., 1988). On the other hand, previous studies have shown that ROS inhibit the motility, capacitation, 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 ROS and antioxidant defenses have been shown to play an important role in fertility and infertility (Aitken et al., 1989; de Lamirande et al., 1997). Bilodeau et al. (2001) reported that thiol compounds prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. Development of bovine embryos was promoted by β-ME, a low molecular weight thiol that is used as a reducing agent in culture medium (Takahashi et al., 2002). However, the effect of β-ME on *in vitro* fertilization under condition of oxidative stress with such a X-XO system, has not been elucidated precisely. Therefore, the present study was undertaken to evaluate effects of β-ME using xanthine (X) - xanthine oxidase (XO) system on *in vitro* fertilization ability and lipid peroxidation in boar spermatozoa frozen-thawed.

Ⅱ. MATERIALS AND METHODS

1. Sperm Viability Assement

The levels of vitality in boar spermatozoa were measured by the eosin-nigrosin (E-N) stain method. For this assay, frozen-thawed spermatozoa were treated with X and/or XO in fertilization medium with or without β-ME and centrifuged twice at 250 × g for 10 min. Subsequently, thawed semen and E-N stain solution were mixed with equal volumes on a spotting plate and leave for 30 seconds. After air-dry, the ratio of live spermatozoa and dead spermatozoa are counted under a phase-contrast microscope at magnification of 400×. This procedure is based on the degree of membrane permeability of dead spermatozoa. Intact cells exclude eosin, whereas dead cells take up the red color of eosin. Nigrosin is used as a counterstain to facilitate visualization of the unstained (white) live cells.

Spermatozoa that are white (unstained) are counted as live and those showing any degree of pink or red coloration as dead. At least 200 spermatozoa were counted for each sample.

2. Chlortetracline Assessment of Spermatozoa

The functional state of the spermatozoa was assessed using the chlortetracycline (CTC) fluorescence assay method described by DasGupta et al. (1993). 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. This solution was kept wrapped in 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 foil-wrapped centrifuge tube and mixed thoroughly. Spermatozoa incubated for 30 min with and without of β-ME (0.001 mM/ml) under X-XO system were then fixed by adding 8 μl 12.5% w/v paraformaldehyde in 0.5 M Tris-HCl (pH 7.4). Slide were prepared by placing 10 μl of the stained, fixed suspension on a slide. One drop of 0.22 M 1,4-diazabicyclo(2.2.2) octane dissolved glycerol:PBS (9:1) was mixed in carefully to retard fading of fluorescence. A coverslip was placed on top. The slide was compressed firmly between tissues to remove any excess fluid and to maximize the number of spermatozoa lying flat on the slide. The coverslip was then sealed and stored wrapped in foil in the cold.

An assessment was carried out on either the same or the following day using an Olympus BHS microscope (BX50F4, Olympus Optical Co. Ltd. Japan) equipped with phase-contrast and epifluorescent optics. Cells were assessed for CTC staining using violet light. The exicitation beam was passed through a 405 nm bandpass filter and fluorescence emission was observed through a DM 455 dichroic mirror. There are three main patterns of CTC fluorescence that can be identified: F, with uniform fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact cells; B, with a fluorescence-free band in the post-acrosomal region, characteristic of capacitated, acrosome-intact cells; and AR, with dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome -reacted cells. At all three stages bright fluorescence on the midpiece could be seen.

3. Oocytes Preparation

Porcine ovaries were collected from a local slaughter-house and kept in saline (NaCl, 0.9% w/v; Penicillin 100,000 IU/L; Streptomycin 100 mg/L and Amphotericin B 250 μg/L; Sigam Chemical, St-Louis, MO, USA) at 32 to 37℃. Cumulus-oocytes complexes were aspirated from 2 to 6 mm follicles with a 10-ml syringe with an 18-G needle. The collected oocytes were washed three times in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium. Oocytes with compact and complete cumulus cells were introduced to in droplets of maturation medium (10 oocytes/50 μl droplet), covered with mineral oil and cultured under an atmosphere of 5% CO_2 in air at 39℃ for 42～44 h. The maturation medium consisted of TCM-199 with Earle's salts (Gibco Lab., NY, USA) supplemented with 3.05 mM glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes (Sigma), 10% fetal bovine serum (FBS; Gibco, Life Technologies, Inc. NY 14072 USA), 0.2 mM Na-pyruvate (Sigma), 50 μg/ml gentamycin (Sigma), 1 μg/ml FSH (from porcine pituitary; Sigma), 5 μg/ml LH (from equine pituitary; Sigma), 1 μg/ml estradiol 17β (Sigma) and 10% (v/v) porcine follicular fluid (PFF). The PFF was aspirated from follicles (2 to 5 mm in diameter) at estrus with a syringe and 18-gauge needle, and centrifuged at 3,850×g for 15 min. The supernatant fluid was frozen at −20℃ until used.

4. Determination of Sperm Penetration

The frozen-thawed spermatozoa were diluted with 2 ml of BTS (Beltsville Thawing Solution) and equilibrated in air-tight tubes at 37℃ in a waterbath for 10 min. After equilibration, the 2 ml of semen was placed over 2 layers of percoll (65 and 70%) and centrifuged at 2,000×g for 15 min at 20℃. The spermatozoa in the 65% percoll layer were carefully collected, washed in preincubation medium (TCM -199) with Earle's salts (Gibco), supplemented with 3.05 mM glucose, 2.92 mM Ca-lactate (Sigma), 10% FCS, 0.2 mM Na-pyruvate, and 50 μg/ml gentamycin (Sigma) by suspension and centrifugation two times at 250×g for 10 min and resuspended in preincubation medium. The fertilization medium with X and/or XO was the same as the preincubation medium, enriched with 2 mM caffeine (Sigma) and adjusted to a pH of 7.2 to 7.4. The final concentration of spermatozoa was adjusted to 1×10^6 cells/ml motile sperm during fertilization. To evaluate the effect of β-ME (1 μM) on *in vitro* penetration, spermatozoa was inseminated in medium with X (0.5 mM) and/or XO (0.05 IU).

At 22～24 h after insemination, the oocytes were examined for spermatozoa penetration *in vitro*. For this assay, cumulus cells were removed by repeated passages though a fine pipette. The cumulus-free oocytes were transferred on to the center of glass slide with four vaseline spots, gently compressed with a cover slide, immersed in 25% acetic alcohol for 2～3 days for complete fixations and stained with 1% orcein in 45% acetic acid. Excess stain was removed by infiltrating aceto-glycerol medium (20% glycerol and 20% glacial acetic acid in distilled water) under the phase-contrast microscope at a magnification of ×200 or ×400.

The oocytes were considered as penetrated when they have a swollen spermatozoon head and male or female pronucleus in the cytoplasm. The oocytes with more than two pronuclei and visible second polar body, even without a spermatozoon tail, were also considered as penetrated.

5. Determination of Lipid Peroxidation

The levels of lipid peroxidation induced in porcine spermatozoa ware assessed by the determination of malondialdehyde (MDA). For this assay, approximately 1×10^6 sperm/ml were treated with X and/or XO in fertilization medium with or without β-ME for 30 min at 39℃. Subsequently, 0.6 ml of reaction solution was mixed with 0.2 ml of 15.2% trichloroacetic acid (Sigma) and centrifuged at 8000 × g for 10 min. Then, 0.6 ml of the supernatant was mixed with 2 ml of 0.6% 2-thiobarbituric acid (Sigma), and incubation was conducted at 95℃ for 40 min. The absorbance of the mixture, measured with a spectophotometer at 534 nm, was used to determine the MDA concentrations.

6. Measurement of Sperm Sulfhydryl(-SH) Groups

The sperm SH content was assessed using a previously described assay (Kodama et al., 1996). After washing of frozen-thawed spermatozoa with and without β-ME in fertilization medium containing X and/or XO, spermatozoa were centrifuged on a 40% and 80% percoll gradient in fertilization medium devoid of FCS. The soft sperm pellet was resuspended into 50 volumes of fertilization medium without FCS and centrifuged for 5 min at 5,000×g. The pellet was resuspended and assayed for -SH group content. The assay mixture contained 180 μl sperm suspension, 10 μl 10% sodium dodecyl sulfate (SDS, Sigma), and 10 μl of 4 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) (stock solution of 10 mM DTNB in dimethylsulfoxide), and the absorbance was measured at 405 nm.

7. Determination of Zona-binding Properties of Spermatozoa

The activity of sperm binding to zona pellucida

was evaluated through binding to salt-stored homologous zona pellucida. Oocytes matured *in vitro* were treated with 0.1% hyaluronidase to dissociate the cumulus oophorus and equilibrated with 1.5 M magnesium chloride containing 1 % dextran. The oocytes were stored at 4℃ for 3~4 days until examined. The oocytes were whased and re-equilibrated in fertilization medium with FCS for 1 hour prior to experimentation. Spermatozoa (1×10^6 sperm /ml) were incubated with and without β-ME for 1 hour at 39 ℃ with oocytes in 50 μl of fertilization medium with X and/or XO covered with mineral oil. The number of spermatozoa attached to the zona pellucida was then evaluated using an inverted microscope.

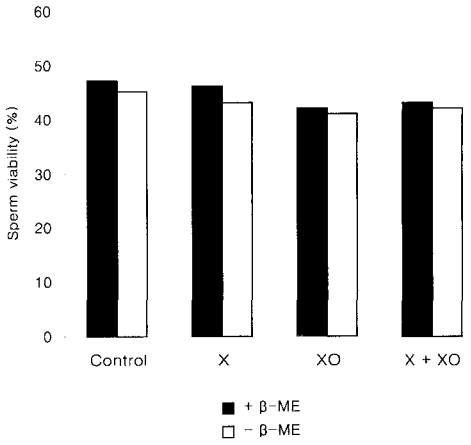

Fig. 1. Effect of β-ME on viability of boar spermatozoa treated by fertilization medium with X and/or XO.

8. Statistics

Data were evaluated by Duncan's multiple-range test. Differences with values of $P<0.05$ were considered to be statistically significant.

Ⅲ. RESULTS

Fig. 1 shows effects of β-ME on viability of boar spermatozoa treated with X and/or XO. In control group, level of viability in boar spermatozoa were higher than in medium with X, XO and X + XO groups. No significant differences, however, were observed between in medium with and without β-ME under the all conditions.

The boar spermatozoa were treated with X and/or XO, and spermatozoa ability were assessed by CTC analysis. The rates of spermatozoa that reached acrosome reaction were significantly (P<

Table 1. Effect of β-ME on *in vitro* penetration in medium with xanthine and/or xanthine oxidase

Cultrue condition for IVF	Presence of β-ME	No. of oocytes examined	No. of oocytes with			No. of polyspermic oocytes(%)
			Total(%)	ESH	BPN(%)	
Control	+	110	59(54)	42	17(29)	25(42)
	-	104	50(48)	34	16(32)	24(48)
X	+	109	53(49)	39	14(26)	20(38)
	-	107	45(42)	35	10(22)	23(51)
XO	+	103	61(59)	49	12(20)	30(49)
	-	109	60(55)	52	8(13)	30(50)
X + XO	+	115	54(47)	43	11(20)	20(37)
	-	123	53(43)	45	8(15)	23(43)

ESH: enlarged sperm head, BPN: both pronuclei.

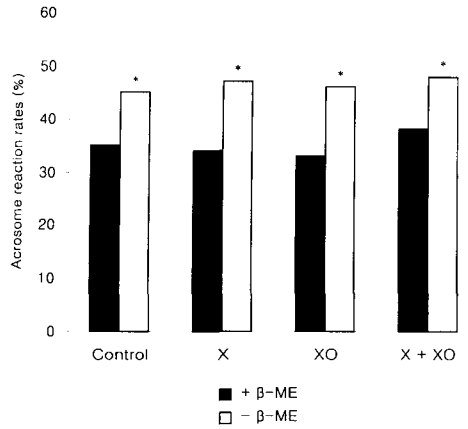

Fig. 2. Effect of β-ME on acrosome reaction of porcine spermatozoa treated with xanthine and/or xanthine oxidase. * P<0.05, difference between with and without β-ME.

0.05) higher in sperm treated without that than with β-ME under the all conditions (Fig. 2).

When spermatozoa were inseminated in medium with X and/or XO, the penetration rates in all conditions were higher in medium with that than without β-ME (Table 1). However, significant differences were not observed between medium with and without β-ME. On the other hand, the polyspermy were lower in medium with that than without β-ME under the all conditions.

In another experiment, sperm sulfhydryl (-SH) group were higher detected in medium with that than without β-ME in medium under the above all conditions (Fig. 3). However, sperm sulfhydryl (-SH) group content were not significantly different between in spermatozoa treated with X and/or XO.

Under the same conditions, the lipid peroxidation of sperm was evaluated on the basis of malondialdehyde (MDA) production. The MDA were higher in sperm treated without that than with β-ME under the above all conditions (Fig. 4). However, significant differences were not observed between medium with and without β-ME.

The activity of sperm binding to zona pellucida was evaluated through binding to salt-stored porcine oocytes (Fig. 5). In control group, sperm binding to zona pellucida were significantly (P<0.05) higher than in medium with X+XO groups. The sperm binding in all conditions were higher in medium with that than without β-ME. However, significant differences were not observed between medium with and without β-ME.

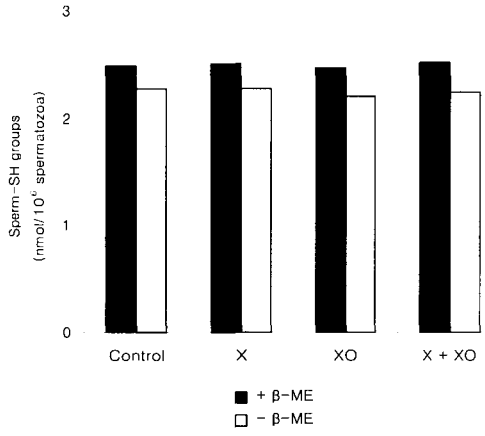

Fig. 3. Effect of β-ME on sulfhydryl (-SH) groups of boar spermatozoa treated with xanthine and/or xanthine oxidase.

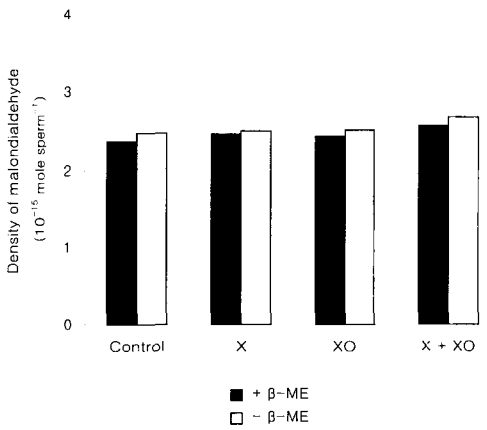

Fig. 4. Effect of β-ME on lipid peroxidation of boar spermatozoa treated with xanthine and/or xanthine oxidase.

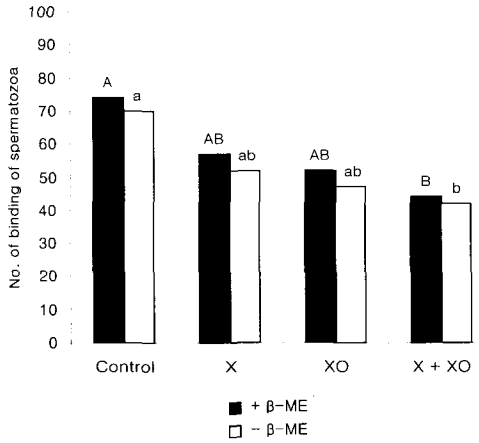

Fig. 5. Effect of β-ME on zona pellucida binding of boar spermatozoa in medium with xanthine and/or xanthine oxidase. Bars with different letter differ in medium with or without β-ME, respectively. $P<0.05$.

Ⅳ. DISCUSSION

Even though many investigators have focused heavily on understanding the effects of free radicals on various cells and organs, the exact mechanism(s) of action of many of antioxidant enzymes in spermatozoa of pig remains unknown. The ROS and antioxidant defenses have been shown to play an important role in fertility and infertility (Aitken et al., 1989; de Lamirande et al., 1997). A combination of X and XO, an enzymatic free radical -generating system, primarily generates the superoxide anoin (O_2^-), which is immediately dismuted in to hydrogen peroxide (Ikeda et al., 1999). The present study were undertaken to examine effects of β-ME on lipid peroxidation and fertilization ability *in vitro* by X and/or XO in boar spermatozoa frozen -thowed.

The results of this study demonstrated that X-XO system are not influence on viability of boar spermatozoa. de Lamirande and Gagnon (1992a,b) reported that the mode of action of ROS on sperm axonemes and motility can be studied with the use of the combination X+XO at concentration sufficiently low not to decrease sperm viability. Thus, the result of this study show that X-XO system of low concentration were not infuence on viability of boar spermatozoa.

Capacitation and acrosome status in boar spermatozoa were evaluated by chlortetracycline fluorescent (CTC) assay. With reference to the CTC analysis of spermatozoa, a recent study (Perry et al., 1995) reported a range of CTC patterns. This method has been used to assess the functional status of mouse (Ward and Storey, 1984), human (DasGupta et al., 1993), bull (Green et al., 1996), goats (Kaul et al., 1997), rams (Perez et al., 1997) and boar spermatozoa (Lim et al., 1997). In the present study, X-XO system had a stimulatory effect on acrosome reaction in boar spermatozoa. However, these stimulatory effects were significantly ($P<0.05$) inhibited by addition of β-ME in medium with X and/or XO. Recent evidences demonstrate that ROS are involved in the process of acrosome reaction. O'Flaherty et al. (1999) reported that O_2^- is required for the capacitation process and that at low concentrations of H_2O_2 participates in enzymatic and membrane modifications leading to the induction of the acrosome reaction in cryopreserved bovine spermatozoa. In this study to examine effect of β -ME in medium with X and/or XO, β-ME may be able to reduce the acrosome reaction of frozen -thowed boar spermaotoza.

When spermatozoa were inseminated in medium with X and/or XO, the penetration rates in all conditions were higher in medium with that than without β-ME (Table 1). Hsu et al. (1997) reported that lead exposure induced ROS generation in rat sperm, which reduced the sperm-oocyte penetration rate, and supplementation with antioxidants, such as vitamin E, vitamin C, protected sperm from loss of motility and increased oocyte penetration capability

(Hsu et al., 1998). In the zona-intact oocytes, the sperm-oocytes penetration rate was decreased as the proportion of acrosome-reacted sperm was very high (Hsu er al., 1999). On the other hand, one of the various ROS, the hydrogen peroxide (H_2O_2) decreased sperm motility in many species (Alvarez et al., 1989; O'Flaherty et al., 1997). However, thiols, including β-ME, prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull spermatozoa (Bilodeau et al., 2001). In addition to its effects on sperm capacity *in vitro*, lipid peroxidation has been detected to decrease by β-ME in medium with X and/or XO. Aitken et al. (1989) reported that lipid peroxidation has also been shown to decrease the sperm-oocyte interaction, as measured by a decreased penetration of zona-free hamster oocytes by human spermatozoa. Because the sperm-zona free hamster oocyte penetration assay is used by some investigators to predict the fertilizing potential of human spermatozoa (Aitken et al., 1991), it was suggested that lipid peroxidation, even at a level that does not affect motility, may decrease the fertilizing potential of spermatozoa. 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 cells. In sperm, increased membrane rigidity may also cause loss of motility.

In the present study, X and/or XO system decreased SH-groups, but this inhibitory effect was improved by β-ME. Sulfhydryl ($-$SH) residues in proteins are highly sensitive to ROS (Snyder et al., 1988; Di-Simplicio et al., 1991). Hecht and Zick (1992) reported that H_2O_2 reduce SH-groups that are essential for enzyme activity. On the other hand, Mammoto et al. (1996) reported that proteins in the sperm plasma membrane that are sensitive to SH-depleting reagents may be involved in sperm -egg fusion in mice. Also, they reported that treatment of sperm with various of SH-depleting reagents, i.e., N-ethylmalemide, $Na_2O_4O_6$ and 5,5' -dithiobis (2-nitro-benzoic acid), specifically blocked sperm-oocyte fusion in mice without affecting motility, the capacity to penetrate the zona pellucida, or sperm-oocyte binding, and that three proteins in mouse sperm were highly sensitive to these SH -depleting reagents. It seems that β-ME, a low molecular weight thiol, can be used as a reducing agent in medium with X and/or XO.

In the study, the activity of sperm binding to zona pellucida was decreased by X and/or XO despite of promoting by β-ME in sperm-zona binding. The decline in sperm motility that occurred in the presence of the X-XO free radical-generating system was not accompanied by a detectable increase in lipid peroxidation (Baumber et al., 2000; de Lamirande and Gagnon, 1992a,b). Also, Lamirande and Gagnon (1992a) suggested that the inhibition of sperm motility after incubation with ROS was caused by a depletion of ATP. These authors subsequently confirmed this hypothesis by demonstrating a significant reduction in sperm ATP levels in the first 1 hour after ROS treatment (de Lamirade and Gagnon, 1992b). These authors proposed that sperm immobilization was due to a decreased phosphorylation of axonemal proteins required for sperm movement. Therefore, it seem that the decreased sperm binding cause loss of sperm motility and that these inhibitory effect can be improved by β-ME.

In summary, the present study suggests that supplement of β-ME in medium with X and/or XO cause an enhancement on fertilization ability *in vitro*. These increase are associated with fertilizing capacity of frozen-thawed boar spermatozoa. These results may suggest that because lipid peroxides are present in membranes of all cells living under aerobic conditions, frozen-thawed spermatozoa could use a strategy involving oxidation and breakdown

of membrane lipids to improve their chances to fertilize oocyte *in vitro*.

Ⅴ. ACKNOWLEDGMENT

We thanks Institute of Animal Resources, Kangwon National University for assistance of this study.

Ⅵ. 요 약

본 연구는 xanthine-xanthine oxidase system하에서 돼지 동결-융해 정자의 lipid peroxidation과 체외수정능력에 대한 β-mercaptoethanol (β-ME)의 영향을 검토하였다. 그 결과 돼지 동결-융해 정자가 X-XO system하에서 처리되었을 때, control구에서 높은 정자생존율이 관찰되었으나 처리구간의 유의차는 인정되지 않았다. 또한 첨체반응이 유기된 정자의 비율은 모든 처리구에서 β-ME 첨가시보다 무첨가시가 유의적($P<0.05$)으로 더 높았다. 한편, X-XO system하에서 체외수정시 난자에 대한 정자의 침입율은 모든 조건하에서 β-ME 첨가시가 무첨가시 보다 높은 경향을 나타냈지만, 유의적인 차이는 인정되지 않았다. 정자의 lipid peroxidation은 malondialdehyde (MDA)의 생성에 기초를 두고 평가하였는데, 모든 조건하에서 β-ME 첨가시 보다 무첨가시에 MDA의 생성이 높게 나타났지만, 유의적인 차이는 인정되지 않았다. 또한 동결-융해된 정자의 sulfhydryl (-SH) group의 함량을 측정한 결과 모든 처리구에서 β-ME 무첨가시 보다 첨가시에 높은 함량이 측정되었지만, 유의적인 차이는 인정되지 않았다. 한편, 체외에서 성숙시킨 난자의 투명대에 대한 동결-융해 정자의 접착 정도를 평가한 결과 모든 처리구에서 β-ME 첨가시 무첨가시에 비해 다소 높은 경향을 보였으며, Control group의 경우 X+XO group에 비해 유의적($P<0.05$)으로 높은 정자접착율이 관찰되었다. 그렇지만, β-ME 첨가 유무에 따른 유의적인 차이는 인정되지 않았다. 본 연구의 결과는 X-XO system하에서 β-ME 첨가가 돼지에서의 체외수정능력 향상에 영향을 미치는 것으로 생각된다.

Ⅶ. REFERENCES

1. Aitken, R. J. 1994. A free radical theory of male infertility. Reprod. Fertil. Dev., 6:19-24.
2. Aitken, R. J. and Clarkson, J. S. 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J. Reprod. Fertil., 81:459-469.
3. Aitken, R. J., Clarkson, J. S. and Fishel, S. 1989. Generation of reactive oxygen species, lipid peroxidation and human sperm function. Biol. Reprod., 40:183-197.
4. Aitken, R. J. and Fisher, H. 1994. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioassays, 16:259-267.
5. Aitken, R. J., Harkiss, D. and Buckingham, D. W. 1993. Analysis of lipid peroxidation mechanism in human spermatozoa. Mol. Reprod. Dev., 35: 302-315.
6. Aitken, R. J., Irvine, D. S. and Wu, F. C. 1991. Prospective analysis of sperm-oocyete fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. Am. J. Obstet. Genecol., 164:542-551.
7. Alvarez, R. J. and Storey, B. T. 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res. 23:77-90.
8. Baumber, J., Ball, B. A., Gravance, C. G., Medina, V. and Davies-Morel, M. C. G. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J. Andro., 21:895-902.
9. Bilodeau, J. F., Blanchette, S., Gagnon, C. and Sirard, M. A. 2001. Thiols prevent H_2O_2-media-

ted loss of sperm motility in cryopreserved bull semen. Theriogenology, 56:275-286.

10. Block, E. R. 1991. Hydrogen peroxide alters the physical state and function of the plasma membrane of pulmonary artery endotherial cells. J. Cell. Physiol., 146:362-369.
11. de Lamirande, E. and Gagnon, C. 1992a. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. J. Androl., 13:368-378.
12. de Lamirande, E. and Gagnon, C. 1992b. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. J. Androl., 13:379-386.
13. de Lamirande, E. and Gagnon, C. 1995. Capacitation associated production of superoxide anion by human spermatozoa. Free Radic. Biol., 18:487-495.
14. de Lamirande, E., Jiang, H., Zini, A., Kodama, H. and Gagnon, C. 1997. Reactive oxygen species and sperm physiology. Rev. Reprod., 2:48-54.
15. DasGupta, S., Mills, C. L. and Fraser, L. R. 1993. Ca^{2+}-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. J. Reprod. Fertil., 99:135-143.
16. Di-Simplicio, P., Cheeseman, K. H. and Slater, T. F. 1991. The reactivity of the SH group of bovine serum albumin with free radicals. Free Radical Res. Commun., 14:253-262.
17. Green, C. M., Cockle, S. M., Watson, P. F. and Fraser, L. R. 1996. Fertilization promoting peptide, a tripeptide similar to thyrotrophin-releasing hormone, stimulates the capacitation and fertilizing ability of human spermatozoa *in vitro*. Human Reprod., 11:830-836.
18. Hecht, D. and Zick, Y. 1992. Selective inhibition of protein tyrosine phosphatase activities by H_2O_2 and vanadate *in vitro*. Biochim. Biophys. Res. Comm., 189:773-779.
19. Hsu, P. C., Hsu, C. C. and Guo, Y. L. 1999. Hydrogen peroxide induces premature acrosome reaction in rat sperm and reduces their penetration of the zona pellucida. 139:93-101.
20. Hsu, P. C., Liu, M. Y., Hsu, C. C., Chen, L. Y. and Guo, Y. L. 1997. Lead exposure causes generation of reactive oxygen species and functional impairment in rat sperm. Toxicology, 122:133-143.
21. Hsu, P. C., Liu, M. Y., Hsu, C. C., Chen, L. Y. and Guo, Y. L. 1998. Effects of vitamin E and/or C on reactive oxygen species-related lead toxicity in the rat sperm. Toxicology, 128: 169-179.
22. Ikeda, M., Kodama, H., Fukuda, J., Shimizu, Y,. Murata, M., Kumagai, J. and Tanaka, T. 1999. Role of radical oxygen species in rat testicular germ cell apoptosis induced by heat stress. Biol. Reprod., 61:393-399.
23. Iwasaki, A. and Gagnon, C. 1992. Formation of reactive oxygen species in spermatozoa of infertile patients. Fertil. Steril., 57:409-416.
24. Kaul, G., Singh, S., Gandhi, K. K. and Anand, S. R. 1997. Calcium requirement and time course of capacitation of goat spermatozoa assessed by chlortetracycline assay. Andrologia, 29:243-251.
25. Kodama, H., Kuribayashi, Y. and Gagnon, C. 1996. Effect of sperm lipid peroxidation on fertilization. J. Androl., 17:151-157.
26. Lim, J. G., Kim, N. H., Lee, H. T. and Chung, K. S. 1997. Effects of extracellular potassium concentration on acrosome reaction and polyspermy during *in vitro* fertilization and subsequent development *in vitro* in the pig. Theriogenology, 48:843-851.
27. Mammoto, A., Masumoto, N., Tahara, M., Yoneda, M., Nishizaki, T., Tasaka, K. and

Miyake, A. 1996. Involvement of a sperm protein sensitive to sulfhydryl-depleting reagents in mouse sperm-egg fusion. Mol. Reprod. Dev. (in press).

28. Myles, D. G. 1993. Molecular mechanisms of sperm-egg membrane binding and fusion in mammalians. Dev. Biol., 158:35-45.
29. O'Flaherty, C., Beconi, M. and Beorlegui, N. 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. 29: 269-275.
30. O'Flaherty, C., Beorlegui, N. and Beconi, M. 1999. Reactive oxigen species requirements for bovine sperm capacitation and acrosome reaction. Theriogenology, 52:289-301.
31. Perez, L. J., Valcarcel, A., de las Heras, M. A., Moses, D. F. and Baldassarre, H. 1997. *In vitro* capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by chlortetracycline assay. Theriogenology, 45:1037-1046.
32. Perry, R. L., Naeeni, M., Barratt C. L. R., Warren, M. A. and Cooke, I. D. 1995. A time course study of capacitation and the acrosome reaction in human spermatozoa using a revised chlortetracycline pattern classification. Fertil. Steril., 64:150-159.
33. Snyder, L. M., Fortier, N. L., Leb, L., McKenney, J., Trainor, J., Sheerin, H. and Mohandas, N. 1988. The role of membrane protein sulfhydryl groups in hydrogen peroxide -membrane damage in human erythrocytes. Biochim. Biophys. Acta., 937:229-240.
34. Takahashi, M., Nagai, T., Okamura, N., Takahashi, H. and Okano, A. 2002. Promoting effect of β-mercaptoethanol on *in vitro* development under oxidative stress and cystine uptake of bovine embryos. Biol. Reprod., 6:562-567.
35. Ward, C. R. and Storey, B. T. 1984. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev. Biol., 104:287-296.

(접수일자 : 2002. 5. 23. / 채택일자 : 2002. 7. 29.)