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Full Length Research Paper

Effect of plasmin and heparin on *in vitro* ovine sperm-oocyte interaction

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This study was conducted to investigate the effect of plasmin and heparin on *in vitro* ovine sperm-oocyte interaction. Different concentrations of plasmin (0, 1, 10, 100 ng/ml) and heparin (0, 5, 10 IU/ml) were added alone or simultaneously into fertilization medium. After sperm and oocyte co-culture, binding and penetration of sperm to zona pellucida (ZP) were assayed. Treatment with 1 and 10 ng/ml plasmin resulted in higher sperm binding to ZP than those in control. The rates of sperm binding to ZP were increased with highest heparin concentrations (10 IU/ml). Heparin had no effect on penetration rate of sperm to ZP. Simultaneously effects of plasmin and heparin were not significant on penetration rate of sperm to ZP. But, 5 or 10 IU/ml heparin in the present of 1 ng/ml plasmin had higher effect on sperm binding to ZP than that in the other groups. These results suggest that plasmin and heparin (alone/simultaneously) might play a role in events related to fertilization in ovine.

Key word: Fertilization, binding, zona pellucida, sperm capacitation.

INTRODUCTION

In mammals, spermatozoa after ejaculation cannot fertilize oocytes *in vivo*; they must gain the capacity to fertilize through a series of changes which is called capacitation (Yanagimachi, 1994). Plasminogen activators are specific proteolytic enzymes which convert the inactive plasminogen to plasmin (Taitzoglu et al., 2004). There are a number of documents suggesting that the plasminogen activator (PA)/plasmin system might also play a role in mammalian sperm motility, oocyte fertilization and acrosome reaction (Taitzoglou et al., 2003, 2004) and alteration of the zona pellucida (ZP) (Cannon and Menino, 1998). Proteolytic enzymes appear to have essential roles in multiple phases of mammalian oocyte fertilization. The roles of proteolytic enzymes in mammalian fertilization, with the exception of acrosin, have not been clearly identified (Sa et al., 2006).

On the other hand, the most familiar member of the glycosaminoglycans (GAGs) is heparin, discovered as far back as 1916, whose action in the prevention of blood clotting has long been widely recognized (Gordon, 2003). Glycosaminoglycans that have been identified as effective inducers of capacitation *in vitro* are abundant in the follicles of cow. They are released by the ovulating follicle into the ampullary region of the oviduct at the time of ovulation (Gordon, 2003). Heparin has been shown to improve the sperm fertilization capacity in cattle (Parrish et al., 1986), sheep (Cox and Saravia, 1992; Li et al., 2006) and goat (Cox et al., 1994). It is thought that heparin may promote capacitation by binding to and removing seminal plasma proteins that are absorbed into the sperm plasma membrane and are normally thought to function to inhibit capacitation (Miller and Ax, 1990; Therien et al., 1995). Cosidering to the involvement of heparin and plasmin in sperm capacitation, we hypothesized that simultaneous adding these biological molecules to *in vitro* fertilization medium may improve fertilization rate. There is no information about simultaneously effect of plasmin and heaprin in fertilization medium on ovine sperm-oocyte interaction. It seems that simultaneous introduction of plasmin and heparin can improve oocyte and sperm interaction. Thus, the objectives of the present study were to assess the effects of plasmin and heparin on: (i) binding of sperm to ZP (ii), and fertilization responses, including penetra-

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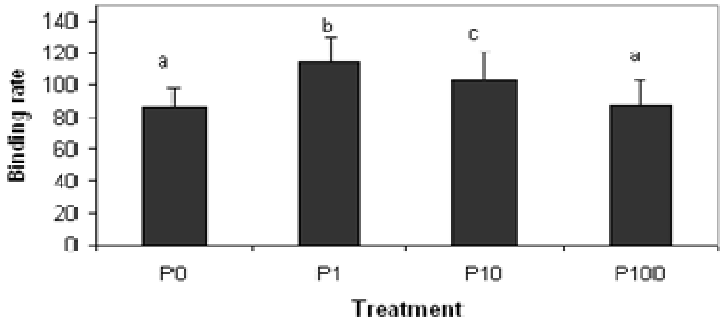

Figure 1. Mean (±SEM) of sperm binding rate to ZP after 6 h of sperm-oocyte co-culture in Fert-TALP containing 0 (P0), 1 (P1), 10 (P10), 100 (P100) ng/ml plasmin. Columns without a common letter differ significantly ($p<0.05$).

tion during *in vitro* fertilization in the sheep.

MATERIALS AND METHODS

Oocyte collection and *in vitro* maturation

Ovaries were collected at a local slaughter-house and transferred to lab in physiological serum containing 100 IU/ml penicillin at 30-37°C within 2 h. In the lab, the ovaries were cleaned of the superfluous tissue and bursa. Cumulusoocyte complex (COCs) was obtained by slicing the whole ovarian surface with the aid of a sterile scalpel blade. Oocytes with uniform ooplasm and a compact cumulus cell mass were selected and collected in H-M199 supplemented with 10% FCS and 50 IU/ml heparin. The collected oocytes were washed five times in maturation medium, which consisted of TCM199 supplemented with FSH (2 µg/ml), LH (1 µg/ml), E_2 (1 µg/ml) and 10% FCS. Ten COCs were transferred into each drop containing 50 µl maturation medium, previously covered with warm mineral oil. The COCs were cultured in maturation medium and placed in a CO_2 incubator maintained at 39°C in a humidified atomsphere of 5% CO_2 and 95% air.

Sperm preparation and in vitro fertilization

Fresh epididemal sperm were collected and washed two times by centrifugation (1000 rpm for 10 min) in sperm-TL (Tyrode lactate solution) supplemented with 1% BSA, 75 µg/ml potassium penicillin G. Sperm were counted by hemocytometer and diluted in Fert-TALP (Fertilization-Tyrodes lactate Pyruvate solution) as a fertilization medium (Parrish et al., 1986). After 24 h of *in vitro* maturation, matured oocytes were washed 4 times in 200 µl of pre-equilibrated Fert-TALP. After washing, 10 oocytes were transferred into 50 µl droplets of Fert-TALP containing various concentrations (alone/simultaneously) of plasmin (0, 1, 10 or 100 ng/ml) and heparin (0, 5, 10 IU/ml) under mineral oil. Sperm were then added at a concentration of 2×10^6/ml in Fert-TALP for 6 h. At 6 h post-insemination, oocytes were washed three times in pre-equilibrated fertilization medium. So, the oocytes were examined for sperm penetration.

Determination of zona-binding properties of sperm

The method used for zona binding properties of sperm was described in detail earlier (Sa et al., 2006). Briefly, after matured oocytes and sperm co-culture in various fertilization mediums, incubated oocytes were washed twice in 200 µl of fertilization medium and pipetted in and out of a wide-bore pipette to remove loosely bound sperm. Incubated oocytes were then placed into 50 µl drops of fresh fertilization medium containing Hoescht 33342 and incubated for 30 min at 39°C, 5% (v/v) CO_2 in air. Incubated oocytes were then mounted, and the number of tightly bound sperm/oocyte counted under a phase-contrast microscope equipped with ultraviolet illumination (excitation at 330–380 nm, emission at 420 nm). Experiment was replicated three times, with 10 oocytes counted from each replicate

Oocytes penetration test

The method used for zona binding properties of sperm was descrybed in detail earlier (Sa et al., 2006). Briefly, matured cumulus-free oocytes, were exposed to various concentrations of plasmin and heparin. Oocytes were placed into 50 µl drops of fertilization medium containing various concentrations of plasmin (0, 1, 10, 100 ng/ml) and heparin (0, 5, 10) and co-incubated with sperm (2×10^6 sperm/ml) for 6 h at 39°C, 5% (v/v) CO_2 in air. After incubation, sperm attached to the zona were removed by washing three times in fertilization medium. Oocytes were then transferred to the center of glass slide with four vaseline spots, gently compressed with a cover slide, immersed in 25% (v/v) acetic acid in ethanol for one day for complete fixations and stained with 1% (w/v) orcein in 45% acetic acid to assess sperm penetration rate under the phase-contrast microscope at a magnification of ×200 or ×400. The oocytes were considered as penetrated when one or more decondensed sperm heads and/or male pronuclei and corresponding sperm tails present. The experiment was replicated three times, with multiple observations for each replicate.

Statistics analyses

Data were analyzed by GLM procedure of the statistical analysis system (SAS Institute Inc., Cary, NC), and Duncan's test for mean differences ($p<0.05$). Data are presented as mean ± standard error of the mean (S.E.M.).

RESULTS

Plasmin effect on number of bound and penetrated sperm to zona pellucida (ZP) is depicted in Figures 1 and

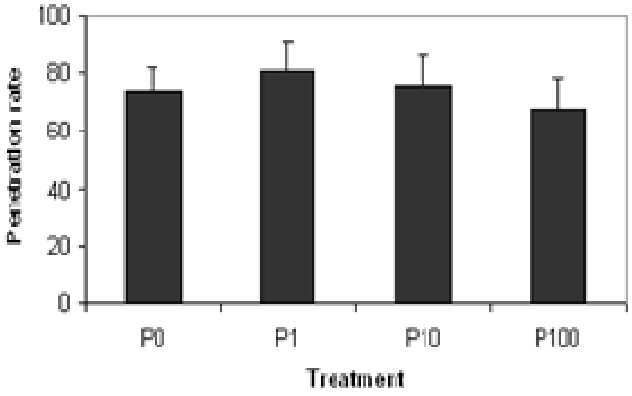

Figure 2. Mean (±SEM) of sperm penetration rate to ZP after 6 h of sperm-oocyte co-culture in Fert-TALP containing 0 (P0), 1 (P1), 10 (P10), 100 (P100) ng/ml plasmin.

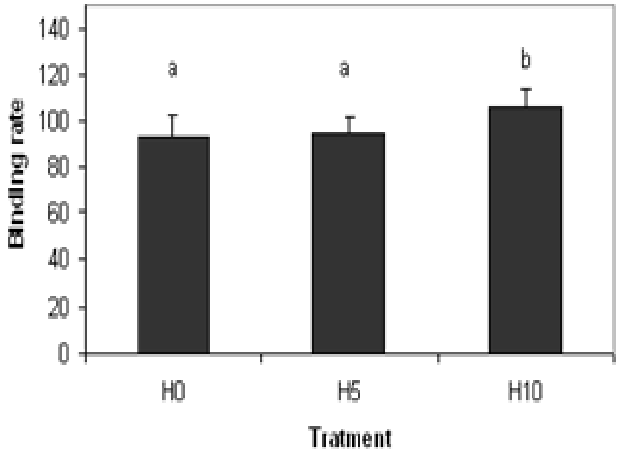

Figure 3. Mean (±SEM) of sperm binding rate to ZP after 6 h of sperm-oocyte co-culture in Fert-TALP containing 0 (H0), 5 (H5), 10 (H10) IU/ml heparin. Columns without a common letter differ significantly (p<0.05).

2, respectively. Binding rate in 1 ng/ml plasmin was higher (p<0.05) than the other concentrations (0, 10, 100). Plasmin had no significant effect (p<0.05) on penetration rate of sperm to ZP.

As it has been shown in Figure 3, 10 IU/ml heparin in Fert-TALP increased (p<0.05) number of bound sperm to ZP. Heparin in this experiment had no effect on number of penetrated sperm to ZP (Figure 4).

Figures 5 and 6 show concurrent effects of plasmin and heparin in Fert-TALP on number of bound and penetrated sperm to ZP. Heparin in 5 and 10 IU/ml along with 1 ng/ml plasmin had the highest effect (p<0.05) on number of bound sperm to ZP. Various concentration of plasmin along with heparin had no effect (p>0.05) on number of bound and penetrated sperm to ZP. But, there was an increased tendency in 10 IU/ml heparin and 1 ng/ml plasmin group.

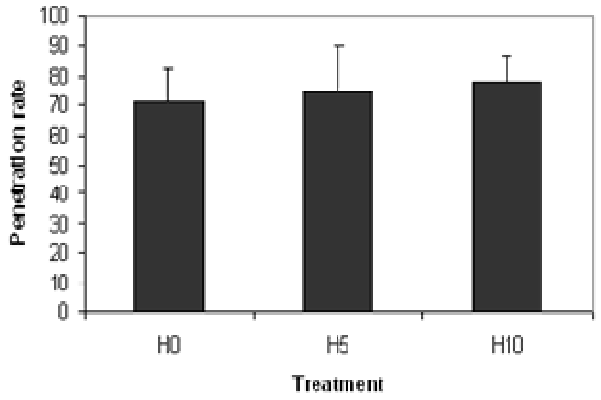

Figure 4. Mean (±SEM) of sperm penetration rate to ZP after 6 h of sperm-oocyte co-culture in Fert-TALP containing 0 (H0), 5 (H5), 10 (H10) IU/ml heparin.

DISCUSSION

Proteases in sperm, oocyte and genital tract secretions participate in several phases of mammalian fertilization, such as acrosome reaction (Meizel, 1985), sperm binding to ZP (Benau and Storey, 1987, 1988), ZP penetration (Fraser, 1982; Brown, 1983), zona hardening (Zhang et al., 1992), hatching (Coates and Menino, 1994), early embryo development and implantation (Zhang et al., 1994). The plasmin, one of the most important members of proteases, is a non-specific, potent protease that cleaves blood fibrin clots and some other extracellular proteins (Dano et al., 1985). Also, cell surface-associated plasmin catalyzes the breakdown of the extracellular matrix and basement membrane molecules, such as fibronectin, laminin (Liotta et al., 1981), vitronectin (Chain et al., 1991), proteoglycans (Mochan and Keler, 1984), fibrin (Dudek et al., 1970) and collagen (Liotta et al., 1981; Vassalli et al., 1991). Plasminogen activators/plasmin can exert directly and/or indirectly extracellular matrix degradation (Smokovitis et al., 1988, 1989). The role of exogenous plasmin, although not clearly demonstrated, would be to hydrolyze the sperm surface glycoprotein coat and thus destabilize the sperm surface membrane (Meizel, 1985). Plasmin is implicated in degradation of the extracellular matrix, tumor invasion, tissue and vascula remodeling and cellular differentiation (Mayer, 1990; Lijnen, 2001). Also, previous research reported that plasmin stimulates the activation of PLA2, a calcium-dependent enzyme that plays an essential role in ZP-induced exocytosis in mammalian sperm (Guerette et al., 1988; Yuan et al., 2003). Based on this information, plasmin, can cleave a wide spectrum of substrates (Dano et al., 1985) may be one of the proteins that might be involved in mammalian fertilization. Also, Huarte et al. (1993) observed enhanced sperm binding to the ZP and fertilization following addition of plasminogen to *in vitro* fertilization medium.

Results of the present study showed that addition of 1

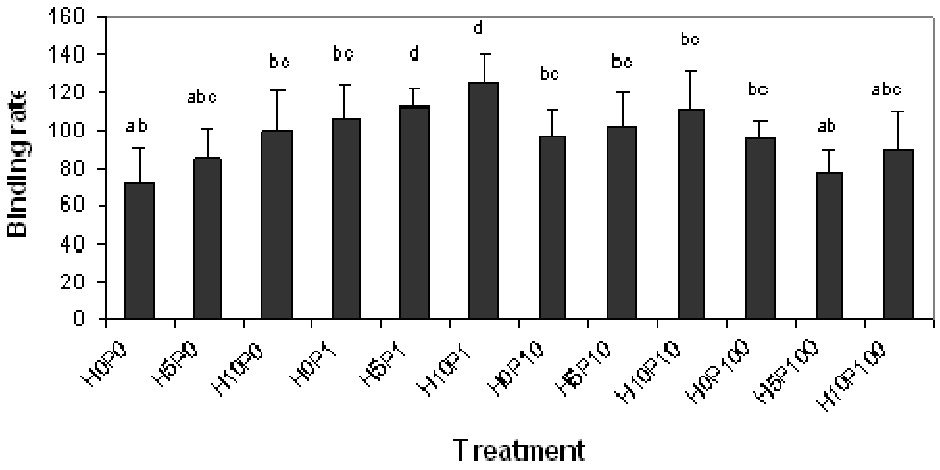

Figure 5. Mean (±SEM) of sperm binding rate to ZP after 6 h of sperm-oocyte co-culture in Fert-TALP containing 0, 1.0, 10.0, 100.0 plasmin along with 0, 5, 10 IU/ml heparin. Columns without a common letter differ significantly ($p<0.05$).

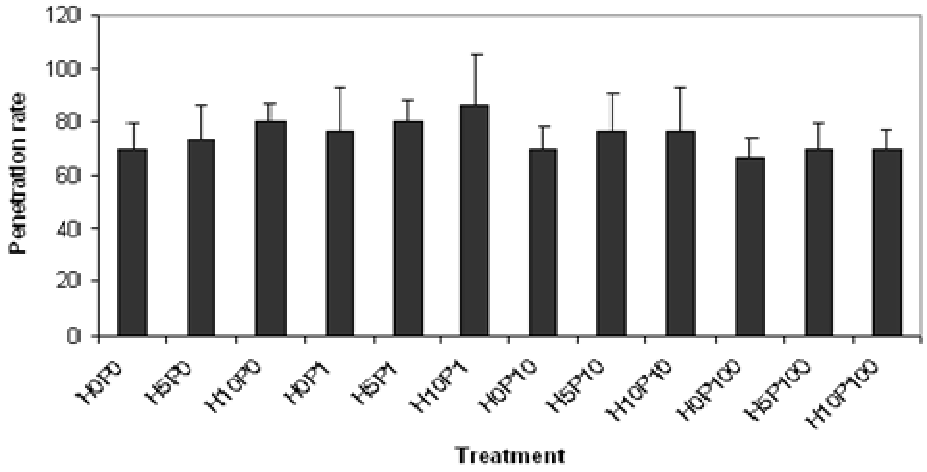

Figure 6. Mean (±SEM) sperm penetration rate to ZP after 6 h of sperm-oocyte co-culture in Fert-TALP containing 0, 1.0, 10.0, 100.0 plasmin along with 0, 5, 10 IU/ml heparin.

and 10 ng/ml plasmin to fertilization medium increase ($p< 0.05$) sperm binding to the ZP in compared with control and 100 ng/ml plasmin groups. But, addition of plasmin in different concentration to fertilization medium had no effect on sperm penetration rate to ZP. One of the plasmin roles is increase of spermatozoa hyperactivation. Taitzoglu et al. (2004) reported that percentage of motile bovine spermatozoa was higher after incubation with plasmin (100, 10 and 1 mU) versus control at the start of incubation. In this research, it was observed that sperm binding to ZP decreased when we used 100 ng/ml plasmin in fertilization medium. It is a surprising result and we have no clear reason for that. Perhaps, 100 ng/ml plasmin alter common its cell signaling pathway in sperm. It has already been proved that plasmin activate Ca^{2+}-related signaling pathway (Taitzoglu et *al*., 2004). These include the activation of platelet PLC and PKC via G-protein (21). Cannon and Menino (1998) reported that plasmin at physiological concentrations is capable of altering the polypeptide profile and structural integrity of the bovine ZP. In mice, ovulated eggs contain and secrete tissue-type PA (tPA) and ejaculated sperm exhibit urokinase-type PA (uPA) (Huarte et al., 1987). Hence, both gametes possess the necessary components to activate plasminogen and produce plasmin. Huarte et al. (1993) reported that addition of plasminogen to fertilization medium increased generation of plasmin and number of sperm bound to the ZP in mice. Huarta et al. (1993) have found that plasminogen is present in the environment in which *in vitro* fertilization takes place; it derived presumably from oviductal fluid in which it must be present at a higher concentration. Other studies have

sugested that fertilization *in vivo* also occurs in an environment relatively rich in plasminogen. Plasminogen is found in pre-ovulatory follicular fluid at a concentration similar to that in plasma (Beers, 1975).

Heparin is used for in vitro capacitation of sperm-atpzoa. It modulates gamete interactions in cattle and sheep IVF system (Wani, 2002). Heparin stimulates the fertilization rate by improving the efficiency of sperm capacitation in sheep (Cox and Saravia, 1992). Also, heparin apparently binds to sperm and plays a role in the sperm uptake of calcium (Parrish et al., 1989).

In our research, addition of heparin increased sperm binding to ZP. The highest effect of heparin on sperm binding to ZP was taken place in 10 IU/ml heparin in fertilization medium. But, different concentration of heparin had no effect on penetration rate of sperm to ZP.

On the other hand, it seems that addition of simultaneously different concentrations of plasmin along with heparin to fertilization medium had no effect on binding and penetration rate of sperm to ZP, unless in 5 or 10 IU/ml heparin in present of 1 ng/ml plasmin that increased ($p<0.05$) binding rate of sperm to ZP.

In conclusion, our results show that adding plasmin and heparin to *in vitro* fertilization medium to increase the number of spermatozoa bound to the ZP. However, the present work provides evidence for the involvement of plasmin and heparin in fertilization, at least under *in vitro* conditions.

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