韓國家畜繁殖學會誌 23(3) : 229~237(1999)
Korean J. Animal Reprod.

Effects of Ascorbic Acid and Ferrous Sulfate on *In-Vitro* Fertility of Frozen-Thawed Spermatozoa in Porcine

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돼지동결정액의 체외수정능력에 있어서 Ascorbic Acid와 Ferrous Sulfate의 영향

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ABSTRACT

The influence of ascorbic acid (Asc) and ferrous sulfate (Fe^{2+}) on capacitation, acrosome reaction and fertility *in vitro* was investigated in boar frozen-thawed spermatozoa with or without preincubation. The addition of 0~1.0 mM Fe^{2+} to sperm suspensions during preincubation increase acrosome reaction ($P<0.05$) and oocyte penetration. These increase are also associated with addition of 0~0.5 mM Asc, but the penetration rates were higher in those without than with sperm preincubation. The addition of 0.1 mM Asc than 0.5 mM in medium with Fe^{2+} were significantly ($P<0.05$) higher on acrosome reaction at 2 h after sperm preincubation. No significant differences, however, were observed in penetration rates among the concentrations of Asc. On the other hand, when preincubation medium containing the Asc was supplemented with 0.1 mM Fe^{2+}, the percentage of spermatozoa acrosome-reacted were significantly ($P<0.05$) higher than in medium wothout Fe^{2+}, on the contrary, the penetration rate was significantly ($P<0.05$) low during in-vitro fertilization. These findings indicate some apparent effects of Fe^{2+} or Asc addition on acrosome reaction and the fertilizing potential by sperm preincubation.
(Key words: Ascorbic acid, Acrosome reaction, Capacitation, Ferrous sulfate, Porcine)

I. INTRODUCTION

The role of reactive oxygen species (ROS) in the pathophysiology of human sperm function has been emphasized in many study (Aitken et al., 1991, 1993; De Lamirande and Gagnon, 1992). Their production in semen has been associated with loss of motility, decreased capacity for sperm-oocyte fusion and loss of fertility (Aitken et al., 1991). The spermatozoa can generate ROS such as the superoxide anion ($O2^{-\cdot}$) and hydrogen peroxide (H_2O_2)(De Lamirande and Gagnon, 1995).

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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). Whereas hydrogen peroxide was shown to be toxic to human spermatozoa (De Lamirande and Gagnon, 1992), the superoxide anion has a positive role (De Lamirande and Gagnon, 1995) in triggering the series of membrane and metabolic transformations known as capacitation (Yanagimachi, 1994) that spermatozoa must undergo before they can fertilize the oocyte.

The dominant role of lipid peroxidation as the agent of oxidative damage to mammalian spermatozoa was established by the extensive and elegant studies (Jones and Mann, 1977) of these reactions in boar and ram spermatozoa, and by their subsequent studies with Sherins (Jones et al., 1979) in human spermatozoa. To ensure that peroxidation was the reaction being studied, the cells were incubated in media containing not only dissolved O_2, but also ascorbate plus Fe^{2+} at concentrations known to induce rapid lipid peroxidation with subsequent degradation. The rapidity of the reaction arises from reaction of Fe^{2+} with formed lipid hydroperoxides to form Fe^{3+} and lipid alkoxyl radicals; the latter in turn are highly reactive initiators of oxidative chain reactions. Reduction by ascorbate of the Fe^{3+} thus formed maintains the supply of Fe^{2+} needed to drive these oxidative chain reactions. Exposures to fatty acid peroxides or to high concentrations of the combination $Fe^{2+}$and Asc to induce excessive lipid peroxidation in sperm membranes results in a rapid loss of motility and viability (Aitken et al., 1989). Although correlations have been reported between the effectiveness of Fe^{2+}/Asc and the duration of sperm motility, the effect of their action in porcine spermatozoa has not been not been elucidated. Therefore, this study was designed to investigated the effects of Fe^{2+} and/or Asc on sperm motility and *in vitro* penetration in porcine.

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 25×106/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 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 *ul* 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 with colourless nail varnish 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 *ul* 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), 1μg/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 2ml 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 was the same as the preincubation medium, enriched with 2 mM ca-

ffeine (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. Experimental Design

The ability of spermatozoa were examined in medium with different concentrations of Fe^{2+} (0, 0.1, 0.5 and 1.0 mM) and Asc (0, 0.01, 0.1 and 0.5 mM). Cells prepared for assessment of sperm function were preincubated in fertilization medium for 0 or 2 h, and sperm ability were assessed by CTC fluorescence assay and oocyte penetration test.

In another experiment, spermatozoa were preincubated with different concentrations of Asc (0, 0.01, 0.1 or 0.5 mM) for 2 h in medium with 1 mM Fe^{2+}. Spermatozoa were also preincubated with different concentrations of Fe^{2+} (0, 0.1, 0.5 or 1.0 mM) for 2 h in medium containing 0.5 mM Asc. Spermatozoa ability *in vitro* were assessed by CTC fluorescence assay and oocyte penetration test as described above.

6. 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 different of Fe^{2+} on *in vitro* capacitation of boar spermatozoa. The Fe^{2+} at concentrations between 0~1.0 mM was added to sperm suspensions which were then incubated for 2 h before being assessed by CTC. Control samples were assessed without preincubation in Fe^{2+} free medium. At concentrations of Fe^{2+} of 0, 0.1, 0.5 and 1.0 mM, the percentage of spermatozoa that reached the capacitated status plus underwent spontaneous acrosome reaction were affected by preincubation and higher than control group. Under control group, high rate in dead spermatozoa were observed when compared to groups preincubated with different concentrations of Fe^{2+}.

To investigate the impact of Fe^{2+} treatment on sperm fertilizing capacity, spermatozoa preincubated with Fe^{2+} at different concentrations that did higher than group without sperm preincubation (Fig. 1). The percentage of oocytes fertilized by spermatozoa preincubated for 2 h with 0.1 mM Fe^{2+} was significantly ($P<0.05$) higher than spermatozoa without preincubation.

Table 1. Effect of concentrations of ferrous sulfate on *in-vitro* capacitation of spermatozoa preincubated for 2 h in porcine

Concentrations of Fe^{2+}(mM)	No. of spermatozoa examined	Capacitated (%)	Acrosomereacted (%)	Non-capacitated and Dead (%)
Control*	500	331(66)[a]	40(8)[a]	129(26)
0	500	332(66)[a]	98(20)[c]	70(14)
0.1	500	338(68)[a]	95(19)[c]	67(13)
0.5	500	360(72)[a]	66(13)[b]	74(15)
1.0	500	280(56)[b]	126(25)[d]	94(19)

* Control : non-preincubated spermatozoa

[a~d] Different superscripts within columns denote significant differences ($P<0.05$).

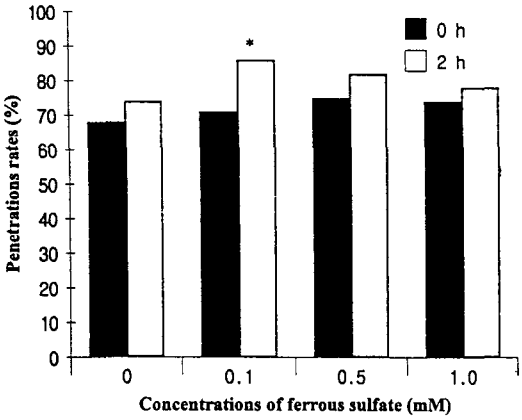

Fig. 1. Effect of concentrations of Fe^{2+} on *in-vitro* penetration by sperm preincubated (2 h) or non-preincubated in porcine. * P<0.05, differences between spermatozoa preicnubated and non-preincubated with 0.1 mM ferrous sulfate.

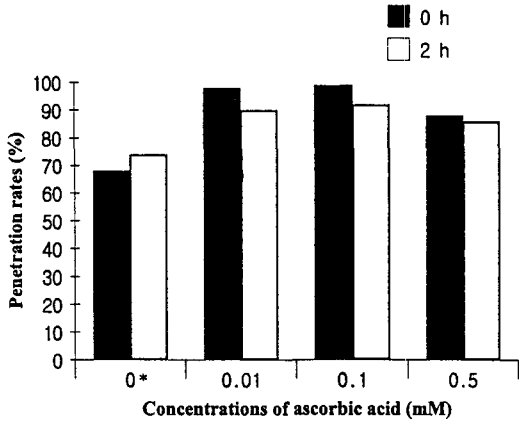

Fig. 2. *In-vitro* penetration by spermatozoa preincubated with various concentrations of ascorbic acid in porcine. * P<0.05, compared with addition of ascorbic acid concentrations.

The boar spermatozoa were incubated with different concentrations of Asc and the spermatozoa were assessed with CTC after 0 and 2 h of incubation. Untreated control suspensions was assessed at time point without preincubation after sperm treatment. The results indicated a significant stimulation of capacitation in the control group when compared with treated by different concentrations of Asc during sperm preincubation (Table 2). However, all concentrations of Asc caused a significant stimulation of acrosome reaction (P<0.05), as shown by a decrease in the proportion of B-pattern cells and an increase in the proportion of AR-pattern cells.

The results indicated that the presence of Asc and fertilizing ability in the porcine oocytes. There was a higher proportion of penetrated oocytes in the suspensions treated with Asc when compared with the group treated in medium wit-

Table 2. Effect of concentrations of ascorbic acid on *in-vitro* capacitation by spermatozoa preincubated for 2 h in porcine

Concentrations of Asc (mM)	No. of spermatozoa examined	Capacitated (%)	Acrosome-reacted (%)	Non-capacitated and Dead (%)
Control*	500	345(69)[a]	60(12)[a]	95(19)
0	500	310(62)[b]	95(19)[b]	95(19)
0.01	500	295(59)[b]	85(17)[b]	120(24)
0.1	500	295(59)[b]	85(17)[b]	120(24)
0.5	500	305(61)[b]	120(24)[c]	75(15)

* Control : Non-preincubated spermatozoa in medium without Asc.

[a~c] Different superscripts within columns denote significant differences (P<0.05).

Table 3. Effects of concentrations of ascorbic acid on in-vitro fertilizing potential of porcine spermatozoa preincubated for 2 h in medium with 1 mM Fe^{2+}

Concentrations of Asc (mM)	No. of spermatozoa capacitated (%)	No. of spermatozoa acrosome-reacted (%)	Oocytes fertilized*
0†	372/500(74)	82/500(16)^{ab}	41/71(58)
0.01	373/500(75)	65/500(13)^{ab}	38/70(54)
0.1	365/500(73)	95/500(19)^{a}	40/71(56)
0.5	394/500(79)	56/500(11)^{b}	40/74(54)

* Oocytes from three trials.

† Medium with 1 mM ferrous sulfate only.

$^{a\sim b}$ Different superscripts within a column denote significant difference ($P<0.05$).

hout Asc during sperm preincubation for 0 and 2 h (Fig. 2).

None of the concentration of Asc during sperm preincubation for 0 and 2 h were significantly ($P<0.05$) inhibited on the percentage of penetrated oocytes when compared to groups with different concentrations.

To investigate the impact of Fe^{2+}/Asc treatment on sperm fertilizing capacity, spermatozoa were preincubated with different concentrations of Asc in medium with 1 mM Fe^{2+}. A concentration of 1 mM Fe^{2+} was used because this was the concentration found to be most effective at stimulating acrosome reaction in boar spermatozoa as shown in Table 1. The results indicated a significant stimulation of acrosome reaction in spermatozoa with 0.1 mM Asc ($P<0.05$) when compared with the group treated with 0.5 mM Asc (Table 3). There was no significant stimulation of capacitation observed after the addition of different concentrations of Asc. There was also not a significantly differences in proportions of fertilized oocytes.

To investigate the effect of Fe^{2+} concentrations in medium with 0.5 mM Asc on sperm fertilizing capacity, spermatozoa were preincubated with different concentrations (0, 0.1, 0.5 and 1.0 mM) of Fe^{2+} for 2 h (Table 4). A concentration of 0.5 mM Asc was used because this was the concentration found to be most effective at stimulating acrosome reaction in boar spermatozoa as shown in Table 2.

Table 4. Effects of concentrations of ferrous sulfate on in-vitro fertilizing potential of porcine spermatozoa preincubated for 2 h in medium with 0.5 mM ascorbic acid

Concentrations of Fe^{2+}(mM)	No. of spermatozoa capacitated (%)	No. of spermatozoa acrosome-reacted (%)	Oocytes fertilized*
0†	360/500(72)	85/500(17)^{a}	30/65(46)^{a}
0.1	338/500(68)	127/500(25)^{b}	18/70(26)^{b}
0.5	328/500(66)	107/500(21)^{ab}	32/75(43)^{a}
1.0	330/500(66)	105/500(21)^{ab}	33/76(43)^{a}

* Oocytes from three trials.

† Medium with 0.5 mM ferrous sulfate only.

$^{a\sim b}$ Different superscripts within a column denote significant difference ($P<0.05$).

The results indicated a significant stimulation of acrosome reaction in spermatozoa with 0.1 mM Fe^{2+} ($P<0.05$) when compared with the group treated without Asc. There was no significant stimulation of capacitation observed after the addition of different concentrations of Asc. However, there was a significantly ($P<0.05$) lower proportion of fertilized oocytes when the suspensions treated with 0.1 mM Fe^{2+} were compared with those treated with 0, 0.5 and 1.0 mM Asc.

Ⅳ. DISCUSSION

We have investigated that lipid peroxidation alters the fluidity of the sperm plasmatic membrane required for capacitation and oocyte penetration of frozen-thawed boar spermatozoa *in vitro*, and that Asc and Fe^{2+} should exert a protective effect against oxidative damage. The results indicated that the presence of Asc or Fe^{2+} stimulated acrosome reaction and fertilizing ability. The experimental results obtained were very similar to those seen with human spermatozoa (Kodama et al., 1996), and suggest that Asc or Fe^{2+} can also stimulate the capacitation and fertilizing ability of boar spermatozoa *in vitro*.

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) and boar spermatozoa (Lim et al., 1997). We have compared the two samples with or without sperm preincubation and have observed the same CTC patterns described originally (DasGupta et al., 1993)

The basis for examining Asc or Fe^{2+} in this study was its established role cofactor on spermatozoa ability *in vitro*. In the present study, Fe^{2+} or Asc had a stimulatory effect on acrosome reaction at all concentrations 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 response was seen early, within 1~3 h of the start of incubation, and did not appear to be concentration dependent; there was no significant stimulation of the acrosome reaction in human spermatozoa (Green et al., 1996). This lack of stimulation of acrosomal exocytosis is of functional importance because *in vivo* the acrosome reaction would be triggered by oocyte-associated agonists. In this study to examine effect of Fe^{2+} and/or Asc concentrations, however, it is possible that Fe^{2+} and/or Asc at all concentrations may be able to stimulate the capacitation of boar spermatozoa after preincubation *in vitro*.

There have been previous reports of the effect of preincubation of frozen-thawed spermatozoa on *in vitro* fertilization in the pig (Park and Sirard, 1996). The present results indicate that preincubation of spermatozoa for 2 h in medium with different concentrations of Fe^{2+} or Asc is helpful in sperm acrosome reaction and spermatozoa preincubated with Fe^{2+} on sperm penetration. However, the fertilizability of spermatozoa preincubated for 2 h in medium with Asc were low than without preincubation, but this was unknown.

The combination of Fe^{2+} and Asc has been used in the past by different investigators to lipid peroxidation for effect of sperm lipid peroxidation on fertilization (Kodama et al., 1996). 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 (Aitken et al., 1993). In the present study, the ad-

dition of 0.01~0.5 mM Fe^{2+} in medium with Asc cause increase in acrosone reaction, and decrease by addition of Asc in medium with Fe^{2+}. However, at two examinations (Table 3 and 4), no significant effect on sperm penetration was observed despite the fact that there was a significant increase and decrease of acrosome reaction from different concentrations of Asc /Fe^{2+} combination.

In summary, the present study suggests that at different concentrations, Fe^{2+} or Asc cause an enhancement in sperm preincubation that is associated with acrosome reaction on spermatozoa ability in vitro. These results may suggest that because lipid peroxides are present in membranes of all cells living under aerobic conditions, spermatozoa could alter plasma membrane features, thus affecting its fluidity or the production of the reactive oxygen species for such processes.

V. 요 약

본 연구는 돼지 동결-융해 정자의 전배양시 ascorbic acid (Asc)와 ferrous sulfate (Fe^{2+})가 정자의 수정능력획득, 첨체반응 및 난자내 침입능력에 미치는 영향을 검토하였다. 정자의 전배양시 0~1.0 mM의 Fe^{2+}의 첨가는 비전배양에 비해 높은 첨체반응($P<0.05$) 및 정자침입율을 얻었다. 이와같은 결과는 0~0.5 mM의 Asc 첨가시 첨체반응율에서는 같은 결과를 나타냈지만 정자침입율은 오히려 정자의 전배양 보다는 비전배양시 높은 비율을 나타냈다. 한편, Fe^{2+}가 함유되어있는 배양액내에서 2시간동안 정자의 전배양시 0.1 mM Asc의 첨가는 0.5 mM Asc의 첨가에 비해 유의적으로 높은 첨체반응율을 나타냈으나 ($P<0.05$), Asc의 농도사이에서 정자침입율에는 차이가 없었다. 또한, Asc가 함유된 배양액내에서 정자의 전배양시 0.1 mM Fe^{2+}를 첨가했을 때 첨체반응율은 Fe^{2+}무첨가시 유의적으로 높았으나($P<0.05$), 오히려 가장 낮은 정자침입율을 나타냈다. 이와같은 결과는 체외에서 돼지정자의 처리시 Fe^{2+} 또는 Asc의 첨가와 정자의 전배양에 의해 첨체반응과 정자침입에 효과적인 작용을 하는 것으로 생각된다.

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(접수일자 : 1999. 8. 18. /채택일자 : 1998. 9. 20.)