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Effects of Ferrous Sulfate and Ascorbic Acid on *In Vitro* Fertility and Sperm Lipid Peroxidation in the Pig

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ABSTRACT

This study investigated the effect of ferrous sulfate (Fe^{2+}) and/or ascorbic acid (Asc) on fertilizing ability *in vitro* of frozen-thawed boar spermatozoa. Using chlortetracycline (CTC) fluorescence, the spermatozoa was treated in preincubation medium with control, Fe^{2+}(1 mM), Asc (0.5 mM) and Fe^{2+}/Asc to assessed for acrosome reaction, and the oocyte penetration test to determine whether the Fe^{2+} and/or Asc can promote the penetration ability *in vitro*. When frozen-thawed spermatozoa was washed with preincubation medium, there were significantly ($P < 0.05$) more acrosome-reacted in medium with Fe^{2+}/Asc (38%) than control (27%). The penetration rates were also significantly ($P < 0.05$) higher in medium with Fe^{2+}/Asc (76%) than control (55%). Next, the lipid peroxidation of sperm was evaluated on the basis of malondialdehyde production following same treatments. The addition of Fe^{2+}/Asc to sperm suspension increases the formation of malondialdehyde. However, there were not significantly different under the all conditions. The sperm suspension were also treated with control, Fe^{2+}, Asc and Fe^{2+}/Asc and assayed for sulfhydryl(-SH) group content. In the Fe^{2+}/Asc group, sperm-SH group were higher than another groups. In spermatozoa treated with Fe^{2+} and/or Asc, however, no changes in sperm -SH-groups were detected when compared to controls. In another experiment, the activity of sperm binding to zona pellucida was evaluated through binding to salt-stored porcine oocytes. In control and Asc treatment groups, sperm binding to zona pellucida were significantly ($P < 0.05$) higher than in medium with Fe^{2+}. On the other hand, there is not a significant increase in binding to zona pellucida with spermatozoa treated by Fe^{2+}/Asc. In summary, the present study suggests that Fe^{2+}/Asc causes an enhancement in fertilizing ability that is associated with penetration rate increased without change of spermatozoa binding capacity to homologous zona pellucida.

(Key words : Ascorbic acid, Ferrous sulfate, Boar spermatozoa, *In vitro* fertility, Lipid peroxidation)

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Ⅰ. INTRODUCTION

Before oocyte fertilization becomes possible, mammalian spermatozoa must complete capacitation, a process that globally includes the series of membrane and metabolic changes that occur in the female genital tract. The concept that capacitation is part of an oxidative process has recently been emphasized (de Lamirande and Gagnon, 1993). Human spermatozoa are very sensitive to oxidative stress, leading to the initiation of peroxidative. This sensitivity is due to the high content of unsaturated fatty acids in their plasma membranes and their small cytoplasmic volume, which limits their scavenging capacities (Alvarez and Storey, 1989).

Oxidative stress induced by reactive oxygen species (ROS) has recently been proposed as one of the major causes of human male infertility (Aitken, 1997; Storey, 1997). High levels of ROS generated by activated leukocytes present in accessory gland infections can negatively affect the function of spermatozoa (Kovalski et al., 1992) and a strong negative correlation was found between the capacity of seminal plasma to produce radicals and sperm progressive motility (Kurpisz et al., 1996). ROS generated *in vitro* are able to produce cytoskeletal modifications (Hindshaw et al., 1986), compromise sperm motility (Thiele et al., 1995) and inhibit sperm-oocyte fusion (Aitken et al., 1993). However, a particular ROS, the superoxide anion radical ($O_2^{\cdot -}$), may also have a physiological function as a signalling molecule in sperm hyperactivation and capacitation (de Lamirande and Gagnon, 1995).

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 (Jeulin et al., 1989; Aitken et al., 1989). Generally, ascorbate is thought to act as an excellent reducing agent since it is able to serve as a donor antioxidant in a variety of radical-mediated oxidative processes. However, as a reducing agent it is also able to reduce catalytically active transition metal irons such as cooper and iron, thereby accelerating vitamin consumption and promoting metal-induced oxidative damage (Buettner and Jurkiewicz, 1996). In extracellular and intracellular compartments the binding of iron and cooper ions to specific proteins prevents any form of metal-dependent catalysis, including ascorbate consumption. However, in the presence of ROS, metals can be released from binding proteins, inducing reactive radical species and ascorbate oxidation (Halliwell and Gutteridge, 1990). Exposures to fatty acid peroxides or to high concentrations of the combination ferrous iron and ascorbic acid to induce excessive lipid peroxidation in sperm membranes result in a rapid loss of motility and viability (Jones et al., 1979; Aitken et al., 1989). Similarly, incubations under conditions that favor oxygen contact with spermatozoa result in high levels of lipid peroxidation and cell death (Alvarez et al., 1987).

The objective of the present studies was examined to evaluate the effects of ascorbic acid and ferrous sulfate on *in vitro* fertilizing ability in boar frozen-thawed spermatozoa.

Ⅱ. MATERIALS AND METHODS

1. Sperm Preparation and Treatments

Pooled ejaculate from boar were frozen, and the straws were thawed by immersion in a 37℃ waterbath for about 30 sec. Spermatozoa were washed twice in TC-199 medium containing 2 mM-caffeine (caffeine-sodium benzoate, Sigma Chemical Co., St Louis, MO, USA) by centrifugation at 833×g each for 10 min. After the final washing, the concentration of motile spermatozoa was adjusted to 25×

10^6/ml.

2. Spermatozoa Assessment

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 treated with control, Asc (0.5 mM), Fe^{2+}(1.0 mM) and Asc/Fe^{2+} 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 the 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. Oocyte 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; Sigma Chemical, St-Louis, MO, USA) at 30 to 32℃. Cumulus -oocyte 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 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 5mm in diameter) at estrus with a syringe and 18-gauge needle, and centrifuged at 3850×g for 15 min. The supernatant fluid was frozen at −20℃ until used.

4. Oocyte Penetration Test

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

minutes. After equilibration, 2 ml semen was placed over 2 layers of percoll (65 and 70%) and centrifuged at 2000×g for 15 minutes. 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 minutes and resuspended in preincubation medium.

The fertilization medium with control, Asc, Fe^{2+} and Asc/Fe^{2+} 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 *in vitro*.

5. Determination of Lipid Peroxidation

The levels of lipid peroxidation induced in porcine spermatozoa were assessed by the determination of malondialdehyde (MDA). For this assay, approximately 1×10^6 sperm/ml were treated with control, Asc, Fe^{2+} and Asc/Fe^{2+} in fertilization medium with or without catlase 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 spectrophotometer at 534 nm, was used to determine the MDA concentration.

6. Measurement of Sperm Sulfhydryl(-SH) Groups

After washing of frozen-thawed spermatozoa with and without catalase in fertilization medium containing control, Asc, Fe^{2+} and Asc/Fe^{2+} , 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), and the absorbance was measured at 405 nm. A standard curve was generated using different concentrations of dithiothreitol.

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 washed and re-equilibrated in fertilization medium with FCS for 1 hour prior to experimentation. Spermatozoa (1×10^6 sperm/ml) were incubated for 1 hour at 39℃ with oocytes in 50 μl of fertilization medium with control, Asc, Fe^{2+} and Asc/Fe^{2+} covered with mineral oil. The number of spermatozoa attached to the zona pellucida was then evaluated using an inverted microscope.

8. Statistics

Data were evaluated by ANOVA and differences between individual means were assessed by the least significant difference test or Turkey's test for unequal sample sizes, as appropriate.

Ⅲ. RESULTS

Table 1 shows effect of Asc and/or Fe^{2+} on acrosome reaction and penetration *in vitro* of frozen-thawed boar spermatozoa. The percentage of spermatozoa that reached acrosome reaction were affected by Asc (31%) and Fe^{2+} (30%) and spermatozoa treated with Asc/Fe^{2+} (38%) was significantly ($P < 0.05$) higher than control group (27%). The penetration rates were also significantly ($P < 0.05$) higher in medium with Asc/Fe^{2+} (76%) than control (55%).

The sperm suspension were also treated with control, Fe^{2+}, Asc and Fe^{2+}/Asc and assayed for sulfhydryl(-SH) group content (Table 2). In the Fe^{2+}/Asc group, sperm-SH group were higher than another groups. In spermatozoa treated with Fe^{2+} and/or Asc, however, no changes in sperm-SH-groups were detected when compared to controls.

Under the same conditions, the lipid peroxidation of sperm was evulated on the basis of malondialdehyde production (Table 3). The addition of Fe^{2+}/Asc to sperm suspension increased the formation of malondialdehyde. However, there were not significantly different under the all conditions.

In another experiment, the activity of sperm binding to zona pellucida was evaluated through binding to salt-stored porcine oocytes. In control and Asc treatment groups, sperm binding to zona pellucida were significantly ($P < 0.05$) higher than in medium with Fe^{2+}. On the other hand, there is not a significant increase in binding to zona pellucida with spermatozoa treated by Fe^{2+}/Asc.

Table 2. Effect of ascorbic acid and/or ferrous sulfate on sulfhydryl (-SH) groups of frozen-thawed spermatozoa in the pig

Treatments	Sperm-SH groups (nmole/10^6 spermatozoa) (Average ±SD)
Control	1.12 ±0.28
Ferrous sulfate (Fe^{2+})	1.36 ±0.26
Ascorbic acid (Asc)	1.14 ±0.26
Fe^{2+} + Asc	1.39 ±0.29

Table 3. Lipid peroxidation of boar spermatozoa treated with ascorbic acid and /or ferrous sulfate

Treatments	Density of MDA (10^{-15} mole $sperm^{-1}$) (Average ±SD)
Control	0.200 ±0.08
Ascorbic acid (Asc)	0.186 ±0.06
Ferrous sulfate (Fe^{2+})	0.195 ±0.04
Asc + Fe^{2+}	0.240 ±0.09

Ⅳ. DISCUSSION

With reference to the CTC analysis of spermatozoa, a recent study (Perry et al., 1995) reported a

Table 1. Effect of ascorbic acid (Asc.) and/or ferrous sulfate (Fe^{2+}) on *in vitro* capacitation and penetration by boar spermatozoa frozen-thawed

Culture conditions	No. of spermatozoa examined	Capacitated (%)	Acrosome-reacted (%)	Non-capacitated (%)	Oocytes fertilized (%)
Control	400	289(72)	109(27)^{a}	2(1)	46/83(55)^{a}
Asc	400	272(68)	125(31)^{ab}	3(1)	50/78(64)^{ab}
Fe^{2+}	400	275(69)	121(30)^{ab}	4(1)	44/73(60)^{ab}
Asc + Fe^{2+}	400	245(61)	152(38)^{b}	3(1)	56/74(76)^{b}

^{a,b} Different superscripts within columns denote significant differences ($P < 0.05$).

Table 4. Effect of ferrous sulfate and/or ascorbic acid on zona pellucida binding of frozen-thawed spermatozoa in the pig

Treatments	No. of oocytes examined	Average no. of sperm binded to zona pellucida
Control	98	89 ± 70^{a}
Ferrous sulfate (Fe^{2+})	99	71 ± 69^{b}
Ascorbic acid (Asc)	101	88 ± 74^{a}
Fe^{2+} + Asc	105	86 ± 77^{a}

[a,b] Different letters are significantly different ($P < 0.05$).

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), and boar spermatozoa (Lim et al., 1997). We have compared the four samples with control, Asc, Fe^{2+} and Asc/Fe^{2+} and have observed the same CTC patterns described originally (DasGupta et al., 1993). In the present study, Asc or Fe^{2+} had a stimulatory effect on acrosome reaction at all conditions used. This was shown by a shift from the F pattern of CTC staining, characteristic of uncapacitated, acrosome- intact cells, to the B pattern of staining, characteristic of capacitated, acrosome-intact cells. The lack of stimulation of acrosomal exocytosis is of functional importance because the acrosome reaction would be triggered by oocyte-associated agonists *in vivo*. In this study to examine effect of Asc and/or Fe^{2+}, it is possible that Asc and/or Fe^{2+} may be able to stimulate the acrosome reaction of boar frozen-thawed spermatozoa.

The combination of Fe^{2+} and Asc has been used in the past by different investigators to induce fertilization and lipid peroxidation. This combination is known to trigger lipid peroxidation, via hydroxyl radical formation, as well as favor the breakdown of lipid peroxides into smaller alkenals like malondialdehyde, the product most often monitored in studies involving lipid peroxides (Aitken et al., 1993; Kodama et al., 1996). The present shows that exposure of frozen-thawed boar spermatozoa to Asc and/or Fe^{2+} lead to an increase of lipid hydroperoxide content and stimulated acrosome reaction and fertilizing ability. These experimental results obtained were very similar to those of human (Kodama et al., 1996), and suggested that Asc or Fe^{2+} should also stimulate the capacitation and fertilizing ability of boar spermatozoa *in vitro*. Kodama et al. (1996) confirms that when added in excess over the defence capacity of spermatozoa, the combination of Asc/Fe^{2+} causes a rapid decrease in sperm motility. In this study, however, significant effect on spermatozoa penetration was observed in medium with Asc/Fe^{2+}.

Alvarez and Storey (1984) established that lipoperoxidative lethal endpoint for mouse spermatozoa, defind as the amount of malondialdehyde produced by a cell population at the point where high percentages of the cells have lost their motility, corresponds to 0.6% thiobarbituric acid-reactive substabces per 10^6 cells. In the present study, the value of malondialdehyde obtained with Asc/Fe^{2+} corresponds to a level of mild peroxidation with no deleterious consequence to sperm ability. This apparent discrepancy between the two studies is likely explained by the fact that in the study of Alvarez and Storey (1984), the value of 0.8 mmol/10^8 cells was obtained after prolonged incubation of mouse spermatozoa in a basic protein-free Tris-buffered isotonic solution, whereas in the present study, spermatozoa were treated with IVF medium (TCM-199).

In addition to its effects on sperm motility, lipid peroxidation has also been shown to decrease the sperm-oocyte interaction, as measured by a decreased penetration of zona-free hamster oocytes by a human spermatozoa (Aitken et al., 1989). 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 (Aitken et al., 1989). Thus the results of the present study significantly differ those obtained with zona-free hamster oocytes, because a percentage increased of fertilization following Asc/Fe^{2+} treatments is obtained in the porcine *in vitro* fertilization assay. This discrepancy may emphasize differences in sperm species and in the mechanism by which oocytes with and without zona pellucida are penetrated by spermatozoa.

Mammoto et al. (1996) determined that treatment of sperm with various types of SH-depleting reagents, i.e., N-ethylmalemide, $Na_2S_4O_6$ and 5,5'-dithiobis, 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. In the present study, Asc or Fe^{2+} increased sperm-SH groups, and its effect was more increased by Asc plus Fe^{2+}. Sinha et al. (1991) showed that in human oligospermia there is a quantitative reduction in the SH-groups in the sperm head membrane compared to that of normal spermatozoa. Thus, oxidation of the fusion-related sperm SH- groups may be one mechanism responsible for the suppressive of oxygen stress on sperm function.

In summary, the present study suggests that when spermatozoa are exposed *in vitro* with Asc and/or Fe^{2+}, Asc plus Fe^{2+} cause an enhancement in lipid peroxidation that is associated increasing in the fertilizing capacity of frozen-thawed boar spermatozoa. However, these increases are not associated with sperm-oocyte binding. These results may suggest that because lipid peroxides are present in cell membranes under aerobic conditions, spermatozoa after ejaculation could use a strategy involving oxidation and breakdown of membrane lipids to improve their chances to fertilization *in vivo*.

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요 약

돼지의 체외수정능력과 정자의 Lipid Peroxidation에 있어서 Ascorbic Acid와 Ferrous Sulfate의 영향

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본 연구는 lipid peroxides를 생산하는 것으로 알려진 ascorbic acid (Asc; 0.5 mM)와 ferrous sulfate (Fe^{2+}; 1 mM)가 돼지 동결-융해 정자의 체외수정능력에 미치는 영향을 검토하였다. 그 결과, 동결정액의 융해 처리시 배양액내 Asc/Fe^{2+} (38%)의 동시첨가시 대조구 (27%)에 비해 정자의 첨체반응이 유의적으로 높게 유기되었다 ($P < 0.05$). 정자침입율 또한 Asc/Fe^{2+} (76%)를 동시에 첨가하므로서 대조구 (55%)에 비하여 유의적으로 높게 나타났다 ($P<0.05$). 한편 정자의 peroxidation은 상기의 처리구에서 malondialdehyde (MDA)의 생성에 기초를 두고 평가하였는데, 정자의 처리시 Asc/Fe^{2+}의 동시첨가에 의해 MDA의 생성이 증가하였으나 처리구 사이에서 유의적인 차이는 인정되지 않았다. 또한 sulfhydryl(-SH) group의 용량을 측정한 결과 Asc/Fe^{2+}를 동시에 첨가한 실험구에서 가장 높게 나타났으나 타처리구와의 사이에서 유의적인 차이는 인정되지 않았다. 또 다른 연구에서 동결-융해정자가 난자의 투명대에 접착하는 정도를 평가한 결과, 대조구와 Asc처리시 Fe^{2+}처리에 비하여 유의적으로 ($P < 0.05$) 높은 정자의 접착을 나타냈으나 Asc/Fe^{2+}의 동시 처리에 의한 정자접착의 증가는 인정되지 않았다. 결과적으로 lipid peroxidation의 증가를 야기시키는 Asc/Fe^{2+}의 동시첨가는 투명대에서 정자의 접착능력을 증가시키지 않았음에도 불구하고 첨체반응과 침입능력을 향상시키는 것으로 나타났다.

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