Use of a Xanthine-Xanthine Oxidase System on *in* vitro Maturation and Fertilization in the Pig*

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돼지난자의 체외성숙과 수정에 있어서
Xanthine-Xanthine Oxidase System의 이용

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ABSTRACT

This study was undertaken to evaluate the effects of catalase using xanthine (X)-xanthine oxidase (XO) system on *in vitro* maturation and fertilization in the pig. When follicular oocytes were cultured with X or XO, the maturation rates were not significantly different between in medium with and without catalase despite of different culture periods. However, significantly ($P<0.05$) higher maturation rates were obtained in culture with X-XO-catalase system. The rates of degenerated oocytes were increased with culture periods prolonged, and were significantly ($P<0.05$) higher in medium without that than with catalase at 120 h of culture. On the other hand, the parthenogenetic oocytes were observed with high proportions at 72 h of culture, but were not different between the medium with and without catalase at various times of culture. In another experiment, the frozen-thawed boar spermatozoa treated with X-XO system for *in vitro* fertilization. The penetration rates were higher in medium with that than without catalase during the *in vitro* fertilization with none ($P<0.05$), XO and X+XO. On the other hand, when sperm were treated with none, X, XO and X+XO, lipid peroxidation were produced with higher rates in medium without that than with catalase, and consequently the changes in sperm penetration and lipid peroxidation showed opposite patterns. Under the above all conditions, however, sperm-SH group were higher detected by catalase. When the activity of sperm binding to zona pellucida was evaluated through binding to salt-stored porcine oocytes, sperm binding to zona pellucida in control group were higher than in medium with X, XO and X+XO groups. No significant differences, however, were observed between medium with and without catalase. In conclusion, the exposure of follicular oocytes and spermatozoa to X-XO-catalase system may be caused stimulating *in vitro* maturation and fertilization in the pig.

(Key words ; Catalase, Fertilization, Maturation, Pig, Xanthine-xanthine oxidase)

Ⅰ. INTRODUCTION

In vitro maturation of mammalian oocytes represents a useful approach for the study of preovulatory oocyte development. It has been studied that the culture conditions employed IVM of porcine oocytes can significantly influence IVF rates and subsequent embryonic development. However, the specific mechanism by which various IVM media components affect oocyte maturation *in vitro* remain speculative.

Toxic metabolities of oxygen, including the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH \cdot$), are important mediators of inflammatory tissue injury (Weiss, 1986). Moreover, these highly toxic oxygen metabolites have been found to be the final common mediator of tissue damage in a large number of disparate processes, including inflammation and post-ischaemic re-perfusion injury (Bulkley, 1987). There are therefore striking similarities between many known actions of oxygen-derived free radicals and the events leading to oocyte maturation.

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, 1992ab), the superoxide anion can induce hyperactivation and capacitation, two cellular phenomena that spermatozoa must go through before they can fertilize oocytes (de Lamirande and Gagnon, 1995). In human, the evidence that ROS can have beneficial effects on sperm functions came from experiments in which spermatozoa that were

incubated with X + XO in the presence of catalase demonstrated levels of hyperactivation and capacitation that surpassed those observed with fetal cord serum (Burkman, 1990) or Ham's F-10 medium alone (de lamirande and Gagnon, 1993).

Sperm-oocyte fusion is mediated by fusion-related proteins in the plasma membranes of both the sperm and oocyte (Myles, 1993). The characteristics of these fusion 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; Synder 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 et al., 1994).

In the present study we have investigated these claims using an experimental system involving the exposure of porcine oocytes and sperm for *in vitro* culture to the powerful oxygen metabolities generated by X and/or XO *in vitro*.

Ⅱ. MATERIALS AND METHODS

1. Oocytes Maturation

Porcine ovaries were collected from a local slaughterhouse and kept in saline (NaCl, 0.9% w/v ; penicillin (100,000 IU/L) ; streptomycin (100 mg/L) ; and amphotericin B (250μg/L; Sigma Chemical, St-Louis, MO, USA) at 32 to 37℃. Cumulus-oocytes complexes were aspirated from 1- to 5-mm follicles with a 10-ml syringe with an 18-G needle. Oocytes were washed 3 times in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium. Oocytes with a compact and

complete cumulus were introduced into 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℃. The maturation medium consisted of TCM-199 with Earle's salt (Gibco, Lab., NY, USA) supplemented with 3.05 mM glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes (Sigma), 10% fetal calf serum (FCS), 0.2 mM Na-pyruvate (Sigma), 50 μg/ml gentamycin (Sigma), 1 μg/ml FSH (Sigma), 5 μg/ml LH (Sigma), 1 μg/ml estradiol 17β (Sigma) and 10% (v/v) porcine follicular fluid. To evaluate the effect of catalase (1 unit/ml) on *in vitro* maturation in medium with X (0.5 mM) and/or XO (0.05 U/ml), the follicular immature oocytes were examined for *in vitro* maturation at 48 h of culture. In another experiment, immature oocytes were cultured for 48, 72, 96 and 120 h. At the end of experiment, oocytes freed from cumulus cells were mounted, fixed and stained for observation of maturation status.

2. Determination of Sperm Penetration

Pooled ejaculate from boar were frozen, and the straws were later thawed by immersion in a 37℃ waterbath for 30 sec. 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 were placed over 2 layers of Percoll (65 and 70%) and centrifuged at 2000×g for 15 min at 20℃. The spermatozoa in the 65% Percoll layer were carefully collected, washed in preincubation medium by suspension and centrifugation twice of 250×g for 10 min and resuspended in preincubation medium. After the final wash, the concentration of motile spermatozoa was adjusted to 25×10^6 cells/ml. The fertilization medium 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 sperm cells during fertilization. To evaluate the effect of catalase on *in vitro* penetration, spermatozoa was inseminated in medium with X and/or XO. At 22~24 h after insemination, the oocytes were examined for spermatozoa penetration *in vitro*.

3. 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 X and/or XO 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.

4. Measurement of Sperm Sulfhydryl(-SH) Groups

After washing of frozen-thawed spermatozoa with and without catalase 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), and the absorbance was measured at 405 nm. A

standard curve was generated using different concentrations of dithiothreitol.

5. 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 with and without catalase 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.

6. Statistics

One-way variance analysis was performed to compare the mean values between control and treated groups. Chi-square analysis was used to compare the results of *in vitro* fertilization assay.

Ⅲ. RESULTS

As shown Table 1, the effect of X and/or XO on *in vitro* maturation were examined. When oocytes were cultured in control medium for 48 h, the maturation rate were not significantly different between with (67%) and without (66%) of catalase in control group. However, the proportions of oocytes matured to metaphase-II stage were lower in oocytes cultured with X or XO than control group. The highest maturation rates were obtained by adding of catalase in medium containing X + XO.

In Table 2, effect of catalase during *in vitro*

Table 1. Effect of catalase on *in vitro* maturation of porcine follicular oocytes in medium with xanthine and/or xanthine oxidase

Culture condition	Presence of catalase	No. of oocytes examined	No. of oocytes matured (%)
Control	+	93	62(67)
	−	95	63(66)
Xanthine(X)	+	95	54(57)
	−	92	56(61)
Xanthine oxidase (XO)	+	94	49(52)
	−	91	46(51)
X + XO	+	103	80(78)
	−	93	66(71)

Table 2. Effect of catalase during the *in vitro* culture with various periods in medium with xanthine and/or xanthine oxidase in porcine follicular oocytes

| Periods of culture (h)	Presence of catalase	No. of oocytes examined	No.(%) of			
			GVBD ～ T-I	M-II oocytes	Degenerated oocytes	Parthenogenetic oocytes
48*	+	103	19(18)	80(78)	4(4)	0(0)
	−	93	18(19)	66(71)	9(10)	0(0)
72	+	93	24(26)	34(37)	14(15)	21(23)
	−	92	13(14)	40(44)	12(13)	27(29)
96	+	100	19(19)	43(43)	22(22)	16(16)
	−	102	9(9)	46(45)	25(25)	22(22)
120	+	98	8(8)	20(20)	33(34)†	37(38)†
	−	97	3(3)	21(22)	53(55)	20(21)

† P<0.05, differences between with and without catalase.
* Data quoted from X + XO in Table 1.

culture of porcine follicular oocytes in medium with X and/or XO were examined for oocyte maturation status. The maturation rates were not significantly different between in medium with and without catalase despite of different culture periods. The rates of degenerated oocytes were increased with culture periods prolonged, and were significantly (P<0.05) higher in medium without that than with catalase at 120 h of culture. On the other hand, the parthenogenetic oocytes were observed with high proportions (16 to 29% for medium with and without catalase) at 72 and 96 h of culture, and were significantly difference (P<0.05) in medium with that and without catalase at 120 h of culture.

To investigate the effect of catalase on *in vitro* fertilization, spermatozoa were inseminated in medium with X and/or XO. The penetration rates in all conditions were higher in medium with that than without catalase (Table 3). There were significant (P<0.05) differences in the penetration rates between with (54%) and without (44%) catalase in medium containing X. Furthermore, the penetrations rates were highest in medium with X + XO despite of presence of catalase. Polyspermy occurs with a similar pattern to that of the sperm penetration rate. There were significantly (P<0.05) higher with

that than without catalase in medium with none, X or X + XO.

Lipid peroxidation of sperm was evaluated on the basis of MDA production. The MDA were higher in sperm treated without that than with catalase in medium with none, X or XO (Fig. 1). In spermatozoa treated with X-XO, however, MDA production was increased by catalase. On the other hand, sperm-SH group were higher detected in medium with that than without catalase under the above all conditions (Fig. 2). The activity of sperm binding to zona pellucida was also evaluated through binding to salt-stored porcine oocytes (Fig. 3).
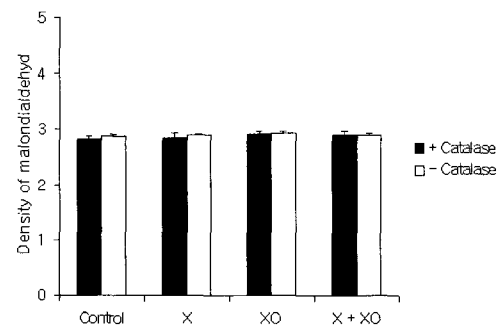

Fig. 1. Effect of catalase on lipid peroxidation of boar spermatozoa treated with X and/or XO.

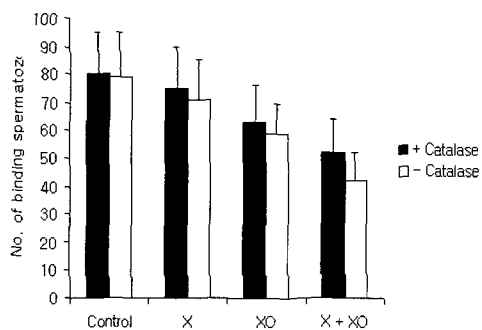Table 3. Effect of catalase on boar spermatozoa penetration *in vitro* in medium with xanthine and/or xanthine oxidase

Culture condition for IVF	Presence of catalase	No. of oocytes examined	No. of oocytes with			No. of polyspermic oocytes (%)
			Total(%)	ESH	BPN(%)	
None	+	197	118(60)	83	35(30)	57(48)†
	−	182	100(55)	72	28(28)	40(40)
X	+	179	96(54)†	58	38(40)	45(47)†
	−	192	84(44)	60	24(29)	28(33)
XO	+	186	106(57)	68	38(36)	56(53)†
	−	185	93(50)	54	39(42)	45(38)
X+XO	+	189	135(71)	82	53(39)	74(55)
	−	202	145(69)	93	52(36)	55(52)

† P<0.05, difference between with and without catalase.
ESH : enlarged sperm head, BPN : both pronuclei.
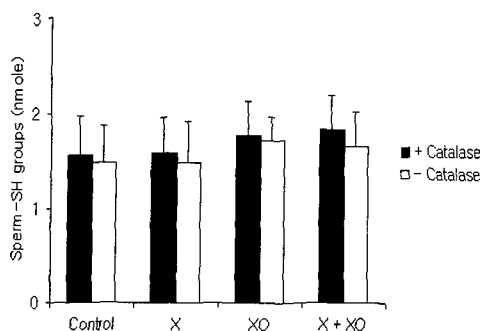
Fig. 2. Effect of catalase on sulfhydryl (-SH) groups of boar spermatozoa treated with X and/or XO.

Fig. 3. Effect of catalase on zona pellucida binding of boar spermatozoa in medium with X and/or XO.

In control group, sperm binding to zona pellucida were higher than in medium with X, XO and X+XO groups. No significant differences, however, were observed between medium with and without catalase.

IV. DISCUSSION

The present experiments were undertaken to examine effects of X and/or XO on *in vitro* maturation in porcine oocytes. The presence of catalase investigated protects oocytes against free oxygen radicals damage for $48 \sim 120$ h after culture. The results of this study show that porcine oocytes

matured with higher proportions in X + XO group than control, X or XO groups. There were not different by addition of catalase during *in vitro* maturation. It is suggested that oocyte maturation is not protected from the oxidative stress under conditions with catalase in medium with X and/or XO. The X-XO system is known to produce reactive oxygen species that are involved in cellular degradation in several cell types. In facts, all the effects of the X-XO system on the enzymatic antioxidant defence systems can be inhibited by the presence of catalase.

In this study, parthenogenetic oocytes were observed at 72 h after culture in medium with X-XO. Kikuchi et al. (1995) reported that when pig oocytes were electrically stimulated, parthenogenetic activation was low during the early stages of maturation, activation increased after 60 h of culture. A similar result was observed in the parthenogenetic activation of mouse (Kubiak, 1989) and cattle (Nagai, 1987) oocytes aged *in vitro*. In this study, when oocytes were cultured in medium with X-XO for 120 h, parthenogenetic oocytes were significantly differences between in medium with and without catalase.

In cattle, prolonged maturation *in vitro* enhances parthenogenetic activation (Nagai, 1987) and ageing results in abnormal cleavage after fertilization *in vitro* (Chian et al., 1992). In pigs, abnormalities of development have been reported in oocytes aged in vivo (Hunter, 1967) and *in vitro* (Sato et al., 1979). These reports indicate a decrease in the ability to be fertilized, poor developmental ability and enhanced cytoplasmic ability for oocyte activation in aged mammalian.

A combination of X and XO, an enzymatic free radical-generating system, primarily generates the superoxide anion, which is immediately dismuted into hydrogen peroxide (Ikeda et al., 1999). In this study, when oocytes were cultured for up to $96 \sim$

120 h and then treated with X and XO, high rates of cultured oocytes had female pronuclei. These seemed to result from a normal oocyte activation process as when fertilization occurs, since the female pronucleus was well developed. However, abnormal activation, resulting in fragmentation, after culture without catalase increased when the duration of maturation was prolonged.

The mode of action of ROS on sperm axonemes and motility can be studied with the use of the combination X+XO at concentrations sufficiently low not to decrease sperm viability. Washed motile human spermatozoa treated with X+XO completely lose motility, but only 3~4 h after the beginning of treatment, even though ROS are generated and then destroyed during the first hour (Chian et al., 1992; de Lamirande and Gagnon, 1992a). In this study, however, the percentage of sperm penetration were increased by X + XO. The evidence that ROS can also beneficial effects on sperm functions came from experiments in which spermatozoa that were incubated with X+XO in the presence of catalase demonstrated levels of hyperactivation and capacitation that surpassed those observed with fetal cord serum (Burkman, 1990), or Ham's F-10 medium alone (de Lamirande and Gagnon, 1993).

In addition to its effects on sperm capacity *in vitro*, lipid peroxidation has been detected to decrease by catalase 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. These results are in agreement with those previous studies in rabbit (Alvarez and Storey, 1982), rats (Fornes et al., 1993), and human (Hong et al., 1994). 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 specifically induced SH-groups, and its effect was increased by catalase. Griveau et al. (1995) reported that the acrosome reaction in human spermatozoa appeared most susceptible to ROS and that H_2O_2 inactivated several enzymatic activities : glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate. In our experiment, the frozen-thawed spermatozoa were used, and the inhibition of sperm binding to zona pellucida was decreased by X and/or XO despite of promoting by catalase in sperm-zona binding. Thus, the promoting of sperm penetration was not attributable to sperm binding.

In summary, the present study suggests that X-XO + catalase system cause an enhancement on *in vitro* oocyte maturation. These increases are also 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. 요 약

본 연구는 xanthine(X)-xanthine oxidase(XO) system하에서 돼지 난자의 체외성숙과 체외수정에 대한 catalase의 영향을 검토하였다. 그 결과 돼지 난포난자가 X 또는 XO하에서 배양되었을 때, 난

포난자의 성숙율은 다른 배양시간에도 불구하고 catalase 첨가 유무에 따른 유의적인 차이를 나타내지 않았다. 그렇지만, X-XO-catalase system하에서 배양한 경우 유의적으로 높은 성숙율을 얻었다 (P<0.05). 퇴행난자의 비율은 배양기간이 늘어남에 따라 증가되었으며, 배양 120시간에서는 catalase 첨가시보다 무첨가시에 유의적으로 높았다. 다른 한편으로, 단위발생 난자들이 배양 72시간에 높은 비율로 관찰되었지만, 다양한 배양시간에서 catalase 첨가유무에 따른 차이는 발견되지 않았다. 또 다른 실험에서, 동결-융해된 돼지 정자가 체외수정을 위해 X-XO system으로 처리되었다. 난자투명대에 대한 정자침입율은 none (P<0.05), XO, X+XO하에서 체외수정시 catalase 무첨가시보다 첨가시에 높게 나타났다. 다른 한편으로, 돼지정자가 none, X, XO, X+XO로 처리되었을 때, lipid peroxidation은 catalase 첨가시보다 무첨가시에 높은 비율로 나타났으며, 그 결과 정자침입과 lipid peroxidation에서의 변화가 상반되는 양상을 보였다. 그렇지만, 모든 조건하에서 정자의 sulfhydryl (-SH) group의 함량은 catalase 첨가시에 높게 측정되었다. 난자의 투명대에 대한 정자의 접착 정도는 salt-stored 돼지 난자에 대한 정자접착을 통해서 평가되었으며, control group의 경우 X, XO, X+XO group에 비해 높은 정자접착율이 관찰되었다. 그렇지만, catalase 첨가유무에 따른 유의적인 차이는 인정되지 않았다. 본 연구의 결과는 X-XO-catalase system에 대한 난포난자와 정자의 노출이 돼지에서의 체외성숙과 체외수정을 촉진시키는 것으로 생각된다.

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