Reprod Dev Biol 32(2) : 97-104 (2008)

Effects of Plasminogen on Sperm-Oocyte Interaction during *In Vitro* Fertilization in the Pig

Soo-Jin Sa[1,2], Tae-Shin Kim[2], Soo-Bong Park[3], Dong-Seok Lee[4,†] and Chun-Keun Park[1,†]

[1]*School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, UK*

[2]*College of Animal Life Sciences, Kangwon National University, Chuncheon 207-701, Korea*

[3]*National Institute of Animal Science, Seonghwan 330-801, Korea*

[4]*College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea*

ABSTRACT

Plasminogen activators (PAs) are serine protease that cleave plasminogen to form the active protease plasmin. PA/plasmin system play a role in mammalian fertilization and motility and acrosome reaction of sperm. The present study was undertaken to identify PAs in porcine gametes and investigate a possible role of plasminogen in *in vitro* fertilization in the pig. When boar spermatozoa were preincubated in a fertilization medium (mTBM) for 0, 2, 4 or 6 h, the activity of tPA-PAI (110~117 kDa), tPA (62~70 kDa), and uPA (34~38 kDa) was observed in the sperm incubation medium and sperm sample. PA activities in the sperm incubation medium significantly ($p<0.05$) increased according to increasing incubation times, while PA activities in sperm significantly ($p<0.05$) decreased at the same times. In addition, the rate of acrosome reaction in spermatozoa increased by increasing culture times. When oocytes were separated from porcine cumulus-oocytes complexes at 0, 22 or 44 h of maturation culture, no PA activities were observed in cumulus free-oocyte just after aspiration from follicles. However, the activity of tPA-PAI (108~113 kDa) and tPA (75~83 kDa) was observed at 22 h of *in vitro* culture and significantly ($p<0.05$) increased as the duration of the culture increased. On the other hand, when porcine oocytes were activated by sperm penetration or calcium ionophore, plasminogen significantly ($p<0.05$) increased ZP dissolution time (sec) in activated oocytes by sperm penetration. These results suggest that supplementation of plasminogen to fertilization medium may play a positive role in the improvement of *in vitro* fertilization ability in the pig.

(Key words : Plasminogen activators, Gametes, Fertilization medium, Pig)

INTRODUCTION

Plasminogen is a ubiquitous zymogen, being abundant in blood plasma and in most extracellular fluids including seminal plasma (Kobayashi *et al.*, 1992), uterine fluid (Bruse *et al.*, 1998), and ovarian follicular fluid (Colgin and Murdoch, 1997). Plasminogen activators (PAs) are specific proteolytic enzymes which convert the inactive proenzyme plasminogen to the active protease plasmin. Plasmin is a trypsin like proteolytic enzyme with broad substrate specificity, which cleaves blood fibrin clots and some other proteinases, such as procollagenase and proelatase (Mullertz, 1988). There are two types of plasminogen activator on the basis of molecular mass: tissue-type PA (t-PA), which is secreted in an active form with a molecular mass of around 70 kDa, and urokinase-type PA (u-PA), which is secreted as an inactive single-chain molecule of 31~54 kDa (Hart and Rehemtulla, 1998).

In humans and other various animals, Smokivitis *et al.* (1987) reported that matured spermatozoa contain a number of protease such as PAs, PA inhibitor (PAI) and plasmin inhibitor (PI). PAs and their inhibitors are both located in the plasma and the outer acrosomal membrane of man and boar spermatozoa (Smokovitis *et al.*, 1992). Trypsin-like protease stimulates the capacitation and/or induces the acrosome reaction (Zaneveld and De Jonge, 1991) or are involved in the sperm binding to zona pellucida (ZP; Benau and Storey, 1987). Ioannis *et al.* (2004) reported that the PA/plasmin system could be involved in the initiation of the hyperactivation of bovine sperm. On the other hand, Huarte *et al.* (1985) reported that the production of tPA was triggered by the resumption of meiosis in mice and rats. PAs activity has also been shown in fo-

* This work was supported by a grant (Code# 20070401034015 and Code# 20070301034040) from BioGreen21 Program, Rural Development Administration, Korea.

† Corresponding author : Phone: +82-53-950-7366, E-mail: lee1@knu.ac.kr, parkck@kangwon.ac.kr

llicular fluid (Smokovitis *et al.*, 1988), oocytes (Huarte *et al.*, 1993), and cumulus cell culture (Liu and Hsueh, 1987; Karakji and Tsang, 1995), while t-PA and its m-RNA have been detected in oocyte cytoplasm (Strickland *et al.*, 1988). Zhang *et al.* (1992) reported that the presence of a tPA in the secretion of activated rat eggs and the inhibitory effects of a tPA antibody on activation-induced zona reaction.

PAs/plasmin system is implicated in a variety of basic biological processes such as extracellular matrix degradation, tumor invasiveness, tissue remodeling and cellular differentiation (Smokovitis *et al.*, 1988). Several observations suggest that the PA/plasmin system might also play a role in mammalian oocyte fertilization, in sperm motility, acrosome reaction of capacitated sperm (Taitzoglou *et al.*, 2004) and alteration of ZP (Cannon *et al.*, 1998). Huarte *et al.* (1993) reported that the addition of plasminogen to fertilization medium has been shown to increase the yield of fertilized mouse eggs.

Therefore, the purposes of the present study were 1) to examine any change of PAs activity in the fertilization medium (mTBM) during pre-incubation of boar spermatozoa, 2) to identify PAs in porcine *in vitro* matured oocytes, and 3) to evaluate a possible role of plasminogen, the major substrate for PAs, on fertilization responses, ZP susceptibility to pronase digestion during *in vitro* fertilization in the pig.

MATERIALS AND METHODS

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). The basic medium used for IVF was essentially the same as that used by Abeydeera and Day (1997). This medium, designated as modified Tris-buffered medium (mTBM), consists of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and no antibiotics. Embryo culture (EC) medium was NCSU-23 containing 0.4% BSA (fraction V; A 8022; Petters and Wells, 1993).

Preparation of Spermatozoa and Oocyte

Ejaculated semen was collected from three Duroc boars by an artificial vagina. The same three boars were used for all experiments. Fresh spermatozoa were washed three times by centrifugation (1,900 ×g for 4 min) in Dulbecco's PBS (DPBS; GIBCO BRL, Grand Island, NY, USA) supplemented with 1% BSA, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate. After washing of the fresh boar spermatozoa with PBS, the washed spermatozoa were resuspended in mTBM.

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 an 18-gauge needle. COCs with uniform ooplasm and a compact cumulus cell mass were prepared in a Hepes-buffered TALP medium containing 0.1 (v/v) polyvinylalcohol (H-TL-PVA). The collected oocytes were washed three times in maturation medium and 10 COCs were introduced to 50 μl drop of the maturation medium under mineral oil. After culture for 22 h, oocytes were washed three times and then cultured in the maturation medium without hormones for another 22 h at 39℃, 5% CO_2, in air.

In Vitro Fertilization

After 44 h of *in vitro* maturation, cumulus cells were removed mechanically by repeated passages though a fine pipette. Nude oocytes were initially washed three times in 500 μl of TL-Hepes-PVA medium and subsequently in pre-equilibrated fertilization medium (mTBM). After washing, ten oocytes were transferred into pre-equilibrated 50 μl drop of fertilization medium (mTBM) with or without 50 μg/ml plasminogen under mineral oil. Fresh spermatozoa were washed three times by centrifugation (1,900 ×g for 4 min) in Dulbecco's PBS (DPBS; GIBCO BRL, Grand Island, NY, USA) supplemented with 1% (w/v) BSA. At the end of the washing procedure, the sperm pellet was resuspended in fertilization medium (mTBM), and then 50 μl of this suspension was added to the fertilization drop containing IVM oocytes to give a final concentration of 1×10^6 spermatozoa/ml. At 6 h post-insemination, oocytes were washed three times in pre-equilibrated embryo culture (EC) medium and cultured in the same medium.

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, a stock solution of 0.5 ng/ml tPA from a human melanoma cell culture (Sigma) was prepared in a sample buffer. Each homogenized sample (20 μl) be-

ing 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 a 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 was 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 was cast into a warmed dish (100× 15 mm; Falcon 1012; Becton and Dickinson) and allowed to cool. Zymograms containing 0 μg/ml of 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, and rinsed under tap water. Zymograms were dried and stained with 0.1% (w/v) Amido Black 10 B for permanent storage. Polyacryamide gels were stained with 0.025% Coomassie Brilliant Blue R-250 in 65:25:10 water : isopropanol : acetic acid and destained with 80:10:10 water : isopropanol : acetic acid.

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.

Assay of Chlortetracycline (CTC) Fluorescence of Spermatozoa

The functional state of the spermatozoa was assessed using the chlortetracycline (CTC) fluorescence assay method described by DasGupta *et al.* (1993). A CTC solution was prepared on the day of use and contained 750 μM CTC (Sigma) in a buffer of 130 mM NaCl, 5 mM cysteine, 20 mM Tris-HCl; the pH was adjusted to 7.8 with 1N HCl. This solution was kept wrapped in aluminum foil at 4℃ until just before use. Hoechst-treated sperm suspension (45 μl) was added to 45 μl of CTC solution at room temperature in an aluminum foil-wrapped centrifuge tube and mixed thoroughly. Spermatozoa were then fixed by adding 8 μl 12.5% (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.4). Slides were prepared by placing 10 μl of the stained, fixed suspension on a slide for observation. One drop of anti-fade solution [0.22 M 1,4-diazabicyclo(2.2.2) octane (DABCO; Sigma) was dissolved in glycerol:PBS (9:1)] was mixed with the drop of semen to retard fading of the fluorescence. After adding a clean coverslip, the slide was compressed firmly between absorbent tissues (Kimwipes) to remove any excess fluid and to maximize the number of spermatozoa lying flat on the slide. The coverslip was then sealed with colorless nail varnish, wrapped in aluminum foil and stored at 4℃ until used for microscopic observation.

An assessment was carried out on either the same or the following day using an Olympus BHS microscope equipped with phase-contrast and epifluorescent optics. Cells were assessed for CTC staining using ultraviolet light. The exicitation beam (halogen lamp) was passed through a 405 nm band pass filter, and CTC emission was observed through a 455 nm dichroic mirror with an additional 375 nm barrier filter. Three kinds of CTC staining patterns were identified: an F pattern, spermatozoa with uniform fluorescence over the entire head (uncapacitated spermatozoa); a B pattern, spermatozoa with a fluorescence-diminished band in the post-acrosomal region and a relatively bright fluorescence in the acrosomal region (capacitated spermatozoa); and an AR pattern, spermatozoa with almost no fluorescence over the whole sperm head except for a thin band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa). At all three stages a bright fluorescence on the midpiece could be seen. At least 200 spermatozoa were counted for each sample. Experiment was replicated three times.

Assessment of Zona Pellucida Solubility of Oocytes Activated

To test the effect of plasminogen on zona pellucida susceptibility to pronase digestion, cumulus-free oocytes matured *in vitro* washed three times in 500 μl drops of fertilization medium (mTBM) and transferred to 50 μl drops of the same medium (mTBM) with or without 50 μg/ml plasminogen for 6 h at 39℃, 5% (v/v) CO_2 in air. Oocytes matured were activated by coincubation with 1×10^6 cells/ml spermatozoa or by treatment with 5 μM calcium ionophore A23187. At 6 h after insemination, oocytes were washed three times in TL-Hepes-PVA (39℃) and placed (n = 5~15 oocytes/treatment) into 100 μl of a 0.1% (w/v) pronase solution in PBS. Zona digestion was observed continu-

ously at room temperature (25℃) with an inverted microscope. When the zona pellucida was no longer visible at ×200 magnification, the zona pellucida dissolution time (sec) was recorded. The experiment was replicated three times.

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).

Experimental Designs

In experimental design 1, to determine the changes of PAs activity and acrosomal status in boar spermatozoa during preincubation, spermatozoa were preincubated in fertilization medium (mTBM) for 0, 2, 4 or 6 h. After preincubation, sperm incubation medium and spermatozoa (40×10^6 cells/ml) were sampled for determination of PAs activity. Also, the functional state of spermatozoa (acrosomal status) was assessed using chlortetracycline (CTC) fluorescence assay.

In experimental design 2, to determine the changes of PAs activity in porcine oocyte during *in vitro* maturation, COCs were cultured in maturation medium. At 0, 24 and 48 h after the onset of maturation culture, the cumulus cells were removed mechanically by repeated passages though a fine pipette. After the removal of cumulus cells from the COCs at each time point, 40 denuded oocytes (DOs) were separately put into microtubes containing 20 μl of sample buffer (5.0 % [w/v] SDS, 20% [v/v] glycerol, and 0.0025% [w/v] bromophenol blue in 0.125 M Tris-HCl buffer) and frozen at −80℃ until used for zymographic analysis.

In experimental design 3, to examine the effects of plasminogen on the fertilizing ability *in vitro* of porcine gametes, *in vitro* fertilization was performed in a fertilization medium (mTBM) with or without 50 μg/ml plasminogen. At 6 h after the onset of insemination, susceptibility to pronase digestion was monitored. At 12 h post-insemination, oocyte penetration tests were performed.

RESULTS

Fig. 1. and 2 show the changes of PAs activity in sperm incubation medium and boar spermatozoa preincubated with different incubation periods. When boar spermatozoa were preincubated in a fertilization medium (mTBM) for 0, 2, 4 or 6 h, three plasminogen-dependent lytic bands (110~117 kDa, 62~70 kDa, and 34~38 kDa) were observed in a sperm incubation medium (Fig. 1 (A)) and spermatozoa sample (Fig. 2 (A)). PAs activities in the sperm incubation medium were significantly ($p<0.05$) increased during increasing preincubation periods (Fig. 1 (A)), whereas PAs activities in spermatozoa were significantly ($p<0.05$) decreased (Fig. 2 (B)).

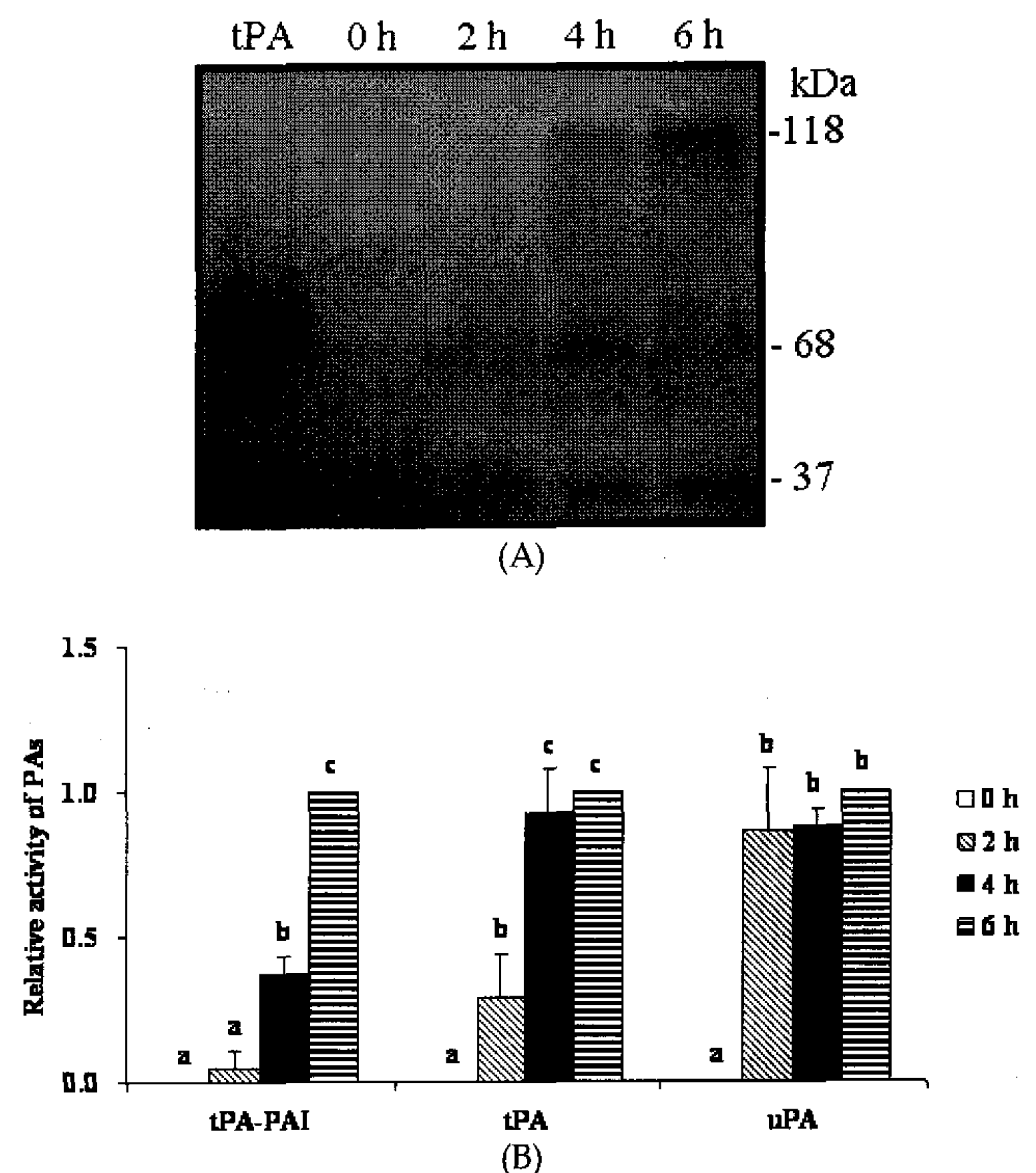

Fig. 1. Changes of PAs activity of sperm incubation medium at 0, 2, 4 and 6 h culture in fertilization medium. (A) Zymographic analysis of sperm incubation medium of culture in fertilization medium (mTBM). (B) Intensity of PAs activity was quantified by densitometric scanning of zymography and expressed relative to the activity at 6 h of culture for tPA-PAI complex, tPA and uPA. Values obtained from three replicates are expressed as mean ± SEM. [a~c] Values with different letters are significantly different ($p<0.05$).

On the other hand, the changes of acrosomal status in boar spermatozoa were examined after preincubation for 0, 2, 4 or 6 h by CTC assay. Fig. 3 shows that the changes of CTC patterns in boar spermatozoa incubated with different periods. The rates of capacitated spermatozoa were significantly ($p<0.05$) decreased (67.0% to 42.3%) and the rates of acrosome reaction (AR) in boar spermatozoa were significantly ($p<0.05$) increased (21.8% to 55.4%) as the preincubation time increased from 0 to 4 h.

This study showed the change of PAs activity in oocytes that were separated from porcine COCs at 0, 22 and 44 h of maturation culture (Fig. 4). No PAs activity was observed in cumulus free-oocyte just after

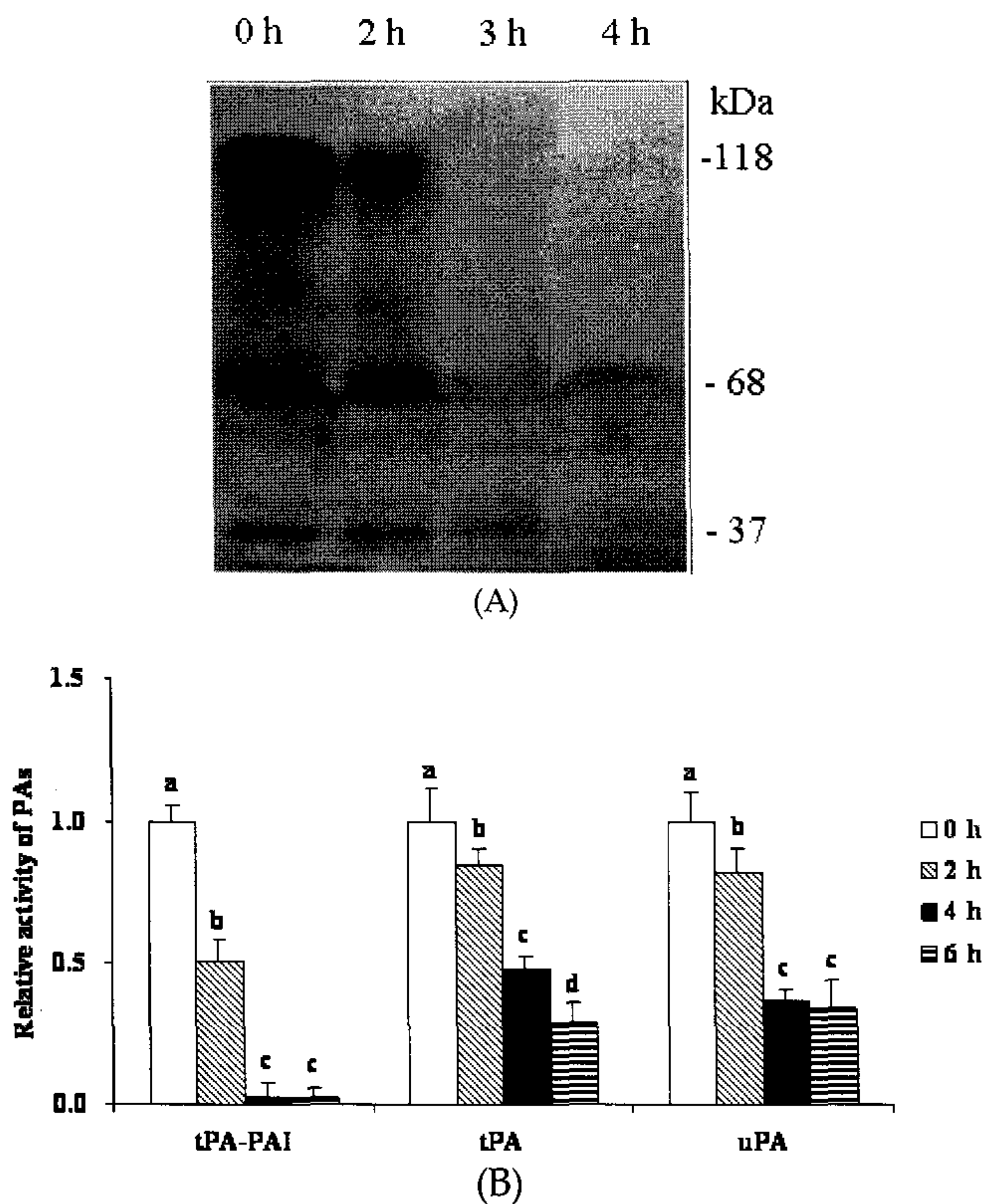

Fig. 2. Changes of PAs activities of sperm at 0, 2, 4 and 6 h culture in fertilization medium. (A) Zymographic analysis of sperm at culture periods in fertilization medium (mTBM). (B) Intensity of PAs activity was quantified by densitometric scanning of zymography and expressed relative to the activity at 0 h of culture for tPA-PAI complex, tPA and uPA. Values obtained from three replicates are expressed as mean ± SEM. [a~d] Values with different letters are significantly different (p<0.05).

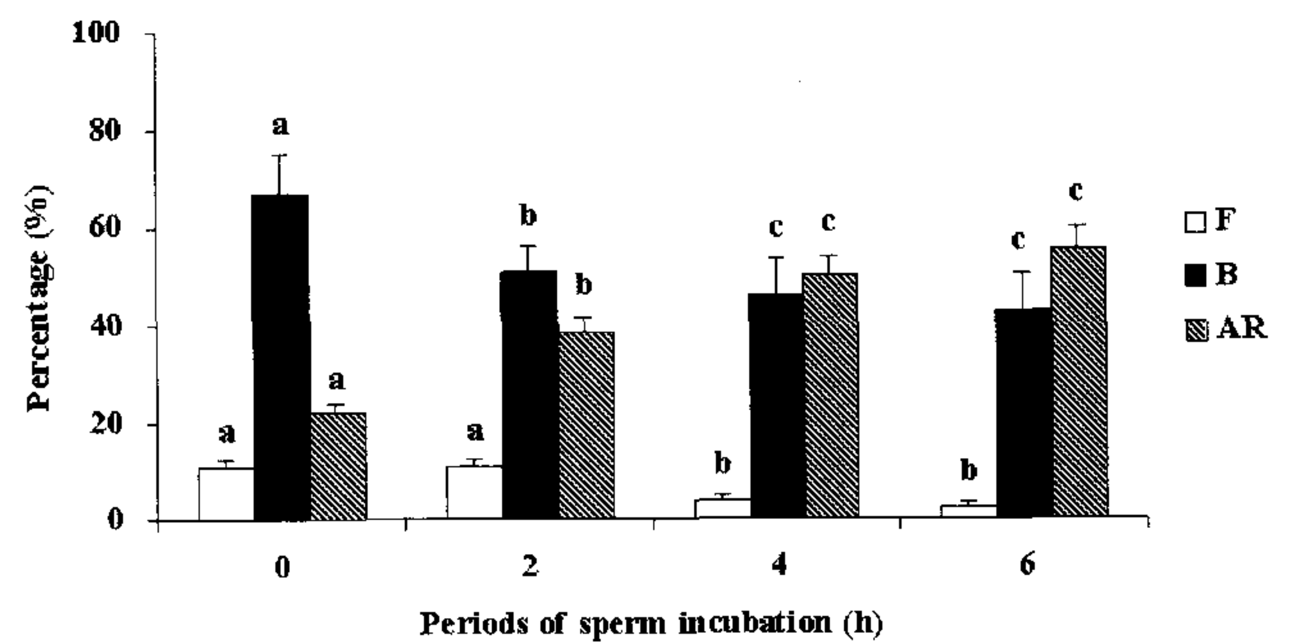

Fig. 3. Change of CTC patterns of sperm at 0, 2, 4 and 6 h culture in fertilization medium (mTBM). F: intact non-capacitated, B: capacitated, AR: acrosome-reacted spermatozoa. Values obtained from three replicates are expressed as mean ± SEM. [a~c] Values with different letters within each CTC patterns differ significantly (p<0.05).

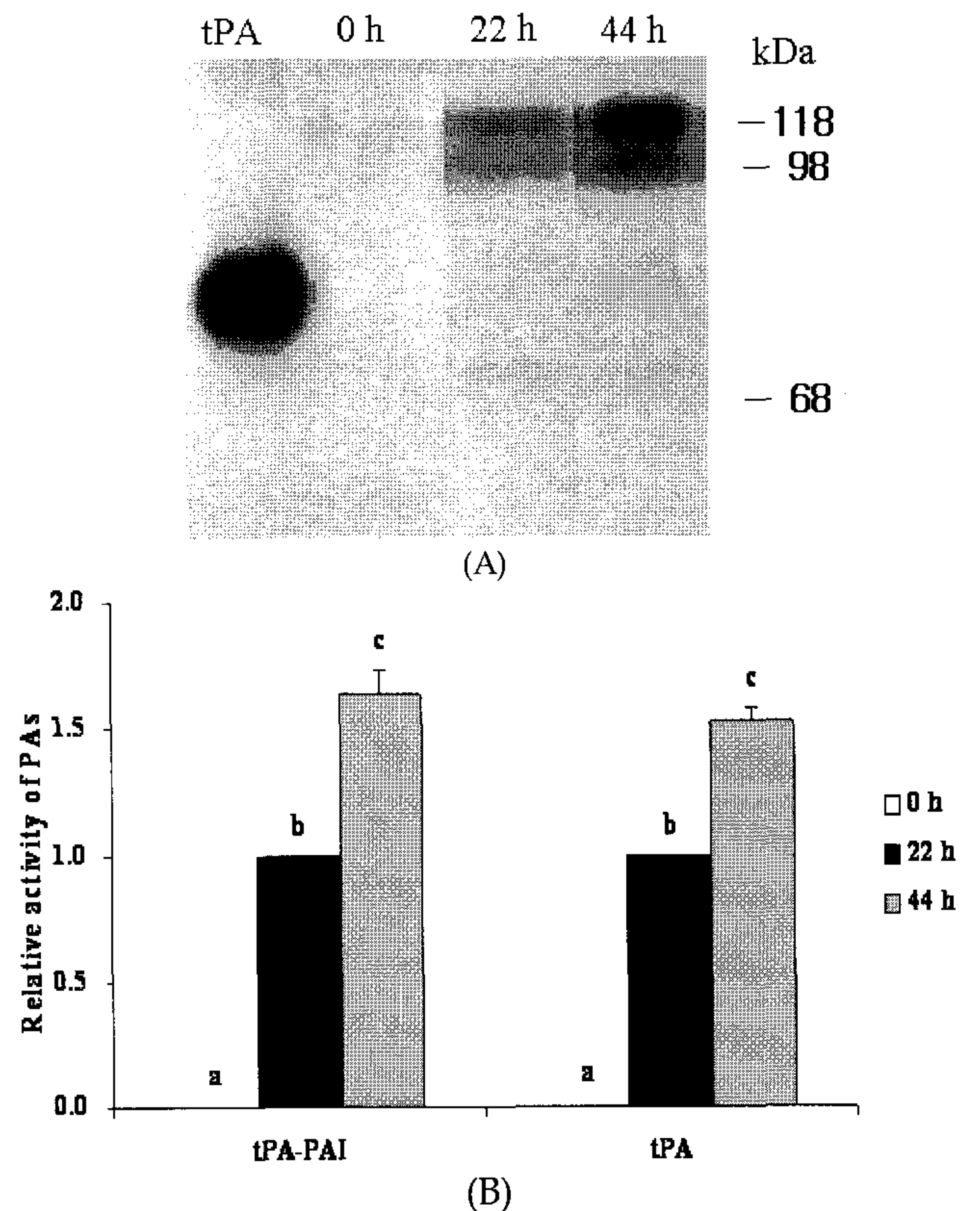

Fig. 4. Changes of PAs activities of porcine COCs at 0, 22 and 44 h of *in vitro* culture. (A) Zymographic analysis of different stage of COCs. (B) Intensity of PAs activity was quantified by densitometric scanning of zymography and expressed relative to the activity at 22 h of culture for tPA-PAI complex and tPA. Values obtained from three replicates are expressed as mean ± SEM. [a~c] Value with different letters are significantly different (p<0.05).

aspiration from follicles. As shown in Fig. 4, the activity of tPA-PAI (108~113 kDa) and tPA (75~83 kDa) was observed at 22 h of culture and significantly (p< 0.05) increased as the duration of the culture increased.

Fig. 5. showed that the effect of plasminogen on ZP solubility in porcine oocyte activated by sperm penetration or by treatment with calcium ionophore. When porcine oocytes were activated, ZP dissolution time (sec) tended to increase when plasminogen was added in fertilization medium (mTBM). Addtion to 50 μg/ml plasminogen significantly (p<0.05) increased ZP dissolution time (sec) in activated oocytes by sperm penetration.

DISCUSSION

The plasminogen activator (PAs) activity of mammalian gametes has been previously described. tPA and uPA are localized to the plasma and the outer acrosomal membrane of human, and boar spermatozoa (Smokovitis *et al.*, 1992), and tPA is detected in the rat (Bicsak and Hsueh, 1989), mouse (Stutz *et al.*, 1998), and pig (Kim and Menino, 1995) oocytes. The present study was undertaken to examine, the changes

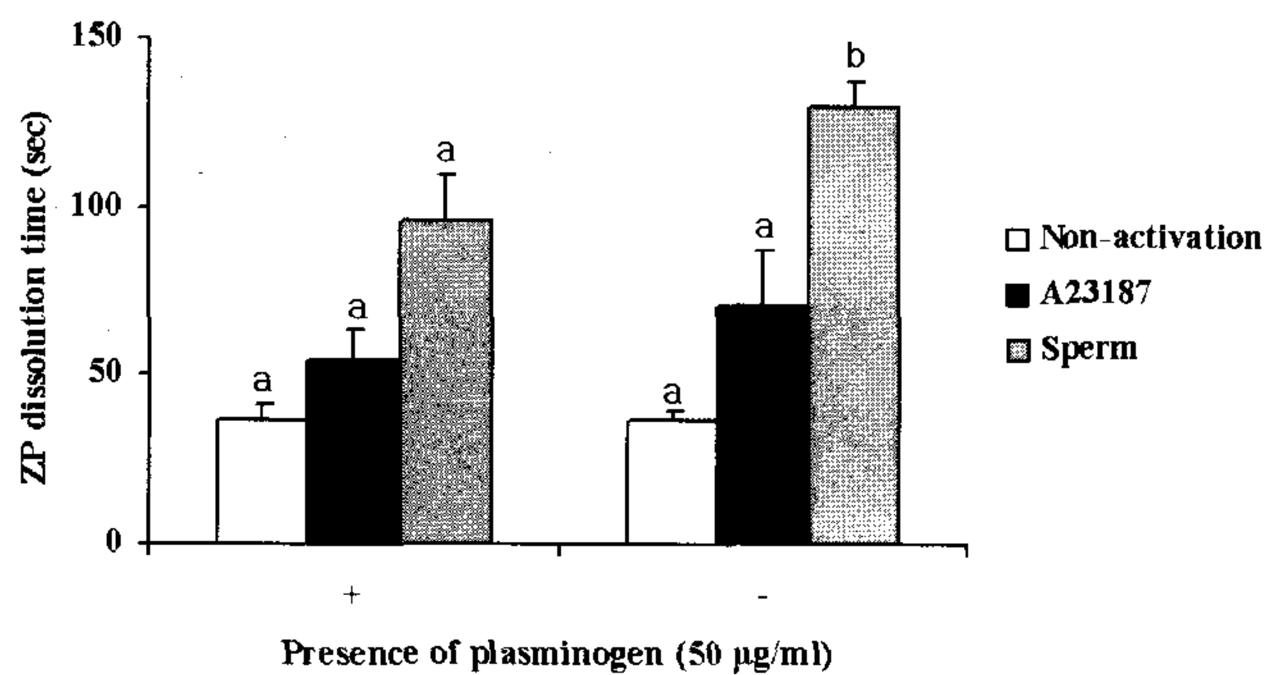

Fig. 5. Effect of plasminogen on zona pellucida (ZP) susceptibility to pronase digestion. Activation of oocytes was achieved by sperm penetration or by treatment with 5 μM calcium ionophore A23187. Values obtained from three replicates are expressed as mean ± SEM. [a,b] Values with different letters within each treatment of oocytes activation differ significantly ($p<0.05$).

of PAs activity in porcine gamates during *in vitro* culture and the effect of plasminogen, the major substrate for PAs, on zona pellucida during *in vitro* fertilization.

Hart and Rehemtulla (1988) reported that tPA is secreted in an active form with a molecular weight of around 70 kDa and uPA is secreted as an inactive single-chain molecule of 31~54 kDa. Both tPA and uPA can form SDS-stable complexes with PAI, a family of specific inhibitors for PA, yielding PA-inhibitor complexes detectable by zymography (Rehemtulla *et al.*, 1990). tPA and PAI form a complex with a molecular weight of approximate 110 kDa (Kruithof *et al.*, 1984; Wagner and Binder, 1986). The research demonstrates that tPA-PAI, tPA, and uPA are released by boar spermatozoa during preincubation in a fertilization medium (mTBM). Although PAs activities in sperm incubation medium increased time-dependently during *in vitro* culture for 6 h, PAs activities in spermatozoa decreased. Also, the rates of acrosome reaction (AR) in boar spermatozoa increased as the preincubation time increased. In addition to several enzymes located in the head of the mammalian spermatozoa (Zaneveld and De Jonge, 1991), the presence of tPA and uPA as well as PA inhibitor (PAI) and plasmin inhibitors (PI) has been detected in the ejaculated spermatozoa of human and various animal species, including bull, ram, buck, boar and stallion (Smokovitis *et al.*, 1987). PAs activity in spermatozoa incubation medium was increased after the induction of acrosome reaction of human, boar, bull and ram spermatozoa by hyamine, triton or calcium ionophore (Taitzoglou *et al.*, 1996). These results are consistent with our results showing that tPA-PAI, tPA, and uPA may be released in boar spermatozoa during acrosome reaction.

This study showed that no PAs activity was detected in porcine oocytes separated from cumulus cells just after collection from the antral follicle, but the activity of tPA-PAI and tPA was increased as the culture time increased from 22 to 44 h. Although the type of PAs detected in COCs seemed to be different according to the different species, the increase of PAs activity during *in vitro* or *in vivo* maturation has been reported in pig and rat COCs. Although uPA activity is not detected in porcine COCs at before and after maturation, other studies have shown that both tPA-PAI and tPA activity increases during *in vitro* maturation (Kim and Menino, 1995).

Results of this study showed that the addition of plasminogen to the fertilization medium increased ZP dissolution time (sec) in activated oocytes by sperm penetration or calcium ionophore. Also, ZP solubility was associated with proteolytic alteration of the ZP polypeptides by the PA/plasmin system. Previous reports suggested that tPA is released from the rat oocytes as a result of oocyte activation and participates in ZP hardening (Zhang *et al.*, 1992). In fertilized or calcium ionophore-activated mouse oocytes, zona hardening may be caused by a proteolytic factor in the cortical granules, which induces limited proteolysis of the zona protein ZP2 (Moller and Wassarman, 1989). Zhang *et al.* (1992) have reported the presence of a tPA-like macromolecule in the secretion of activated rat eggs and the inhibitory effect of a tPA antibody on activation-induced zona reaction, indicating that the ZP molecules could be modified by PA/plasmin system.

In summary, this study provides information concerning the production of PAs by pig gametes during *in vitro* culture and the effect of the plasminogen, the major substrate for PAs, on various fertilization phases, such as ZP susceptibility to pronase digestion during fertilization in the pig. It was found that boar spermatozoa release tPA-PAI, tPA and uPA during preincubation and pig oocytes produce tPA-PAI and tPA during *in vitro* maturation. Also, addition of plasminogen to the fertilization medium increases ZP digestion time (sec) by pronase. These results suggest that supplementation of plasminogen to the fertilization medium may play a positive role in the improvement of fertilization ability in the pig.

ACKNOWLEDGEMENTS

We thank the Director, Young-Seo Artificial Insemination Center, Wonju-si, Republic of Korea for providing the boar semen during the course of the study.

REFERENCES

1. Abeydeera LR, Day BN (1997). *In vitro* penetration

of pig oocytes in a modified Tris-buffered medium: Effect of BSA, caffeine and calcium. Theriogenology 48:537-544.
2. Benau DA, Storey BT (1987). Characterization of the mouse sperm plasma membrane zona-binding site sensitive to trypsin inhibitors. Biol Reprod 36: 282-292.
3. Bicsak TA, Hsueh AJ (1989). Rat oocyte tissue plasminogen activator is a catalytically efficient enzyme in the absence of fibrin endogenous potentiation of enzyme activity. J Biol Chem 264:630-634
4. Bray BA, Hsu KC, Wigger HJ, Le Roy EC (1975). Association of fibrinogen and microfibrils with trophoblast basement membrane. Connect Tissue Res 3:55-71.
5. Bruse C, Bergqvist A, Carlstrom K, Fianu-Jonasson A, Lecander I, Astedt B (1998). Fibrinolytic factors in endometriotic tissue, endometrium, peritoneal fluid, and plasma from women with endometriosis and in endometrium and peritoneal fluid from healthy women. Fertil Steril 70:821-826.
6. Canipari R, Epifano O, Siracusa G, Salustir A (1995). Mouse oocytes inhibit plasminogen activator production by ovarian cumulus and granulosa cells. Dev Biol 167:371-378.
7. Cannon MJ, Menino AR Jr (1998). Changes in the Bovine zona pellucida induced by plasmin or embryonic plasminogen activator. Mol Hum Reprod 51:330-338.
8. Colgin DC, Murdoch WJ (1997). Evidence for a role of the ovarian surface epithelium in the ovulatory mechanism of the sheep: Secretion of urokinase-type plasminogen activator. Anim Reprod Sci 47:197-204.
9. DasGupta S, Mills CL, Fraser LR (1993). Ca^{2+}-related changes in the capacitation state of human spermatozoa assessed with a chlortetracycline fluorescence assay. J Reprod Fertil 99:135-143.
10. Dyk AR, Menino AR Jr (1991). Electrophoretic characterization of the plasminogen activator produced by bovine blastocysts. J Reprod Fertil 93:483-489.
11. Hart DA, Rehemtulla A (1988). Plasminogen activator and their inhibitors: Regulators of extracellular proteolysis and cell function. Com. Biochem Physiol 90:691-708.
12. Huarte J, Belin D, Vassalli J-D (1985). Plasminogen activator in mouse and rat oocytes: Induction during meiotic maturation. Cell 43:551-558.
13. Huarte J, Vassalli J-D, Belin D, Sakkas D (1993). Involvement of the plasminogen activator/plasmin proteolytic cascade in fertilization. Dev Biol 157:539-546.
14. Ioannis AT, David AC, Ioannis AZ, Gary JK (2004). Effect of plasmin on movement characteristics of ejaculated bull spermatozoa. Theriogenology 62:553-561.
15. Karakji EG, Tang BK (1995). Growth hormone releasing factor and vasoactive intestinal peptide stimulate rat granulosa cell plasminogen activator activity *in vitro* during follicular development. Mol Cell Endocrinol 107:105-112.
16. Kim NH, 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.
17. Kobayashi T, Matsuda Y, Park J, Hara I, Kaneko S, Fujimoto Y, Nozawa S, Akihama S (1992). Trypsin-like arginine amidases including plasminogen and plasmin in human seminal plasma by affinity adsorption and elution. Arch Androl 28:165-170.
18. Kruithof EKO, Tran-Thang C, Ransjin A, Bachmann F (1984). Demonstration of a fast acting inhibitor of plasminogen activators in human plasma. Blood 64:907-913.
19. Lee SL, Wei YH (1994). The involvement of extracellular proteinases and proteinase inhibitors in mammalian fertilization. Biotechnol Appl Biochem 19:31-40.
20. Liu K, Liu YX, Du Q, Zhou HM, Lin X, Hu ZY, Zhang GY, Zhang GH (1996). Preliminary studies on the role of plasminogen activator in seminal plasma of human and rhesus monkey. Mol Hum Reprod 2:99-104.
21. Liu YX, Hsueh AJ (1987). Plasminogen activator activity in cumulus-oocyte complexes of gonadotropin-treated rats during the periovultory period. Biol Reprod 36:1055-1062.
22. Moller CC, Wassarman PM (1989). Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. Dev Biol 132:103-112.
23. Mullertz S (1988). Fibrinolysis: general aspects characteristic features and perspectives. Fibrinolysis 1: 3-12.
24. Petters RM, Wells KD (1993). Culture of pig embryos. J Reprod Fertil 48:61-73.
25. Rehemtulla A, Arndt A, DA (1990). Induction of plasminogen activator inhibitor type 2 expression during differentiation of human K562 cells towards a macrophage phenotype. Biochem Cell Biol 68:1337-1343.
26. Smokovitis A, Kokolis N, Alexopoulos C, Alexaki E, Eleftheriou E (1987). Plasminogen activator activity, plasminogen activator inhibition and plasmin inhibition in spermatozoa and seminal plasma of man and various animal species. Effect of plasmin on sperm motility. Fibrinolysis 1:253-257.
27. Smokovitis A, Kokolis N, Alexaki-Tzivanidou E (1988). The plasminogen activator activity is markedly increased mainly at the area of the rupture of the

follicular wall at the time of ovulation. Anim Reprod Sci 10:285-294.

28. Smokovitis A, Rekkas C, Vainas E, Kokolis N, Taitzoglou I (1992). The effect of chromosomal anomalies on the plasminogen activator activity, plasminogen activator inhibition and plasmin inhibition in spermatozoa and seminal plasma. I. Chromosomal chimaerism XX/XY in the ram. Anim Reprod Sci 29:241-254.
29. Strickland S, Huarte J, Belin D, Vassalli A, Rickles RJ, Vassalli JD (1988). Antisense RNA directed against the 3' noncoding region prevents dormant m-RNA activation in mouse oocytes. Science 241:680-684.
30. Stutz A, Conne B, Huarte J, Gubler R, Volkel V, Flandin P, Vassalli JD (1998). Masking, unmasking, and regulated polyadenylation cooperate in the translational control of a dormant mRNA in mouse oocytes. Genes Dev 12:2535-2548.
31. Taitzoglou IA, Chapman DA, Zervos IA, Killian GJ (2004). Effect of plasmin on movement characteristics of ejaculated bull spermatozoa. Theriogenology 62:553-561.
32. Vassalli JD, Sappino AP, Belin D (1991). Review: The plasminogen activator/plasmin system. J Clin Invest 88:1067-1072.
33. Wagner O, Binder B (1986). Purification of an active plasminogen activator inhibitor immunologically related to the endothelial type plasminogen activator inhibitor from the conditioned media of human melanoma cell line. J Biol Chem 261:14474-14481.
37. Zaneveld LJD, De Jonge CJ (1991). Mammalian sperm acrosomal enzymes and the acrosome reaction. In: A comparative overview of mammalian fertilization. Dunbar BS, O'Rand MG (Eds). New York: Plenum Press pp 63-79.
35. Zhang X, Rutled J, Khamsi F, Armstrong DT (1992). Release of tissue-type plasminogen activator by activated rat eggs and its posbsible role in fertilization. Mol Reprod Dev 32:28-32.

(Received; 5 May 2008/ Accepted: 11 June 2008)