Analysis of Relationship Between Spermatozoa Ability and Reactive Oxygen Species in Porcine : I. Sperm Preincubation by Xanthine and Xanthine Oxidase

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돼지정자의 수정능력과 Reactive Oxygen Species의 관계분석 I. Xanthine과 Xanthine Oxidase에 의한 정자의 전배양

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Four tables

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= 국문초록 =

본 연구는 xanthine과 xanthine oxidase가 첨가된 배양액내에서 전배양된 돼지동결정액의 수정능력에 있어서 catalase의 영향을 검토하였다. 체외수정을 위한 기본 배양액내에서 0 또는 30분간 전배양한 정자는 catalase를 첨가(40 및 15%)한 경우 보다는 무첨가(66 및 38%)시 유의적으로 높은 정자침입율을 나타냈지만(P<0.05), 배양액내에 xanthine을 첨가해 정자를 0, 30 및 60분간 전배양했을 때에는 catalase 무첨가(33, 41 및 19%) 보다는 첨가시(68, 70 및 49%) 유의적으로 높은 정자침입율이 인정되었다(P<0.05). 그러나 xanthine oxidase를 첨가하여 정자의 전배양을 행하지 않은 경우는 catalase의 첨가(13%) 보다는 무첨가(51%)시 유의적으로 높은 정자침입율을 나타냈지만(P<0.01), 전배양(30 및 60분)후에는 catalase의 존재유무와 정자전배양시간에 관계없이 매우 낮은 정자침입율 (10-21%)을 나타냈다. xanthine과 xanthine oxidase를 동시에 첨가하여 0, 30 및 60분간 정자를 전배양한 경우 catalase의 무첨가(14, 4 및 8%)보다 첨가(75, 55 및 52%)시 유의적으로 높은 정자침입율을 나타냈다(P<0.001). 한편, 다정자침입율은 xanthine, xanthine+xanthine oxidase의 첨가시 정자전배양기간이 길어짐에 따라 감소하였으며, catalase의 첨가보다는 무첨가시 낮은 다정자침입율을 나타냈다. 본 연구의 결과로부터 xanthine과 xanthine oxidase를 동시에 첨가시 catalase의 존재는 정자의 전배양후에도 다정자침입을 억제하면서 수정능력의 유지를 위해 매우 효과적인 것으로 추측되었다.

Key wards : catalase, porcine IVF, sperm preincubation, xanthine, xanthine oxidase

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INTRODUCTION

Spermatozoa acquire the ability to successfully fertilize the oocyte during their transit in the female genital tract. Thus the female reproductive tract must be responsible for supporting motility to extend the fertile life span of sperm in the female reproductive tract by secreting motility factor(s) and/or by reducing sperm metabolism in certain locations to allow better survial. The spermatozoa, like all cells living under aerobic conditions, constantly faces the oxygen paradox. Oxygen is clearly required to support life, but its metabolites modify cell functions and/or can endanger cell survival.

The generation of reactive oxygen species in sperm preparations became a real concern because of the toxic effects they have at high concentrations on sperm functions, and of their possible involvment in made idopathic infertility. Reactive oxygen species producrion in semen has been associated with loss of motility, decreased capacity for sperm-oocyte fusion and loss of fertility (Aitken et al., 1991). High concentrations of reactive oxygen species produced by spermatozoa themselves (Aitken & Clarkson, 1987 ; Alvarez et al., 1987) or by the combinations of xanthine plus xanthine oxidase (Aitken et al., 1993) induce the formation of toxic lipid peroxides (Windsor et al., 1993) and compromise sperm viability. Reactive oxygen species also effect the sperm axoneme as a result of ATP depletion (De Lamirand and Gagnon, 1992), inhibit mitochondrial functions, and synthesis of DNA, RNA and proteins (Comporti, 1989), produce cytoskeletal modifications (Hindshaw et al., 1986) and inhibit sperm-oocyte fusion (Aitken et al., 1993). However, the spermatozoa have enzymatic defence systems such as superoxide dismutase, glutathione peroxidas/reductase and catalase (Griveau et al., 1995) to counteract the toxic effects induced by reactive oxygen species. Although correlations have been reported between the effectiveness of reactive oxygen species and the duration of sperm motility (Alvarez & Storey, 1989 ; De Lamirande & Gagnon, 1997), the importance of their action in vitro has not been fully elucidated.

It is known that fresh sperm have higher fertilization potential than frozen-thaw sperm (Hunter, 1990) and a lower survival rate is possibly the principle explanation for the reduced capacity. Polyspermy refers to the penetration of more than one spermatozoon into the cytoplasm of oocytes (Hunter, 1991), which can result in a polyploid zygote, a condition that results in abnormal embryonic development (Birkhead et al., 1993). Therefore, the present study was undertaken to determine the whether incubation of frozen-thawed boar sperm with xanthine and/or xanthine oxidase in medium with or without catalase would allow in vitro fertilization.

MATERIALS AND METHODS

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 100mg/L and Amphotericin

B 250㎍/L ; Sigma Chemical, St-Louis, MO, USA) at 30 to 32℃. 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 a compact and complete cumulus cells were introduced to droplets of maturation medium (10 oocytes/50㎕ droplet), covered with mineral oil and were 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 3.05mM glucose, 0.32mM Ca-lactate, 2.5mM Hepes (Sigma), 10% fetal calf serum(FCS), 0.2 mM Na-pyruvate(Sigma), 50㎍/ml gentamycin(Sigma), 1㎍/ml FSH(Sigma), 5㎍/ml LH(Sigma), 1㎍/ml estradiol 17β(Sigma) and 10%(v/v) porcine follicular fluid.

Sperm Preparation

Pool of ejaculates from boar were frozen, the straws were thawed by immersion in a 35-37℃ waterbath for 30 seconds. 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, the 2ml semen were 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 by suspension and centrifugation two times 250×g for 10 minutes and resuspended in preincubation medium. After the final wash, the concentration of motile spermatozoa was adjusted to 25×10^6. The medium for fertilization and sperm preincubation was TCM-199 supplemented with 3 mM glucose, 3 mM Ca-lactate, 0.2 mM Na-pyruvate and 10% FCS. The final concentration of spermatozoa was adjusted to 1×10^{6} cells/ml motile spermatozoa during fertilization in vitro.

Experimental Design

In the first experiment, suspensions of spermatozoa were added to a droplet of 50μl fertilization medium with or without catalase (0.1mg/ml, Sigma). Spermatozoa were preincubated for 0, 30 and 60 min then mixed with five oocytes each for fertilization in vitro. In the second experiment, to evaluate the effect of xanthine and catalase on penetration in vitro, spermatozoa were preincubated in 50μl fertilization medium with or without catalase in the presence of xanthine (0.7mg/ml) for 0, 30 and 60 min, then mixed with oocytes. In the third experimental, the effect of catalase on in vitro penetration of spermatozoa preincubated with xanthine oxidase (1mg/ml) for 0, 30 and 60 min was examined. In the final experiment, to evaluated the effects of xanthine plus xanthine oxidase on spermatozoa penetration during in vitro fertilization, spermatozoa were preincubated in fertilization medium with or without catalase in the presence of xanthine plus xanthine oxidase for 0, 30 and 60 min, then mixed with oocytes.

Evaluation of Oocyte Fertilization

At 20-22 h after insemination, the oocytes were mounted, fixed (acetic acid :

ethanol 1:3) for 2-3 days and stained with 1% aceto-orcein in 40% acetic acid water solution. The proportions of penetration and polyspermy were examined with the light microscope at 200 and 400 × magnification. Oocytes were considered as penetrated when spermatozoa with a swollen head or pronuclei were found in the vitellus. Oocytes penetrated by only one spermatozoon were judged to be monospermic oocytes.

Statistics

Chi-square analysis with the Yates correction was used to test the significance of individual comparisons for the rates of penetration and polyspermy.

RESULTS

As shown in Table 1, oocytes were inseminated with spermatozoa preincubated in medium with or without catalase for various duration. The penetrations rates were significantly ($P<0.05$) higher in spermatozoa preincubated without (66 and 38% for 0 and 30 min) than with (40 and 15% for 0 and 30 min) catalase. The proportions of polyspermy had a tendency to decrease as time of sperm preincubation was prolonged. No polyspermy was observed when spermatozoa were preincubated for 60 min.

The oocytes were inseminated with spermatozoa preincubated in medium with or without catalase in the presence of xanthine. Table 2 shows that the penetration rates were significantly ($P<0.05$) higher in spermatozoa preincubated with (68, 70 and 49%) than without (33, 41 and 19%) catalase for 0, 30 and 60 min. The proportions of polyspermy were lower in the absence of catalase regardless of preincubation duration, not significantly different in medium with or without catalase.

As shown in Table 3, oocytes cultured with spermatozoa preincubated with or without catalase in the presence of xanthine oxidase. The penetration rates had a tedency to decrease as time of sperm preincubation was prolonged. When spermatozoa were not preincubated, the penetration rate was significantly ($P<0.01$) higher in medium without catalase(51%) than with catalase(13%). However, Penetration rates in spermatozoa preincubated for 30 and 60 min were not significantly different. On the other hand, polyspermy rates were very low, but not significantly different in medium with or without catalase during fertilization.

In another experiment, oocytes were inseminated with spermatozoa preincubated in medium with xanthine plus xanthine oxidase in the presence or absence of catalase for 0, 30 and 60 min. Table 4 shows that the penetration rates were significantly ($P<0.001$) higher in spermatozoa preincubated with than without catalase for various periods. The proportions of polyspermy were also higher in the presence of catalase at 0, 30 and 60 min (28 vs 0%, 17 vs 0% and 20 vs 0%, respectively), but no differences were observed in durations of spermatozoa preincubation.

DISCUSSION

Capacitation is now considered by most investigators as an event or a series of events preceding the acrosome reaction (Bavister, 1986). To determine the time required for capacitation of mammalian spermatozoa, they are first preincubated in an approproate medium and examined to see whether or not acrosome reaction is induced and penetration has occurred. There have been previous reports of spermatozoa preincubation on fertilization in porcine (Nagai & Moor, 1990 ; Park & Sirard, 1996). The present results indicate that preincubation of frozen-thawed spermatozoa with catalase for 0-60 min (Table 1) is not helpful and results in low penetration rates (10-40%) of porcine oocytes in medium with catalase. Sperm capacitation induced by biological fluids is also prevented by superoxide dismutase (De Lamirande & Gagnon, 1993). Bize et al. (1991) proposed that H_2O_2 is involved in hamster sperm capacitation. Catalase, but not superoxide dismutase, strongly reduces the rate of acrosome reaction of spermatozoa incubated for 5 h in the presence of adrenaline, a substance known to stimulate this process, but also to generate H_2O_2 when kept in aerobic conditions. Furthermore, addition of H_2O_2, either directly or through enzymatic generation by the combination of glucose and glucose oxidase, stimulates the acrosome reaction by 85-150%, depending on the time of observation (Bize et al., 1991). Addition of catalase to human spermatozoa incubated in B2 Menezo medium reduces both the hyperactivation (by 43%) and the A23187-induced acrosome reaction (by 46%) without affecting the percentage of motile or viable cells

(Griveau et al., 1994).

The spontaneous formation of reactive oxygen species by cells present in semen has been associated with reduced sperm motility (Alvarez et al., 1987 ; Iwasaki and Gagnon, 1992)), abnormal sperm morphology (Rao et al., 1989)), decreased sperm-oocytes interaction (Aitken et al., 1989)), reduced fertility in vivo (Aitken et al., 1991). In the present study, when spermatozoa were preincubated and inseminated in medium with xanthine, the rates of sperm penetration decreased in the absence of catalase (Table 2). To the contrary, penetration rates in medium with xanthine oxidase were higher in presence of catalase regardless of duration of spermatozoa preincubation (Table 3). The xanthine plus xanthine oxidase system known to produce reactive oxygen species that are involved in cellular degradation in several cell types, including spermatozoa. In mice, the combination of xanthine plus xanthine oxidase cause a significant increase in sperm hyperactivation and capacitation (De Lamirande et al., 1997). Individually, superoxide dismutase or catalase completely prevent these effects, whereas together they decrease capacitation to rates even lower than those observed in control spermatozoa. These results suggest that, under these conditions, $O_2^{\cdot -}$ and H_2O_2 may be needed to promote mouse sperm hyperactivation and capacitation. However, this study showed that reactive oxygen species can have beneficial effects on sperm functions came from experiments in which spermatozoa that were incubated with xanthine plus xanthine oxidase in the presence of catalase (Table 4). There are probably many different capacitation inducers in the various biological fluids but the data presented in this study suggest that they may all act through a common mechanism, possibly by promoting $O_2^{\cdot-}$ generation at the level of the sperm membrane.

The high incidence of polyspermy in porcine oocytes matured in vitro have been repeatedly reported by many investigators (Wang et al., 1991 ; Suzuki et al., 1994 ; Park & Sirard, 1996). In the present study, the proportions of oocytes penetrated with more than one sperm were low (0-37%) than previous study (0-63%) by spermatozoa preincubated with oviductal cells (Park & Sirard, 1996). Since a high proportion of the oocytes inseminated with spermatozoa preincubated with xanthine plus xanthine oxidase for 0-60 min were polyspermic in the medium with(17-28%) versus without catalase (0%), it is believed that catalase dose have a role for inducing capacitation and penetration of porcine spermatozoa in the presence of xanthine and xanthine oxidase. Park et al. (1997) reported that polyspermy rates were high between in medium with (22-40%) and without (40%) superoxide dismutase, but no difference. In our study, when oocytes were inseminated in the presence of xanthine, although not significantly, the polyspermy rates were below the rates observed without (21, 10 and 0%) the with (37, 20 and 13%) catalase in spermatozoa preincubated for 0, 30 and 60 min. On the other hand, no differences were observed in polyspermy rates in the presence of xanthine oxidase. It seems that catalase can complete advantageously with xanthine plus xanthine oxidase to induce high fertilization rates and moderate polyspermy. This property should allow a further reduction of the number of sperm to be added at fertilization. However, there are no reports to date showing the penetrability of porcine oocytes in medium with xanthine and/or xanthine oxidase. Future studies should be aimed at demonstrating how high polyspermy rates reduce by spermatozoa treated in the presence of reactive oxygen species.

In conclusion, experimental approaches of this study were used to demonstrate the advantage of the preincubation with xanthine plus xanthine oxidase in the presence of catalase to maintain penetration potential with suppress in the polyspermy rates during in vitro fertilization in porcine.

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SUMMARY

The objective of this study was to test the effect of catalase on penetration in vitro by spermatozoa preincubated with xanthine and/or xanthine oxidase. The penetration rates were significantly ($P<0.05$) higher in spermatozoa preincubated without (66 and 38%) than with (40 and 15%) catalase for 0 and 30 min. When spermatozoa were preincubated and inseminated in medium with xanthine, the penetration rates were significantly higher ($P<0.05$) in medium with (68, 70 and 49% for 0, 30 and 60min) than without (33, 41 and 19% for 0, 30 and 60min) catalase. However, in oocytes were inseminated with spermatozoa preincubated with or without

catalase in the presence of xanthine oxidase, no decrease in penetrations rates were observed for up to 60 min of preincubation. In another experiment, the penetration rates were significantly ($P<0.001$) higher in medium with (75, 55 and 52%) than without(14, 4 and 8%) catalase when oocytes were inseminated with spermatozoa preincubated for 0, 30 and 60 min in the presence of xanthine plus xanthine oxidase. On the other hand, The rate of polyspermy in oocytes penetrated in medium without catalase in the presence of xanthine or xanthine plus xanthine oxidase decreased with time of spermatozoa preincubation. However, no differences were observed in polyspermy rates in the medium with xanthine oxidase alone despite presence of catalase. These results indicate the advantages of spermatozoa preincubated with xanthine plus xanthine oxidase in the presence of catalase to increase penetration potential and with suppressed polyspermy in porcine.

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Table 1. Effect of catalase on penetration in vitro by spermatozoa preincubated for various periods in fertilization medium

Periods of spermatozoa preincubation (min)	Presence of catalase (0.1mg/ml)	No. of oocytes examined	No. of oocytes penetrated with Total (%)	enlarged sperm head	both pronuclei	No. of polyspermic oocytes(%)†
0	+	63	25(40)*	25	0	6(24)
	−	67	44(66)	39	3	15(34)
30	+	68	10(15)*	10	0	1(10)
	−	55	21(38)	19	2	2(10)
60	+	87	9(10)	9	0	0(0)
	−	68	14(21)	13	1	0(0)

† Percentage of total number of oocytes penetrated

* $P<0.05$, differences between with and without catalase

Table 2. The role of catalase on penetration in vitro by spermatozoa preincubated for various duration in fertilization medium with xanthine

Periods of spermatozoa preincubation (min)	Presence of catalase (0.1mg/ml)	No. of oocytes examined	No. of oocytes penetrated with			No. of polyspermic oocytes(%)†
			Total (%)	enlarged sperm head	both pronuclei	
0	+	56	38(68)*	31	7	14(37)
	−	43	14(33)	14	0	3(21)
30	+	57	40(70)*	29	11	8(20)
	−	51	21(41)	20	1	2(10)
60	+	49	24(49)*	2	1	3(13)
	−	47	9(19)	7	2	0(0)

† Percentage of total number of oocytes penetraqted

* $P<0.05$, differences between with and without catalase

Table 3. Effect of catalase on penetration in vitro by spermatozoa preincubated for various duration in fertilization medium with xanthine oxidase

Periods of spermatozoa preincubation (min)	Presence of catalase (0.1mg/ml)	No. of oocytes examined	No. of oocytes penetrated with			No. of polyspermic oocytes(%)†
			Total (%)	enlarged sperm head	both pronuclei	
0	+	75	10(13)*	10	0	1(10)
	−	65	33(51)	31	2	4(12)
30	+	79	8(10)	8	0	0(0)
	−	66	14(21)	14	0	1(7)
60	+	83	7(8)	7	0	1(14)
	−	67	12(18)	10	2	1(8)

† Percentage of total number of oocytes penetrated

* P<0.01, differences between with and without catalase

Table 4. Effect of catalase on penetration in vitro by spermatozoa preincubated for various duration in fertilization medium with xanthine plus xanthine oxidase

Periods of spermatozoa preincubation (min)	Presence of catalase (0.1mg/ml)	No. of oocytes examined	No. of oocytes penetrated with			No. of polyspermic oocytes(%)†
			Total (%)	enlarged sperm head	both pronuclei	
0	+	53	40(75)*	29	11	11(28)
	−	57	8(14)	8	0	0(0)
30	+	53	29(55)*	24	5	5(17)
	−	55	2(4)	2	0	0(0)
60	+	57	30(52)*	30	0	6(20)
	−	59	4(8)	4	0	0(0)

† Percentage of total number of oocytes penetrated

* $P<0.001$, differences between with and without catalase