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Effect of Sperm Preincubation Medium with Ascorbic Acid and/or Ferrous Sulfate on Porcine *In-Vitro* Fertilization

Park, C. K.†, H. S. Nam, J. H. Lee, I. C. Kim, H. T. Cheong, B. K. Yang and C. I. Kim
College of Animal Resource Science, Kangwon National University

돼지의 체외수정시 Ascorbic Acid와 Ferrous Sulfate의 첨가하에서 정자 전배양의 영향

박춘근† · 남희선[1] · 이장희[2] · 김인철[2] · 정희태 · 양부근 · 김정익
강원대학교 동물자원과학대학

ABSTRACT

The aim of this work was to study the effects of ascorbic acid (Asc) and/or ferrous sulfate (Fe^{2+}) and spermatozoa preincubation on the *in vitro* fertilization in porcine. Porcine follicular oocytes matured in culture were inseminated with frozen-thawed boar spermatozoa preincubated for 0, 1, 2, 3, 4 and 5 h. The penetration rates (37~51%) were not significantly different between durations of spermatozoa preincubation in medium with 0.1 mM Asc. The addition of 1.0 mM Fe^{2+} during spermatozoa preincubation were not significantly affecting the penetration rates (41~56%). When spermatozoa were preincubated with Asc and Fe^{2+}, the penetration rates had a tendency to increase with time of spermatozoa preincubation, and were significantly ($P<0.05$) higher in spermatozoa preincubated with that than without Asc and Fe^{2+} for 5 h. On the other hand, when spermatozoa were preincubated in fertilization medium without Asc and/or Fe^{2+}, the penetration rates were significantly ($P<0.05$) higher in medium with Fe^{2+} than with Asc or Asc and Fe^{2+} for *in vitro* fertilization. The rate of polyspermy in penetrated oocytes in medium with Asc and Fe^{2+} decreased with the period of spermatozoa preincubation. Despite different culture conditions for spermatozoa preincubation, no differences were observed in polyspermy rates in the presence of Asc and/or Fe^{2+}. These results indicate the advantage of preincubating spermatozoa with Asc and Fe^{2+} and an addition of Fe^{2+} during *in vitro* fertilization with spermatozoa preincubated maintain penetration potential without increased polyspermy rates on *in vitro* fertilization in porcine oocytes.

(Key words : Ascorbic acid, Ferrous sulfate, *In vitro* penetration, Preincubation, Porcine)

Ⅰ. INTRODUCTION

Capacitation is a term used for the series of preparatory changes that occur during the period of

[1] 강남 미즈메디병원
[2] 축산기술연구소 종축개량부
† Corresponding Author : College of Animal Resource Science, Kangwon National University, E-mail : parkck@kangwon.ac.kr

mammalian sperm incubation required for successful triggering of the acrosome reaction by egg-associated factors. During the acrosome reaction, the overlying plasma membrane fuses at multiple sites with the outer acrosomal membrane (Barros et al., 1967). Capacitation is accompanied by changes in membrane lipid and composition of protein and localization and changes in ion fluxes, including calcium, sodium, and potassium, and proteins. These subcellular changes regulate changes in motility patterns and the onset of the fusion that must occur in the vicinity of the egg for successful gamete interaction.

Several investigators have demonstrated the ability of porcine oocytes matured and fertilized *in vitro* to develop normally(Grupen et al., 1995; Nagai, 1994; Niwa, 1993), and the birth of piglets from embryos produced *in vitro* has been reported (Kameyama et al., 1997; Yoshida et al., 1993). Despite these achievements, the *in vitro* development of *in vitro* matured and fertilized porcine oocytes to the blastocyst stage is poor. The failure of most *in vitro* matured and fertilized porcine oocytes to develop normally is attributable to abnormalities of fertilization, including polyspermy and asynchronous pronuclear formation (Grupen et al., 1995).

Reactive oxygen species are harmful to spermatozoa. Their production in spermatozoa has been associated with loss of motility, decreased capacity for sperm-oocyte fusion and loss of fertility(Aitken et al., 1987, 1991). Hydrogen peroxide produced by the dismutation of superoxide anion has been recognized as the most toxic oxidising species for human spermatozoa(Aitken et al., 1993; de Lamirande and Gagnon, 1992). Polyunsaturated fatty acids are sensitive to lipid peroxidation. 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(Aitken et al., 1989).

The aim of the this study was to determine whether preincubation of spermatozoa in the presence of Asc and/or Fe^{2+} can effect the penetration and polyspermy of oocytes by frozen-thawed porcine spermatozoa.

Ⅱ. MATERIALS AND METHODS

1. 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 17 β (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.

2. 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 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*.

3. Experimental Design

Suspensions of spermatozoa were added to a droplet of 50 μl fertilization medium with or without 0.1 mM ascorbic acid. Spermatozoa were preincubated for 0, 1, 2, 3, 4 and 5 h then mixed with drops containing 5 oocytes each. To evaluate the ferrous sulfate on sperm penetration *in vitro*, the sperm were preincubated in 50 μl of fertilization medium with or without ferrous sulfate (1mM) for 0, 1, 2, 3, 4 and 5 h and then combined with oocytes. In another experiment, effect of ascorbic acid and ferrous sulfate on sperm penetration *in vitro* were examined. Spermatozoa were preincubated in 50 μl of fertilization medium for 0, 1, 2, 3, 4 and 5 h and then mixed with 5 oocytes. Finally, spermatozoa were preincubated in basic fertilization medium for 1, 2, 3, 4 and 5 h. After preincubation, spermatozoa were mixed in fertilization with ascorbic acid and/or ferrous sulfate and 5 oocytes and the cultured for 20~22 h.

4. Evaluation of Fertilization

At 20 to 22 h after insemination, the oocytes were mounted, fixed (acetic acid : ethanol 1:3) for 2 to 3 days and stained with 1% aceto-orcein in 40% acetic acid:water (v/v) solution. The proportions of penetration and polyspermy were examined under a light microscope at × 200 and 400 magnification. Oocytes were considered penetrated when spermatozoa with a swollen head pronuclei were found in the vitellus. Oocytes penetrated by only 1 spermatozoon were judged to monospermic oocytes. Chi-squire analysis with the Yates correction was used to test the signification of individual comparisons for the rates of penetration and polyspermy.

Ⅲ. RESULTS

As shown Table 1, penetration rate were higher in medium with (46%) than without (36%) Asc in spermatozoa without preincubation. When oocytes were inseminated with preincubated spermatozoa, the penetration rates were not different in medium with or without Asc. Polyspermy rates were not also different between in medium with or without Asc.

Oocytes were inseminated with preincubated spermatozoa in medium with Fe^{2+} for 0, 1, 2, 3, 4 and 5 h (Table 2). Penetration rates were increased with preincubation periods of spermatozoa. The proportions of polyspermy were 23, 7, 35, 13, 29 and 8% for 0, 1, 2, 3, 4 and 5 h of spermatozoa preincubation.

As shown in Table 3, oocytes were also inseminated with spermatozoa preincubated with Asc and Fe^{2+}. The penetration rates had a tendency to increase with time of spermatozoa preincubation.

Table 1. *in vitro* penetration of porcine oocytes matured in culture by frozenthawed spermatozoa preincubated in medium with ascorbic acid (Asc)

Periods of sperm preincubation	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%)
		Total(%)	ESH	BPN	
0	81	37(46)	33	4	11(30)
1	81	35(43)	27	8	13(37)
2	75	28(37)	25	3	6(21)
3	80	41(51)	37	4	13(32)
4	78	36(46)	32	4	7(19)
5	74	32(43)	30	2	6(19)

ESH: enlarged sperm head, BPN: both pronuclei

Table 2. *in vitro* penetration of porcine oocytes matured in culture by frozenthawed spermatozoa preincubated in medium with ferrous sulfate (Fe^{2+})

Periods of sperm preincubation	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%)
		Total(%)	ESH	BPN	
0	73	30(41)	27	3	7(23)
1	74	30(41)	28	2	2(7)
2	62	29(47)	29	0	10(35)
3	62	31(50)	27	4	4(13)
4	75	38(51)	34	4	11(29)
5	72	40(56)	34	6	3(8)

ESH: enlarged sperm head, BPN: both pronuclei

The penetration rate was significantly ($P<0.05$) higher in spermatozoa preincubated without(34%) that than with(13%) Asc + Fe^{2+}. When spermatozoa were preincubated for 5 h, however, the penetration rate was significantly($P<0.05$) higher in medium with that than without Asc+Fe^{2+}. On the other hand, polyspermy rates were significantly ($P<0.05$) higher in spermatozoa preincubated for 0 and 1 h than 2 to 5 h in medium with Asc+Fe^{2+}.

In the last experiment (Table 4), spermatozoa were preincubated for 1, 2, 3, 4 and 5 h in control medium, and then spermatozoa were mixed with oocytes in medium Asc and/or Fe^{2+}. The penetration rates by spermatozoa preincubated for 1, 2, 4 and 5 h were significantly ($P<0.05$) higher in medium with Fe^{2+} than with Asc or Asc+Fe^{2+}.

Ⅳ. DISCUSSION

There have been previous reports of the effect of preincubation of spermatozoa on fertilization in the pig (Nagai and Moor, 1990; Park and Sirard, 1996). The present results indicate that preincubation of frozen-thawed spermatozoa in medium with Asc or Fe^{2+} for 1 to 5 h (Table 1) is not helpful. Nagai et al. (1984) reported that frozen ejaculated spermatozoa could not penetrate porcine oocytes matured in culture. In their study, the spermatozoa were preincubated for 4 h before insemination, which resulted in great reduction of sperm motility.

Table 3. *in vitro* penetration of porcine oocytes matured in culture by frozenthawed spermatozoa preincubated in medium with Asc and Fe^{2+}

Periods of sperm preincubation	Presence of Asc+Fe^{2+}	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%)
			Total(%)	ESH	BPN	
0	+	83	35(42)	23	12	15(43)[a]
	−	78	28(36)	25	3	7(25)
1	+	80	10(13)*	7	3	4(40)[b]
	−	76	26(34)	26	0	6(23)
2	+	72	22(31)	20	2	5(23)[b]
	−	81	35(43)	30	5	8(23)
3	+	76	39(51)	36	3	7(18)[b]
	−	80	29(36)	27	2	7(22)
4	+	68	42(62)	38	4	11(19)[b]
	−	77	37(48)	32	5	8(22)
5	+	69	46(67)*	38	8	8(17)[b]
	−	69	29(42)	25	4	7(24)

ESH : enlarged sperm head, BPN: both pronuclei

* $P<0.05$, different between with and without Asc+Fe^{2+}

[ab] $P<0.05$, different between preincubation periods

The reduced motility of frozen ejaculated spermatozoa during the preincubation was also reported by Clark and Johnson (1987).

A variety of evidence in different species suggests that the Asc and/or Fe^{2+} may play a role in these events by providing an environment that facilitates both capacitation of spermatozoa and fertilization. In the present study, the results show that it is possible to maintain the rate on penetration with spermatozoa preincubated in medium with Asc and Fe^{2+}. It has been shown that the motility and the fertilizing capacity of bovine spermatozoa could be maintained with ascorbic acid (Dalvit et al., 1998). Kodama et al. (1996) also reported that at low concentrations, Asc/Fe^{2+} cause an enhancement in lipid peroxidation/breakdown that is associated with a 50% increase in the fertilizing capacity of mouse spermatozoa. They confirms that when added in excess over the defense capacity of spermatozoa, the combination Asc/Fe^{2+} causes a rapid decrease in sperm motility. In this study, at concentrations of 0.1/1.0mM for Asc/Fe^{2+}, significant effect on penetration was observed in spermatozoa preincubated for 5 h. However, under these conditions, low penetration rate in spermatozoa preincubated for 1 h was observed in medium with (13%) that and without (34%) Asc/Fe^{2+}.

In another experiment, when spermatozoa were preincubated in fertilization medium without Asc and/or Fe^{2+}, the penetration rates in medium with Fe^{2+} after preincubation for 1 to 5 h were higher than in medium with Asc or Asc and Fe^{2+}. In results of this study (Table 4), the increase in fertilizing ability of spermatozoa treated with Asc/Fe^{2+} after preincubation does not appear. It suggests that Fe^{2+} enhance and prolongs motility of porcine spermatozoa and that the addition of Fe^{2+} during *in vitro* fertilization with spermatozoa preincubated results in increasing penetrations *in*

Table 4. Effects of Asc and/or Fe^{2+} on *In-Vitro* penetration after spermatozoa preincubation in control medium

Periods of sperm preincubation	Conditions of culture	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%)
			Total(%)	ESH	BPN	
1	Asc	70	26(37)[ab]	25	3	7(27)
	Fe^{2+}	71	30(42)[a]	26	4	5(17)
	Asc+Fe^{2+}	73	15(20)[b]	14	1	0(0)
2	Asc	76	26(34)[a]	25	1	3(12)
	Fe^{2+}	74	42(57)[b]	38	4	6(14)
	Asc+Fe^{2+}	71	30(42)[ab]	25	5	4(13)
3	Asc	74	22(30)	21	1	1(5)
	Fe^{2+}	72	33(46)	30	3	5(15)
	Asc+Fe^{2+}	70	31(44)	29	2	3(10)
4	Asc	69	30(33)[a]	19	11	6(20)
	Fe^{2+}	72	40(56)[b]	25	15	10(25)
	Asc+Fe^{2+}	73	29(40)[ab]	17	12	5(17)
5	Asc	70	30(43)[a]	20	10	9(30)
	Fe^{2+}	75	48(64)[b]	33	15	17(35)
	Asc+Fe^{2+}	73	27(41)[a]	8	19	7(26)

ESH: enlarged sperm head, BPN: both pronuclei

[ab] $P<0.05$, different between culture conditions in same preincubation periods

vitro. In the present systems, penetration rates were higher in medium with Fe^{2+} regardless of the duration of preincubation.

The high incidence of polyspermy in porcine oocytes matured *in vitro* has been reported by several investigators (Suzuki et al., 1994; Wang et al., 1993). In the present study, the proportion of oocytes penetrated with more than one sperm cell was high (0 to 43%). Since a high proportion of the oocytes inseminated with spermatozoa preincubated for 0 to 1 h was polyspermic in medium with (43 and 40%) versus without (25 and 23%) Asc/Fe^{2+}, it is believed Asc/Fe^{2+} does play a role in the induction of capacitation and penetration of porcine spermatozoa under this experimental conditions.

Park and Sirard (1996) showed when oocytes were inseminated in the presence of oviductal vesicles, the polyspermy rates were below the rates observed with caffeine in spermazoa without preincubation. In this study, the presence of Asc and/or Fe^{2+} did not increase the polyspermy rate. and had a tendency to decrease with time of sperm preincubation. It seems that spermatozoa preincubation can compete advantageously with Asc and Fe^{2+} to induce high fertilization rates and low polyspermy. This property should allow for a further reduction in the number of spermatozoa to be added at fertilization, and a further reduction of polyspermy with frozen-thawed ejaculated boar sperm.

In conclusion, experimental approaches of this

study were used to demonstrate the advantage of preincubation of spermatozoa with Asc and/or Fe^{2+} to maintain penetration potential with no increase in the polyspermy rate during *in vitro* fertilization in the porcine.

Ⅴ. 요 약

본 연구는 돼지난자의 체외수정시 Asc와 Fe^{2+}의 첨가하에서 정자 전배양의 영향을 검토하기 위하여 수행되었다. 체외에서 성숙시킨 돼지난포난자를 0, 1, 2, 3, 4 및 5시간 전배양된 돼지동결-융해 정액을 이용하여 수정한 결과, 정자침입율(37~51%)은 0.1mM Asc의 첨가하에서 정자의 전배양 기간사이에서 유의적인 차이는 인정되지 않았다. 또한 정자의 전배양 기간동안 1.0mM Fe^{2+}의 첨가시에도 정자침입율에는 커다란 차이를 나타내지 않았다. 그러나 정자 전배양시 Asc와 Fe^{2+}를 동시에 첨가했을 때 정자의 전배양기간이 길어짐에 따라 정자침입율이 증가하는 경향을 나타냈으며, 5시간 전배양시 이들 물질의 첨가시 무첨가에 비해 유의적으로 높은 정자침입율을 나타냈다 ($P<0.05$). 한편 Asc와 Fe^{2+}가 첨가되지 않은 배양액내에서 전배양된 정자를 이용하여 수정했을 때, 수정배양액내에 Asc 또는 Asc+Fe^{2+}가 첨가된 경우 보다는 Fe^{2+}첨가시 유의적($P<0.05$)으로 높은 정자침입율을 나타냈으며, 다정자침입율은 정자의 전배양기간이 길어짐에 따라 감소하는 경향을 나타냈지만 이들 물질이 첨가된 배양조건하에서는 그 차이가 인정되지 않았다. 본 연구의 결과로부터 정자의 전배양시 Asc 와 Fe^{2+}의 첨가는 정자침입에 효과적으로 작용했으며, 전배양된 정자를 이용한 체외수정시 Fe^{2+}의 첨가는 다정자침입을 억제하면서 수정능력이 계속 유지되는 것으로 나타났다.

Ⅵ. REFERENCES

1. 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.
2. Aitken, R.J., Harkiss, D. and Buckingham, D.W. 1993. Analysis of lipid peroxidation mechanisms in human spermatozoa. Mol. Reprod. Dev. 35:302-315.
3. Aitken, R.J., Irvine, D.S. and Wu, F.C. 1991. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. Am. J. Obstet. Gynecol. 164:542-551.
4. Alkan, I., Sims, F. and Haklar, G. 1987. Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: Relationship to seminal plasma antioxidants. J. Urol., 157:140-143.
5. Barros, C., Bedord, J.M., Franklin, L.F. and Austin, C.R. 1967. Membrane vesiculation as a feature of the mammalian acrosome reaction. J. Cell Biol., 34:c1-c5.
6. Clark, R.N. and Johnson, L.A.. 1987. Effect of liquid storage and cryopreservation of boar spermatozoa on acrosomal integrity and the penetration of zona-free hamster ova *in vitro*. Gamete Res., 16:193-294.
7. Dalvit, G.C., Cetica, P.D. and Beconi, M.T. 1998. Effect of α-tocopherol and ascorbic acid on bovine *in vitro* fertilization. Theriogenology, 49:619-627.
8. De Lamirande, E. and Gagnon, C. 1992. Reactive oxygen species and human spermatozoa: I. Effects on the motility of intact spermatozoa and on sperm axonemes. J. Androl. 13:368-378.
9. Grupen, C.G., Nagashima, H. and Nottle, M.B. 1995. Cysteamine enhanced *in vitro* development of porcine oocytes matured and fertilized *in vitro*. Biol. Reprod., 53:173-178.
10. Kameyama, K., Kudoh, O., Takedomi, T. and Fukawa, K. 1997 Transfer of cryopreserved pig embryos. Theriogenology, 47:366 (abstr.).

11. Kodama, H., Kuribayashi, Y. and Gagnon, C.. 1996. Effect of sperm lipid peroxidation on fertilization. Am. Soc. Andro. 17:151-157.
12. Nagai T. 1994. current status and perspectives in IVM-IVF of porcine oocytes. Theriogenology, 41:73-78.
13. Nagai, T. and Moor, R.M.. 1990. Effect of oviduct cells on the incidence of polyspermy in pig. Mol. Reprod. Dev., 26:377-382.
14. Nagai, T., K. Niwa and A. Iritani. 1984. Effect of sperm concentration during preincubation in a defined medium on fertilization *in vitro* of pig follicular oocytes. J. Reprod. Fertil., 70: 271-275.
15. Niwa, K. 1993. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs. J. Reprod., Fertil., 48:49-59.
16. Park, C.K. and M.A. Sirard. 1996. The effect of preincubation of frozen-thawed spermatozoa with oviductal cells on the *in vitro* penetration of porcine oocytes. Theriogenology 46:1181-1189.
17. Suzuki, K., Mori, T. and Shimizu, H. 1994. *in vitro* fertilization of porcine oocytes in the chemistry defined medium. Theriogenology, 42:1457-1468.
18. Wang, W.H., Niwa, K. and Okuda, K.. 1993. *in vitro* penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. J. Reprod. Fertil., 93:491-496.
19. Yoshida, M., Mizoguchi, Y., Ishizaki, K., Kozima, T. and Nagai, T.. 1993. Birth of piglets derived from *in vitro* fertilization of pig oocytes matured *in vitro*. Theriogenology, 39:1303-1311.

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