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Gelatinases in Boar Seminal Plasma and Their Relation to Semen Indicators

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Abstract

Matrix metalloproteinases were detected in reproductive tissues and seminal plasma of various animal species. The aim of this study was to determine for the first time the presence of gelatinases and metalloproteases in boar seminal plasma and to correlate the results with semen indicators. Gelatin zymography was used for simultaneous identification and measurement of gelatinase enzyme activity associated with their molecular weights.

Several gelatinase forms were identified in seminal plasma of boars. Those that were stimulated by $CaCl_2$ and inhibited by EDTA and phenanthroline were considered as metalloproteases. Negative correlation between semen indicators (sperm index, sperm concentration and concentration of progressive motile sperm) and the concentrations of metalloprotease at 78 kDa and 66 kDa means that higher values of semen indicators correlate with lower concentrations of these metaloproteases in seminal plasma. Gelatinases with molecular weight of 225, 78 and 66 kDa correlated with higher levels of acrosome damage. Samples with sperm index above 110 M/ml contained gelatinases of significantly lower band intensities at 78 and 66 kDa compared to samples with SI less than 110 M/ml. Bands with 225, 78 and 66 kDa are suggested to belong to a dimer of MMP-9, proMMP-2 and mature MMP-2.

Matrix metalloproteinases, boar semen evaluation, semen characteristics

Numerous proteolytic enzymes and their inhibitors are localized in both spermatozoa and seminal plasma in the mammalian semen. The spermatozoa are rich in different proteases, which are mainly localized within the acrosomal vesicle (Tulsiani et al. 1998). Seminal plasma contains many proteinases originating either from testicular cells or prostate and other accessory sex glands. Most proteinases described for seminal plasma are serine proteinases, however, proteinases from other classes are present in semen from human and domestic mammals (Metayer et al. 2002).

The fertilization process requires breakdown of the physiological barrier during sperm penetration through the zona pellucida and the egg plasma membrane, but a detailed molecular mechanism is still unclear. It is suggested that matrix metalloproteinases (MMPs) can be involved in this process (Buchman-Shaked et al. 2002). The MMPs are a family of proteolytic enzymes capable of degrading specific components of extracellular matrix (ECM) at physiological pH in a zinc-dependent manner. MMPs and their tissue inhibitors (TIMPs) play a key role in many physiological processes, including ovulation and implantation. Although little is known about MMP expression and their exact function in the male reproductive tract, they are believed to be involved in the regulation of spermatozoal function (Hulboy et al. 1997).

Matrix-metalloproteinases (MMPs) are zinc-dependent proteinases involved in tissue remodelling, cell migration, and cell-cell interaction (Robinson et al. 2001), and are known as the main enzymes digesting the extracellular matrix. MMPs are secreted in inactive forms that are activated by cleavage of the inhibitory pro-peptide of about 10 kDa (Nagase and Woessner 1999). Keeping sperm proteases in an inactive form is critical for maintaining cell integrity in order to ensure reproductive function of sperm cells (Zheng et al. 1994). A complex mechanism controlling MMP activation includes regulation at the level of gene expression, cleavage of inactive forms and inhibition of active MMPs

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by endogenous inhibitors, primarily tissue inhibitors of MMPs (Nagase and Woessner 1999).

Gelatinases have been detected in epididymal fluids of ram, stallion and boar (Metayer et al. 2002). Metalloproteases, serine proteinases and serine proteinase inhibitors have been detected in turkey seminal plasma (Kotlowska et al. 2005). Complexes of gelatinases and TIMP-1 and TIMP-2 (Shimokawa et al. 2003) or MMP-2 and MMP-9 (Shimokawa et al. 2002) were found in human seminal plasma. The first report of gelatinase in human sperms indicated higher 28-kDa gelatinase activity and lower 92-kDa MMP activity in normal compared to abnormal semen (Buchman-Shaked et al. 2002). TIMP-2 in bovine seminal plasma has been described as a factor influencing the bull fertility (McCauley et al. 2001). The role of TIMPs for human sperm function is most probably based on interaction with MMPs in spermatozoa (Baumgart et al. 2002). MMP-2 and MMP-9 were evaluated in human seminal plasma, where latent forms of both MMPs predominate, and pro-MMP-9 was detected in samples of low sperm concentration (Tentes et al. 2007).

The aim of this study was to examine the presence of gelatinases and metalloproteases in boar seminal plasma.

Materials and Methods

Semen samples and their analysis

Twenty-one semen samples were obtained from 12 to 24-month-old boars of various breeds. While the boar mounted a dummy sow, semen was collected with gloved hand using a clean semen collecting flask that filters gel, dust and bristles out. Semen was kept at the room temperature and analysed within 1 h. Computer-assisted semen analysis (Hamilton Thorne IVOS 10.2; Hamilton Thorne Research, MA, USA) was performed with a Makler counting chamber (Sefi Medical Instruments, Israel) to determine sperm concentration and motility characteristics. Sperm morphology was examined in Giemsa-stained samples (Hafez 1993). Metabolic activity of spermatozoa was assayed using a spectrophotometric application of the resazurin reduction assay (Zrimšek et al. 2004; Zrimšek et al. 2006). Sperm index was calculated by multiplying the total sperm concentration by the square root of its motility multiplied by the percentage of normal sperm (Mahmoud et al. 1994).

Seminal plasma preparation

Samples of seminal plasma were prepared at the same time as evaluation of the semen. Following centrifugation at 818 g for 10 min at room temperature, the supernatant was further centrifuged at 13,000 *g* for 15 min at 4 ºC to separate seminal plasma, which was then aliquoted and frozen at –80 ºC until assayed.

Gelatin zymography

Gelatin zymography was performed on seminal plasma using a modified method of Laemmli (1970). The 7.5% separating polyacrylamide gel (0.75 mm thickness) contained 0.12% pig skin type I gelatin (Sigma, Germany), whereas the 4% stacking gel contained no gelatin. The samples of seminal plasma, diluted 1:5 in PBS, were denatured with SDS but not reduced. The mixture of molecular weight markers was diluted 1:4 with SDS reducing buffer and heated in a boiling water-bath for 5 min prior to loading on the gel. MMP-2 from human fibroblasts (Sigma) and MMP-9 from human fibroblasts (Sigma) were used as controls. Each sample of 10 µl was loaded into a well and samples were electrophoresed at a constant 200 V for 45 min. After electrophoresis, the proteins were allowed to renature by removing SDS by washing the gel in 2.5% Triton X-100 with gentle shaking for 30 min at room temperature. Gels were incubated overnight at 37 ºC in a reactivation buffer of pH 7.6 containing 50 mM Tris/HCl, 10 mM $CaCl_2$, 150 mM NaCl and 0.2% Brij 35. Following incubation, the gels were stained for 20 min with 0.1% Coomassie brilliant blue R-250 (Bio-Rad, Germany) in a solvent mixture containing 40% methanol and 10% acetic acid. After staining, the gels were de-stained in the same solution in the absence of dye until gelatinolytic bands became white against a blue background. De-stained gels were scanned using a Model GS-700 Imaging Densitometer (Bio-Rad). Relative intensities of gelatinolytic bands, representing the gelatinolytic activity, were quantified using GIMP 2.2.10 software. Average pixel intensities of the bands were reduced for the pixel intensity of the gel's background.

To examine the inhibition of enzyme activity, gels were also incubated in reactivation buffer without $CaCl_2$, and reactivation buffer containing 10 mM EDTA or 2 mM o-phenanthroline.

Statistical analysis

Spearman rank correlation coefficients were calculated to correlate the concentrations of gelatinases with each other and with sperm indicators such as sperm concentration, motile sperm concentration, sperm index, metabolic activity, % of morphological normal spermatozoa and % of spermatozoa with damaged acrosome.

In order to investigate the possible association of gelatinases with the above sperm indicators, semen samples were divided into two groups according to a sperm index (SI) of 110 M/ml: group A (SI $\leq$ 110 M/ml; n = 9) and

group B (SI > 110 M/ml; n = 12). A sperm index was calculated by multiplying sperm concentration by the square root SQRT of percentage sperm motility multiplied by morphology (% normal forms) using the lowest values of normal range of semen parameters (Hafez 1993).

The statistical analysis included descriptive statistics for the above indicators. Comparison between the groups of semen samples was performed with Mann-Whitney U test using SigmaStat 3.5 (SYSTAT Software Inc.) software.

Results

Boar seminal plasma contains several proteinase activities that are capable of hydrolysing gelatine. Bands of gelatinolytic activity were observed in a gelatin polyacrylamide gel with Mr values of 30, 32, 34, 40, and 45 kDa, a double band at 59 kDa and bands at 64, 66, 78 and 225 kDa (Fig.1, lane D). Gelatinase forms that were stimulated by $CaCl_2$ and inhibited by EDTA and phenanthroline were considered as metalloproteases.The presence of Ca^{2+} was required for gelatinases with a molecular weight from 59 to 225 kDa – if $CaCl_2$ was omitted from the reactivation buffer, these activities were not observed (Plate IV, Fig.1, lane A). EDTA inhibited gelatinases with the molecular weight higher than 45 kDa (Fig. 1, lane B), whereas phenanthroline inhibited all detected gelatinases (Fig. 1, lane C).

Table 1. Summary of spermatoanalytical and enzymatic examination

	Mean	SD	Min	Max
Spermatoanalytical examination				
Sperm concentration (M/ml)	176.67	56.03	62.83	254.33
Progressive motile sperm conc. (M/ml)	84.26	34.95	18.85	136.13
Sperm index	105.88	40.72	27.32	165.02
Normal morphology (%)	74.42	5.70	63.00	83.67
Acrosome damage (%)	6.84	3.05	1.00	11.00
Resazurin reduction assay (A_{610})	0.0875	0.0418	0.0310	0.1510
Enzymological examination				
64 kDa (pixels)	128.10	41.62	30.33	187.67
66 kDa (pixels)	111.38	53.18	1.00	174.67
78 kDa (pixels)	27.90	15.91	1.00	57.00
225 kDa (pixels)	16.48	9.87	5.83	53.67

Spermatoanalytical and enzymatic examinations are summarized in Table 1. Band intensities at 78, 66 and 64 kDa representing gelatinolytic activity correlate with each other significantly ($P < 0.05$). These metalloproteases also strongly correlated with the percentage of acrosome damage ($P < 0.05$). Activity of 66 kDa also correlated significantly with metabolic activity (resazurin reduction assay). Gelatinolytic activity of 225 kDa correlated only with % acrosome damage. There was a negative correlation between the intensities of 78 and 66 kDa bands and sperm concentration, progressive motile sperm concentration and sperm index (Table 2).

Both bands at 78 and 66 kDa were negatively related to sperm index, sperm concentration and concentration of progressive motile sperm ($P < 0.07$). Other gelatinases with Mr between 32 and 45 kDa correlated with each other (P between 0.008 and < 0.001). Gelatinase at 59 kDa correlated only with gelatinase at 45 kDa ($P = 0.05$). No correlation was observed between sperm characteristics and activity of gelatinases with Mr below 59 kDa ($P > 0.05$).

Significant differences in band intensity of metalloprotease at 66 kDa and metalloprotease at 78 kDa (Fig. 2) were observed between the groups with sperm index below and above 110 M/ml ($P < 0.05$). Furthermore, these groups differed significantly in terms of

Table 2. Spearman rank correlations between semen characteristics and metalloprotease levels

Sperm characteristic and gelatinase	Gelatinase (metalloprotease) Mr			
	64 kDa	66 kDa	78 kDa	225 kDa
Sperm concentration	-0.318 (0.157)	**-0.563 (0.0079)**	**-0.418 (0.0583)**	-0.043 (0.850)
Progressive motile sperm conc.	-0.179 (0.431)	**-0.485 (0.0259)**	**-0.405 (0.0673)**	-0.070 (0.758)
SI	-0.203 (0.370)	**-0.521 (0.0156)**	**-0.422 (0.0439)**	-0.080 (0.724)
Normal morphology (%)	-0.146 (0.520)	-0.338 (0.132)	**-0.442 (0.0558)**	- 0.043 (0.850)
Acrosome damage (%)	**0.441 (0.0452)**	**0.759 (< 0.001)**	**0.747 (< 0.001)**	**0.498 (0.0214)**
Resazurin reduction assay (A_{610})	0.228 (0.314)	**0.455 (0.0375)**	0.355 (0.111)	0.009 (0.694)
66 kDa	**0.800 (< 0.001)**		**0.777 (< 0.001)**	0.236 (0.297)
78 kDa	**0.604 (0.0038)**	**0.777 (< 0.001)**		0.358 (0.109)
225 kDa	0.0914 (0.690)	0.236 (0.297)	0.358 (0.109)	

Statistical significance (P) is shown in brackets. $P < 0.1$ are bolded. $P < 0.05$ was set as significant

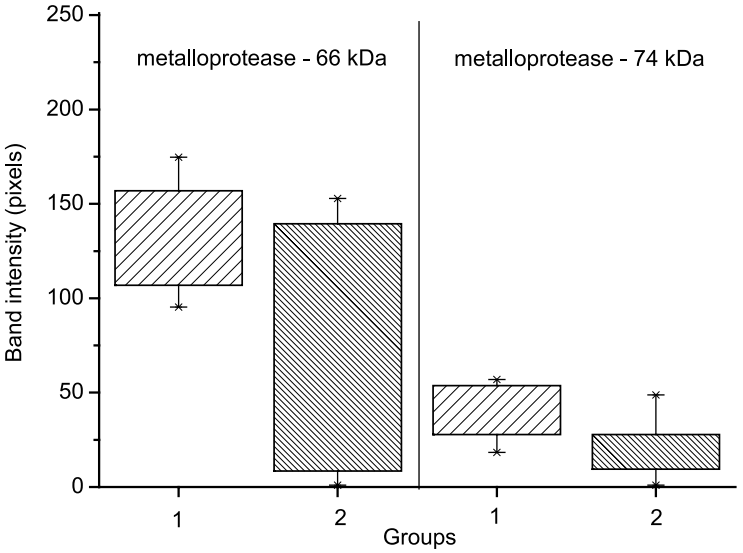

Fig. 2. Band intensity for metalloprotease of 66 kDa and 78 kDa in groups with different sperm indices

sperm concentration, concentration of progressive motile sperm, and metabolic activity ($P < 0.001$), morphology and percentage of acrosome damage ($P < 0.005$).

Discussion

Although gelatinases have been detected in boar epididymal fluids (Metayer et al. 2002), this is the first report of gelatinases in boar seminal plasma and their correlation with semen indicators. The influence of boar breed and age was not the aim of this study. Seasonal effect that could influence the electrophoretic profile of the zymograms was also not a part of this study; all samples were collected in spring.

Using gelatin zymography, we detected several gelatinases in boar semen. The method used can also detect the inactive proforms of gelatinase because SDS causes activation of the enzymes without proteolytic cleavage of the inhibitory N-terminal sequence (Birkedal-Hansen and Taylor 1982).

All gelatinases with molecular weights higher than 45 kDa were metalloproteases, based on inhibition of their enzyme activities by the metalloprotease inhibitors EDTA and phenanthroline, and stimulation of their activity by Ca^{2+}. Based on previous reports (Metayer et al. 2002; Shimokawa et al. 2002; McCauley et al. 2001; Tentes et al. 2007), the bands with 225, 78 and 66 kDa correspond to dimer-MMP-9, proMMP-2 and active MMP-2. The variation in molecular weights (by 10 to 15 kDa) of gelatinases of different origins can be result of different glycosylation (Metayer et al. 2002). Bands with lower molecular weights can represent the degradation products of metalloproteases with enzymatically active domain (Shimokawa et al. 2002). This can be explained because both MMP-2 and MMP-9 have gelatin binding domains in their catalytic domains (Banyai et al. 1994).

Higher values of semen indicators correlate with lower concentrations of metalloproteases in seminal plasma. Similarly, in human seminal plasma a higher concentration of pro-MMP-9 was detected in semen with abnormally low sperm concentration (Tentes et al. 2007). The same study reported that inactive or active forms of MMP-2 and MMP-9 do not correlate with semen indicators. Our results indicate that higher concentrations of metalloproteases of 78, 66 and 225 kDa, that correspond to active MMP-2, pro-MMP-2 and dimer MMP-9, correlate with higher concentrations of acrosome damage. In human sperm samples, MMP-2 and MMP-9 were detected by immunofluorescence in the acrosome region and midpiece (Buchman-Shaked et al. 2002). In the case of abnormal sperms, MMP-9 was also found in the acrosome region (Buchman-Shaked et al. 2002). Since the acrosome is important for the penetration of the oocyte, its integrity is vital for optimal fertilising capacity. In spermatozoa, different proteases are mainly localized within the acrosomal vesicle (Tulsiani et al. 1998). All measured semen indicators were significantly lower in a group with SI < than in a group with SI above 110 M/ml. A negative correlation was observed between metalloproteases (proposed active MMP-2 and pro-MMP-2) and sperm concentration, as well as motility. A higher degree of acrosome damage was observed in a group of semen with SI < 110 M/ml, with significantly higher concentrations of proposed MMP-2 and pro-MMP-2. The acrosome reaction, namely the release of enzymes to penetrate the zona pellucida, should take place at the place of fertilization (Woelders 1991). Keeping these sperm proteases in an inactive form is crucial for maintaining cell integrity to ensure good reproductive function (Uhrin et al. 2000). Therefore, our results suggest that a higher concentration of 66 and 78 kDa metalloproteases (proposed as MMP-2 and pro MMP-2) is the result of acrosome damage. On the other hand, no correlation was observed between sperm characteristics and enzyme activity of gelatinases without metalloprotease activity.

Our results demonstrate that metalloprotease activities correlate with sperm index, which combines sperm concentration, motility and morphology and therefore provides a better evaluating of semen quality than assessing the characteristics mentioned above independently. Therefore, the examination of metalloproteases in seminal plasma by the method used here could be a valuable tool for semen evaluation. Further studies will be focused on the function of metalloproteases in the process of capacitation and maturation of boar spermatozoa.

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Plate IV
Zakošek Pipan M. et al.: Gelatinases in ... pp. 491-496

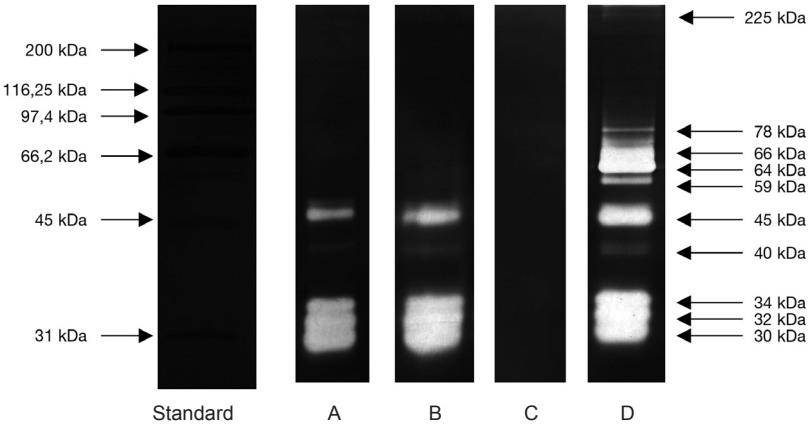

Fig. 1. The presence of gelatinases in boar seminal plasma

Molecular weight of standards is shown on the left. Lanes A, B and C correspond to the gelatinolytic band patterns obtained after reactivation of the enzyme activity without $CaCl_2$, in the presence of EDTA and in the presence of phenanthroline, respectively. Lane D represents different forms of gelatinases with their molecular weight (arrows).