Effects of Cumulus Cells and Reactive Oxygen Species (ROS) on Plasminogen Activator Activity during *In Vitro* Maturation of Porcine Oocytes

Soo-Jin Sa[1,*], Chun-Keun Park[2], In-Cheul Kim[1], Seung-Hoon Lee[1], Oh-Sub Kwon[1], Myung-Jick Kim[1], Kyu-Ho Cho[1], Du-Wan Kim[1], Kyoung-Min So[1], Hee-Tae Cheong[3] and Bob Webb[4]

[1]*Swine Science Division, National Institute of Animal Science, RDA, Cheonan 330-801, Korea*
[2]*College of Animal Life Science, Kangwon National University, Chuncheon 200-701, Korea*
[3]*Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea*
[4]*School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK*

ABSTRACT

Plasminogen activators (PAs) are serine proteases that convert plasminogen to plasmin. The PA/plasmin system has been associated with a number of physiological processes such as fibrinolysis, ovulation and fertilization. Although correlations have been reported between reactive oxygen species (ROS) and oocyte maturation, the relationship between PA activity and ROS is unknown. The present study was undertaken to determine the effects of cumulus cells on PA activity in matured porcine oocytes under xanthine (X)-xanthine oxidase (XO) system. When oocytes were matured under the X-XO system, the proportion of oocytes remaining GV stage was higher ($p<0.05$) in oocytes without cumulus cells. The incidence of degenerated oocytes was higher ($p<0.05$) in the X+XO (11.1±6.1 and 21.6±3.4%) than in the control group (2.9±1.8 and 4.0±1.6%). The proportion of TUNEL-positive oocytes and activity of caspase-3 were higher ($p<0.05$) in cumulus-free oocytes and oocytes exposed to ROS. Tissue-type plasminogen activator-plasminogen activator inhibitor (tPA-PAI) and tissue-type plasminogen activator (tPA) activity were detected in oocytes that were separated from cumulus-oocytes complexs (COCs) at 44 h of maturation culture, and only tPA was produced in oocytes that were denuded before the onset of maturation culture. On the other hand, the activities of PA were increased ($p<0.05$) when oocytes were cultured under the X-XO system. The higher activity of tPA was observed in denuded oocytes (DOs) underwent apoptotic changes by oxidative stress. In COCs, however, tPA-PAI as well as tPA activity was detected and apoptotic changes such as DNA cleavage or caspase-3 activation were not observed. These results suggest that tPA may be relevant to apoptotic cell death in porcine oocytes by oxidative stress.

(Key words : plasminogen activators (PAs), reactive oxygen species (ROS), cumulus cell, oocyte maturation, pig)

INTRODUCTION

The processes of cytoplasmic maturation as well as nuclear maturation in mammalian oocytes are prerequisites for normal fertilization and subsequent embryonic development (Thibault, 1977; Armstrong, 1991). Niwa (1993) also reported that the deficiency of cytoplasmic maturation during IVM is reflected in an oocyte's low ability to form a male pronucleus (MPN) and to develop to the blastocyst stage after *in vitro* insemination. In mammalian, immature oocytes were commonly matured under higher (20%) concentrations of O_2 than that those that matured *in vivo* and, therefore, result in increased accumulation of reactive oxygen species (ROS) in the cytoplasm of developing embryos (Luvoni *et al.*, 1996). The ROS, toxic metabolites of oxygen, including the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxidase (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) are important mediators of inflammatory tissue injury (Weiss, 1986) and can damage cell membranes (Aitken *et al.*, 1989) and DNA (Halliwell and Aruoma, 1991) and may play a role in apoptosis (Yang *et al.*, 1998). Moreover, these ROS 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 action of oxygen-derived free radicals and the events leading to oocyte maturation. Gutteridge and Halliwell (1988) reported that low levels of ROS may act as "trigger" molecules. In bovine, the ROS produced with the hypoxanthine-xanthine oxidase system

† This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-352-F00015).
* Correspondence : E-mail : soojinsa@korea.kr

plays a role in the induction of oocyte nuclear and/or cytoplasmic maturation (Patrick *et al.*, 1997). Hideki *et al.* (2000) also reported that COCs have less sensitivity to apoptotic signals triggered by oxidative stress during *in vitro* maturation.

Oxidative stress is determined by the balance between the generation and degradation of ROS within a tissue. In initial studies, many investigators have used indirect means to characterize free radical mechanisms in physiological processes. An indirect approach frequently employed is the use of the highly specific inhibitor of free radicals, such as superoxide dismutase (SOD), catalase and β-mercaptoethanol (β-ME). SOD is the initial enzyme that induces the conversion of the $O_2^{\cdot -}$ to H_2O_2 which in turn is removed by catalase and glutathione peroxidase (Meister, 1983). Addition of these antioxidants during culture of mammalian embryos improved embryonic development with up-regulation of glutathione (GSH) synthesis controlling the redox environment (Caamano *et al.*, 1998; Iwata *et al.*, 1999; Orsi and Leese, 2001).

Plasminogen activators are serine protease that cleave plasminogen to form the active protease plasmin. Mammalian have two forms of plasminogen activator, tissue (tPA) and urokinase types (uPA) (Dano *et al.*, 1985). Although these PAs are believed to be important in extracellular tissue remodeling in many physiological process, including fibrinolysis, ovulation, mammary involution, implantation (Dano *et al.*, 1985), and fertilization (Huarte *et al.*, 1993) and pathological process, including cardiovascular diseases (Sowers, 1998) and tumor metastasis (Schmitt *et al.*, 1997), the unrestrained generation of plasmin from plasminogen by the action of PA is potentially hazardous to cells (Irigoyen *et al.*, 1999). The pericellular activation of plasminogen is a powerful proteolytic pathway able to trigger apoptosis (Rossignol *et al.*, 2004). Also, Kwaan *et al* (2000) reported that addition of plasminogen activator inhibitor-1 (PAI-1), a known inhibitor of PAs, to culture media inhibits spontaneous and induced apoptosis of multiple cell lines. Although correlations have been reported between PA production and oocyte maturation, the relationship between apoptotic cell death and PAs activity during *in vitro* maturation has not been elucidated in porcine oocytes.

Therefore, the present study was conducted to determine the effect of cumulus cells on plasminogen activator activity in matured porcine oocytes under X-XO system. After treatment of cumulus-oocytes complexes (COCs) and denuded oocytes (DOs) with X-XO system for 44 h, meiotic maturation, DNA cleavage, caspase-3 activity and PA production in oocytes were determined in each experimental group.

MATERIALS AND METHODS

1. Culture Media

All chemicals used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA) unless otherwise stated. The medium used for oocyte maturation was BSA-free North Carolina State University-23 (NCSU-23; Petters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.6 mM cysteine, 10 IU/ml human chorionic gonadotropin (hCG) and 10 IU/ml pregnant mare's serum gonadotropin (PMSG).

2. Preparation of Oocytes

Porcine ovaries were collected at a local slaughterhouse and kept in saline (NaCl, 0.9% w/v ; penicillin 100,000 IU/l ; streptomycin 100 mg/l and amphotericin B 250 μg/l) at 30 to 32℃. Cumulus-oocytes complexes (COCs) were aspirated from 2 to 6 mm follicles with a 10 ml syringe with 18-gauge needle. COCs with uniform ooplasm and a compact cumulus cell mass were prepared in Hepes-buffered TALP medium containing 0.1 (v/v) polyvinylalchol (H-TL-PVA). The collected oocytes were washed three times with a specified maturation medium and each group of 10 COCs were matured in 50 μl drop of BSA-free NCSU-23 (NCSU-23; Petters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.6 mM cysteine, 10 IU/ml human chorionic gonadotropin (hCG; Sigma) and 10 IU/ml pregnant mare's serum gonadotropin (PMSG; Sigma) under mineral oil. After culture for 22 h, oocytes were washed three times and then cultured in maturation medium without hormones for another 22 h at 39℃, 5% CO_2, in air.

3. Generation of Reactive Oxygen Species (ROS)

Reactive oxygen species were generated by the xanthine-xanthine oxydase (X-XO) system described by McCord and Fridovich (1968). Xanthine oxidase catalyzes the univalent and divalent reduction of ground-state oxygen to generate both $O_2^{\cdot -}$ and H_2O_2 with the oxidation of xanthine to uric acid. Treatments in present experiment were done as follows: 1) oocytes alone, 2) oocytes+X (0.5 mM)+XO (0.05 U/ml).

4. Experimental Design

To evaluate the relationship between PA production and apoptotic changes by oxidative stress during *in vitro* matura-

tion in the porcine oocytes, The COCs and DOs were matured in maturation medium (NCSU-23) containing xanthine (X, 0.5 mM) and xanthine oxidase (XO, 0.05 U/ml). Cumulus cells were removed from COCs at 44 h after or before the onset of maturation culture. To determine the effect of cumulus cells on PA production in oocytes during *in vitro* maturation, oocytes were cultured with or without cumulus cells for 44 h in maturation medium (NCSU-23). After maturation culture, 40 COCs and DOs, which were denuded at 44 h after or before maturation culture, were sampled for determination of PA activity, respectively.

5. SDS-PAGE and Zymography

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and zymography were performed using procedures described by Dyk and Menino (1991) with a slight modification. Frozen samples were thawed and homogenized with a sonicator. As a standard of tPA, stock solution of 0.5 ng/ml tPA from human melanoma cell culture (Sigma) was prepared in sample buffer. Each homogenized sample (20 μl) being compared in each experiment, a stock solution of human tPA (7 μl), and molecular mass markers (10 μl; Bio-Rad Lab., Hercules, CA) were placed in a castellated well in a 4.5% stacking gel with a 12.5% separating gel. Electrophoresis was conducted at 20 mA for 2 h.

After electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 for 45 min, rinsed with distilled water three times, and incubated for 30 min at 39℃ in phosphate-buffered saline (PBS). Each gel was carefully laid on a casein-agar gel (zymogram) containing purified human plasminogen supported in a plastic chamber. For preparing zymograms, 0.4 g of nonfat dry milk was dissolved in 10 ml of buffer containing 0.0013 M $CaCl_2 \cdot 2H_2O$, 0.1 M glycine, 0.038 M Tris, and 0.005 M sodium azide. The nonfat dry milk mixture were heated to 55℃ and combined with 9.9 ml of 1% (v/v) melted agarose maintained at 55℃. Purified human plasminogen stock solution (100 μl) was added to 19.9 ml of the warmed mixture to yield a final plasminogen concentration of 50 μg/ml and 10 ml of this mixture were cast into a warmed dish (100×15 mm; Falcon 1012; Becton and Dickinson) and allowed to cool. Zymograms containing 0 μg/ml plasminogen were used for detection of any nonspecific proteolytic activity. Polyacrylamide gels and zymograms were incubated at 39℃ for 24～48 h to allow the development of lytic bands. After the distance from the edge of the separating gel to the center of the clear lytic bands in each lane was measured, incubation of zymograms was terminated by separating the gels. Then the zymograms were fixed with 3% (v/v) acetic acid for 10 min, rinsed under tap water.

All experiments measuring PA activity were repeated at least three times. Protease activity was quantified by densitometric scanning of the zymograph using NIH Image 1.62 (Center for information Technology National Institutes of Health, Maryland, USA). PA activities were expressed relative to the activity in a fixed sample, which was different in each experiment.

6. Assessment of Meiotic Maturation

After maturation with X-XO system, oocytes were freed from cumulus cells by washing three times of Hepes-buffered TALP medium containing 0.1 (v/v) polyvinylalcohol (H-TL-PVA) with a small-bore pipette. Groups of 30～40 oocytes were then transferred to the center of glass slide with four spots of vaseline and paraffin (9:1), gently compressed with a cover slide, immersed in 25% (v/v) acetic acid in ethanol for 2～3 days for complete fixations and stained with 1% (w/v) orcein in 45% (v/v) acetic acid. The maturation stages of oocytes were examined under a phase-contrast microscope at a magnification of ×200 or ×400. The oocytes were classified according to chromatin configuration as germinal vesicle (GV), condensed chromatin (CC), metaphase Ⅰ (MⅠ) and metaphase Ⅱ (MⅡ).

7. Analysis of DNA Cleavage

The COCs and DOs cultured with X-XO system were transferred into 0.1% (w/v) protease solution in TL-Hepes-PVA at room temperature to remove the zona pellucida, and washed quickly in PBS (Gibco BRL, Grand Island, NY, USA) containing 3 mg/ml polyvinylalcohol (PBS-PVA) and fixed overnight at 4℃ in 4% (w/v) paraformaldehyde diluted in PBS. After fixation, the oocytes were washed three times in PBS-PVA and incubated for 1 h in PBS containing 0.5% (v/v) Triton X-100. Oocytes were then washed three times in PBS-PVA and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagents; *In situ* apoptosis detection kit; Roche Applied Science, Penzberg, Germany) at 38.5℃ for 1 h in the dark. After TUNEL, oocytes were washed three times in PBS-PVA and counterstained with 25 μg/ml bis-benzamide (Hoechst 33342; Sigma) for 30 min. The oocytes were then washed three times in PBS-PVA and mounted between a coverslip and a nonfluorescence glass slide supported by four spots of vaseline and paraffin (9:1). The slides were sealed with clear nail polish and labelled oocytes were examined using an Olympus BHS micro-

scope equipped with phase-contrast and epifluorescent optic. Two standard filter sets were use: (1) a filter with an excitation wavelength of 520~560 nm and a barrier filter of 580 nm to detect the DNA strand breaks by Terminal deoxynucleotidyl transferase (TdT); and (2) a filter with an exitation wavelength of 330~380 nm and a barrier filter of 420 nm to detect the nuclear status of oocytes stained by Hoechst 33342.

8. Assay of Caspase-3 Activity

After maturation under X-XO system, oocytes were washed three times in TL-Hepes-PVA, and groups of 40 oocytes were put into 0.6 ml microfuge tubes containing 20 μl STKM buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ and 0.25% Triton X-100), and lysed for 15 min on ice. Cell lysates were stored at −20℃ until assay. Caspase-3 activity was colorimetrically measured using Ac-Asp-Glu-Val-Asp 4-methyl-coumaryl-7-amide (Ac-DEVD-MCA) (Peptide Institute Inc., Osaka, Japan) as a substrate. The assay was performed by incubating 20 μl of cell lysates with 178 μl of reaction buffer (100 mM Hepes, pH 7.5, 20% [v/v] glycerol, 5 mM dithiothreitol, 0.5 mM EDTA) and 2 μl of 10 mM substrate at 37℃ for 2 h. Release of 7-amino-4-methyl- coumarin (AMC) from Ac-DEVD-MCA by the enzyme reaction was spectrophotometrically monitored at 370 nm with a UV spectrophotometer (Perkin Elmer, USA) and its quantity determined based on its standard curve.

9. Statistics

Data were analyzed by ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). When *F*-test results were significant in ANOVA, individual data were further tested by Duncan's multiple-range test. Differences with values of $p<0.05$ were considered to be statistically significant. Data are presented as mean±standard error of the mean (SEM).

RESULTS

This study was shown that the effect of cumulus cells on meiotic maturation of matured porcine oocytes under X-XO system (Table 1). The proportion of oocytes remaining GV stage was significantly higher ($p<0.05$) in oocytes without cumulus cells and the maturation rates of oocytes with cumulus cells were significantly higher ($p<0.05$) than in oocytes without cumulus cells regardless of the addition of X and XO. The incidence of degenerated oocytes was significantly higher ($p< 0.05$) in the X+XO (11.1±6.1 and 21.6±3.4%) than in the control group (2.9±1.8 and 4.0±1.6%). The proportion of TUNEL-positive oocytes and activity of caspase-3 were significantly higher ($p<0.05$) in cumulus-free oocytes and oocytes exposed to ROS (Table 2 and 3). The activities of tPA-PAI and tPA were detected in oocytes that were separated from porcine COCs at 44 h of maturation culture, and only tPA was produced in oocytes that were denuded before the onset of maturation culture. On the other hand, the activities of PA were significantly increased ($p<0.05$) when porcine oocytes were cultured under the X-XO system (Fig. 1).

DISCUSSION

Although type of PAs detected in COCs seemed to be different according to the different species, the increase of PAs

Table 1. Effect of cumulus cells on *in vitro* maturation in medium with xanthine (X) and xanthine oxidase (XO)

Culture condition for IVM	Presence of cumulus cells	No. of oocytes examined	No. (%) of oocytes matured with				No. (%) of oocytes degenerated
			GV	CC	M-I	M-Ⅱ	
Control	+	151	5 (3.1±1.6)^{a}	6 (3.8±1.5)^{a}	13 (9.2±5.2)^{a}	123 (81.0±3.8)^{a}	4 (2.9±1.8)^{a}
	−	145	27 (18.8±3.0)^{c}	7 (5.2±4.1)^{a}	23 (15.9±5.6)^{a}	82 (56.1±4.9)^{b}	6 (4.0±1.6)^{a}
X+XO	+	144	10 (6.9±2.9)^{ab}	6 (4.3±2.2)^{a}	18 (12.0±4.4)^{a}	95 (65.7±4.9)^{b}	15 (11.1±6.1)^{b}
	−	143	48 (32.6±9.4)^{d}	8 (5.4±3.2)^{a}	18 (12.6±3.3)^{a}	40 (27.7±4.7)^{c}	32 (21.6±3.4)^{c}

X, xanthine; XO, xanthine oxidase; GV, germinal vesicle; CC, condensed chromatin; M-Ⅰ, metaphase I; M-Ⅱ, metaphase-Ⅱ. Cumulus cells were removed from COCs at 44 h after or before the onset of maturation culture and the data are expressed as mean±SEM of five replicates. $^{a\sim d}$ Value with different superscript letters in the same column differ significantly ($p<0.05$).

Table 2. Effect of cumulus cells on DNA fragmentation in porcine oocytes matured in medium with xanthine (X) and xanthine oxidase (XO)

Culture condition for IVM	Presence of cumulus cells	No. of oocytes examined	TUNEL-positive oocytes (%)
Control	+	145	12 (8.5±3.5)[a]
	−	147	42 (28.7±4.5)[b]
X+XO	+	146	21 (14.3±4.1)[a]
	−	144	103 (71.5±5.2)[c]

Cumulus cells were removed from COCs at 44 h after or before the onset of maturation culture. The results are expressed as mean±SEM of five replicates. [a~c] Value with different superscripts are significantly different ($p<0.05$).

Table 3. Effect of cumulus cells on caspase-3 activity of porcine oocytes matured in medium with xanthine(X) and xanthine oxidase(XO)

Culture condition for IVM	Presence of cumulus cells	AMC released (pmol/oocyte)
Control	+	46.7±4.6[a]
	−	56.8±4.8[b]
X+XO	+	49.9±5.3[a]
	−	77.5±3.8[c]

Cumulus cells were removed from COCs at 44 h after or before the onset of maturation culture. For the analysis, 60 cumulus-free oocytes were used. The results are expressed as mean±SEM of five replicates. [a~c] Value with different superscripts are significantly different ($p<0.05$).

activity during maturation *in vitro* or *in vivo* has been reported in pig (Kim and Menino, 1995) and rat (Liu and Hsueh, 1987). Huarte *et al.* (1985) reported that tPA, but not tPA-PAI and uPA, activity was increased in cumulus-free mouse oocytes during maturation *in vitro*. In rats and mice, oocyte accumulates tPA mRNA during the maturation process and translation of this mRNA is triggered upon resumption of meiotic maturation (Huarte *et al.*, 1985; 1987). In our previous study, we reported that activities of tPA-PAI, tPA and uPA were observed in porcine oocytes (COCs) that were attached with cumulus cells during maturation *in vitro* (Ann *et al.*, 2006). However, when porcine oocytes were separated from COCs at 44 h of maturation culture, tPA-PAI and tPA, but not uPA,

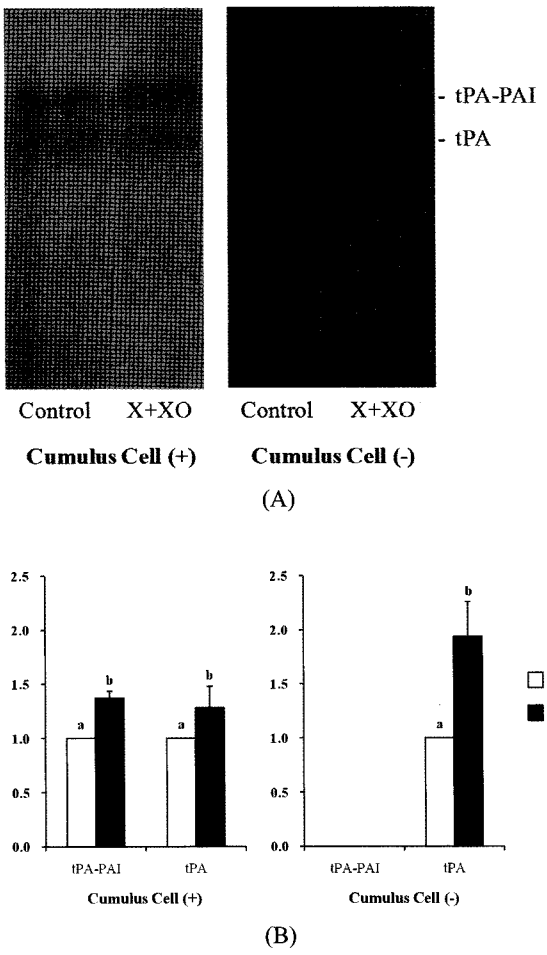

Fig. 1. Effect of cumulus cells on PA activities of porcine oocytes matured in medium with xanthine(X) and xanthine oxidase (OX). (A) Zymographic analysis of porcine oocytes matured in medium with X+XO. (B) Intensity of PAs activity was quantified by densitometric scanning of zymography and expressed relative to the activity in the control group for tPA-PAI and tPA. Values obtained from three replicates are expressed as mean±SEM. [a,b] Value with different letters are significantly different ($p<0.05$). Cumulus cells were removed from COCs at 44 h after or before the onset of maturation culture. For the analysis, 40 cumulus-free oocytes were used.

activities were detected in cumulus-free oocytes. Also, only tPA was produced in pig denuded oocytes that were matured *in vitro* without cumulus cells. In the present study, uPA, but not tPA-PAI and tPA, activity was not detected in porcine oocytes when cumulus cells were removed from COCs after maturation, suggesting that uPA may be produced by cumulus cells during maturation *in vitro*. Also, the presence of cumulus cells during maturation *in vitro* of oocytes may be relevant in

production of tPA-PAI in porcine oocytes because activity of tPA-PAI was not detected in DOs that were matured without cumulus cells.

PAs are serine protease that cleaves plasminogen to form the active protease plasmin. Although these enzymes are believed to be important in extracellular tissue remodeling in many physiological and pathologic process (Stricland *et al.*, 1976; Ossowski *et al.*, 1979; Dano *et al.*, 1985; Sappino *et al.*, 1989), the unrestrained generation of plasmin by plasminogen activation is potentially hazardous to cells. Thus, the process of plasminogen activation is strictly controlled through the availability of PAs, localized activation, and interaction with specific inhibitor (PA inhibitors) (Irigoyen *et al.*, 1999). Results of this study showed that addition of xanthine and xanthine oxidase to maturation medium increased the proportion of oocytes remaining GV stage and degenerated oocytes. The oocytes cultured without cumulus cells were more sensitive to oxidative stress, and the incidence of degeneration, DNA cleavage, activation of caspase-3 were significantly increased in DOs exposed to ROS generated by the X-XO system. The higher activity of tPA was observed in porcine DOs exposed to ROS compared with those of oocytes cultured without X+XO. In COCs exposed to ROS, the increased activity of tPA-PAI as well as tPA was observed. The maturation rate was reduced but apoptotic changes such as DNA cleavage and caspase-3 activation were not observed in COCs. These results indicate that the enhanced activity of tPA may be associated with apoptosis triggered by oxidative stress during porcine oocytes maturation periods. Rossignol *et al.* (2004) have demonstrated that pericellular activation of plasminogen is a powerful proteolytic pathway able to trigger apoptosis and that protease nexin-1, a potent serine proteinase inhibitor, inhibited the activity of plasmin and tPA via the formation of inhibitory complexes and prevented cell detachment and apoptosis. PA inhibitor type-1 (PAI-1), a known inhibitor of plasminogen activators may play a significant role in regulating plasmin formation at the cell surface (Lee *et al.*, 1996) and increased expression of PAI-1 is associated with decreased apoptosis of neoplastic cells (Chen *et al.*, 2004). Recent observations suggest apoptosis and expression of PAI-1 may be linked through an interaction between caspase-3 and PAI-1. Activation of caspase- 3 and induction of apoptosis of neurons were seen when cells were cultured in media deficient in PAI-1 (Soeda *et al.*, 2001). Chen *et al.* (2004) reported that PAI-1 forms a high affinity complex with caspase-3 and thereby inhibits the functional activity of caspase-3.

In summary, the present study provides information concerning the effect of cumulus cells on maturation *in vitro* and PA activity in cultured porcine oocytes under the X-XO system. The proportion of oocytes remaining GV stage, oocytes degenerated, DNA cleavage and the activity of caspase-3 were increased in the DOs exposed to ROS generated by the xanthine-xanthine oxidase system, which resulted in apoptotic cell death. The higher activity of tPA were observed in porcine DOs underwent apoptotic changes by oxidative stress. In COCs, however, tPA-PAI complex as well as tPA activity was detected and apoptotic changes such as DNA cleavage or caspase-3 activation were not observed. These results suggest that free radical may induce the PA activity in porcine oocytes during *in vitro* maturation and PA activity may be relevant to apoptotic cell death in porcine oocytes by oxidative stress.

REFERENCES

Aitken RJ, Clarkson JS and Fishel S. 1989. Generation of reaction oxygen species, lipid peroxidation and human sperm function. Biol. Reprod. 40:183-197.

Ann JY, Sa SJ, Cao Y, Lee SY, Cheong HT, Yang BK and Park CK. 2006. Effects of cumulus cells and follicular fluid on plasminogen activator activity during *in vitro* maturation of porcine oocytes. Reprod. Dev. Biol. 30:135-141.

Armstrong DT, Zhang X, Vanderhyden BC and Khamsi F. 1991. Hormonal actions during oocyte maturation influence fertilization and early embryonic development. Ann. NY. Acad. Sci. 626:137-158.

Bulkely GB. 1987. Free radical-mediated reperfusion injury: A selective review. Br. J. Cancer (Suppl) 5:66-73.

Caamano JN, Ryoo ZY and Youngs CR. 1998. Promotion of development of bovine embryos produced *in vitro* by addition of cysteine and beta-mercaptoethanol to a chemically defined culture system. J. Dariy Sci. 81:369-374.

Chen Y, Kelm RJ, Budd RC, Sobel BE and Schneider DJ. 2004. Inhibition of apoptosis and caspase-3 in vascular smooth muscle cells by plasminogen activator inhibitor-type 1. J. Cell Biochem. 92:178-188.

Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS and Skriver L. 1985. Plasminogen activator, tissue degradation and cancer. Adv. Cancer Res. 44:139-266.

Gutteridge J and Halliwell B. 1988. The antioxidant proteins of extracellular fluids. In: Chow CK, ed. Cellular Antioxi-

dant Defense Mechanism. Boca Raton, Florida: CRC Press Inc., pp. 2-19.

Halliwell B and Aruoma OI. 1991. DNA damage by oxygen derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. 281:9-19 [review].

Hideki T, Noriaki S and Norio M. 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during *in vitro* maturation: Role of cumulus cells. Biol. Reprod. 63:805-810.

Huarte J, Belin D and Vassalli J-D. 1985. Plasminogen activator in mouse and rat oocytes: Induction during meiotic maturation. Cell 43:551-558.

Huarte J, Belin D, Bosco D, Sappino AP and Vassalli JD. 1987. Plasminogen activator and mouse spermatozoa: Urokinase synthesis in the male genital tract and binding of the enzyme to the sperm cell surface. J. Cell Biol. 104:1281-1289.

Huarte J, Vassalli J-D, Belin D and Sakkas D. 1993. Involvement of the plasminogen activator/plasmin proteolytic cascade in fertilization. Dev. Biol. 157:539-546.

Irigoyen JP, Munoz-Canoves, Montero L, Koziczak M and Nagamine Y. 1999. The plasminogen activator: Biology and regulation. Cell Mol. Life Sci. 56:104-132.

Iwata H, Akamatsu S, Minami N and Yamada M. 1999. Allopurinol, an inhibitor of xanthine oxidase, improves the development of IVM/IVF bovine embryos (>4 cell) *in vitro* under certain culture conditions. Theriogenology 51:613-622.

Janjic D and Wollheim CB. 1992. Effect of 2-mercaptoethanol on glutathione levels, cystine uptake and insulin secretion in insulin-secreting cells. Eur. J. Biochem. 210:297-304.

Kim NH and Menino AR Jr. 1995. Effects of stimulators of protein kinase A and C and modulators of phosphorylation on plasminogen activator activity in porcine oocyte-cumulus cell complexes during *in vitro* maturation. Mol. Reprod. Dev. 40:364-370.

Lee E, Vaughan DE, Parikh SH, Grodzinsky AJ, Libby P, Lark MW and Lee RT. 1996. Regulation of matrix metalloproteinases and plasminogen activator-1 synthesis by plasminogen in cultured human vascular smoothe muscle cells. Circ. Res. 78:44-49.

Liu YX and Hsueh AJ. 1987. Plasminogen activator activity in cumulus-oocyte complexes of gonadotropin-treated rats during the periovultory period. Biol. Reprod. 36:1055-1062.

Luvoni GC, Keskintepe L and Brackett BG. 1996. Improvement in bovine embryo production *in vitro* by glutathione-containing culture media. Mol. Reprod. Dev. 43:437-443.

Meister A. 1983. Selective modification of glutathione metabolism. Science 220:472-477.

Niwa K. 1993. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pig. J. Reprod. Fertil. (Suppl) 48:49-59.

Orsi NM and Leese HJ. 2001. Protection against reactive oxygen species during mouse preimplantation embryo development: Role of EDTA, oxygen tension, catalase, superoxide dismutase, and pyruvate. Mol. Reprod. Dev. 59:44-53.

Ossowski L, Biegel D and Reich E. 1979. Mammary plasminogen activator: Correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. Cell 16:929-940.

Patrick B, Karine C and Marc-andre S. 1997. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. J. Androl. 18:454-460.

Petters RM and Wells KD. 1993. Culture of pig embryos. J. Reprod. Fertil. 48:61-73.

Rossignol P, Ho-Tin-Noe B, Vranckx R, Bouton MC, Meilhac O, Lijnen HR, guillin MC, Michel JB and Angles-Cano E. 2004. Protease nexin-1 inhibits plasminogen activation-induced apoptosis of adherent cells. J. Biol. Chem. 279:10346-10356.

Sappino AP, Huarte J, Belin D and Vassalli JD. 1989. Plasminogen activators in tissue remodelling and cell invasion: mRNA localization in mouse ovaries and implanting embryos. J. Cell Biol. 109:2471-2479.

Schmitt M, Harbeck N, Thomssen C, Wilhelm O, Magdolen V, Reuning U, Ulm K, Hofler H, Janicke F and Graeff H. 1997. Clinical impact of the plasminogen activation system in tumor invasion and metastasis: Prognostic relevance and target for therapy. Thromb. Haemost. 78:285-296.

Sowers JR. 1998. Obesity and cardiovascular disease. Clim. Chem. 44:1821-1825.

Strickland S, Reich E and Sheman MT. 1976. Plasminogen activator in early embryogenesis: Enzyme production by trophoblast and parietal endoderm. Cell 9:231-240.

Thibault C. 1977. Are follicular maturation and oocyte maturation independent processes? J. Reprod. Fertil. 5:1-15.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW and Oh KS. 1998. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum. Reprod. 13:998-1002.

(접수: 2010. 8. 10 / 심사: 2010. 8. 17 / 채택: 2010. 8. 26)