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Quantitative differences in matrix metalloproteinase (MMP)-2, but not in MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1 or TIMP-2, in seminal plasma of normozoospermic and azoospermic patients

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BACKGROUND: Matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), have been detected in reproductive tissues and seminal plasma. The purpose of this study was to quantify MMP-2, MMP-9, TIMP-1 and TIMP-2 in human seminal plasma and to evaluate their association with sperm. METHODS: Seminal plasma was analysed using ELISA assays for all four analytes in 12 normozoospermic and 12 azoospermic patients and then for MMP-2 only in another 114 men with azoospermia ($n = 16$), after vasectomy ($n = 20$) and with sperm counts within the following ranges: 0.3–19 × 10^6/ml ($n = 20$), 20–23 × 10^6/ml ($n = 11$), 49–57 × 10^6/ml ($n = 12$), 96–110 × 10^6/ml ($n = 12$), 139–161 × 10^6/ml ($n = 12$) and 215–346 × 10^6/ml ($n = 11$). Additional zymographic analyses using SDS–PAGE were performed. RESULTS: All investigated MMPs and TIMPs were detected. MMP-9, TIMP-1 and TIMP-2 were not significantly different in normozoospermia and azoospermia. Only the MMP-2 concentration was significantly decreased in azoospermic compared with normozoospermic patients (mean ± SD: 650.6 ± 288.9 versus 1677 ± 910.4 ng/ml respectively; $P = 0.0002$) and significantly correlated with the number of sperm ($r = 0.54$; $P < 0.0001$). CONCLUSION: MMP-2 in seminal plasma was strongly correlated to the sperm count in a linear fashion. Its origin and potential function remain to be elucidated.

Key words: male fertility/MMP/seminal plasma/TIMP/zymography

Introduction

Matrix metalloproteinases (MMPs) form a family of structurally-related, zinc-dependent proteases which are capable of restructuring tissue components by proteolytic degradation of extracellular matrix and basement membrane components (Birkedal-Hansen, 1995). It is for this reason that they are considered important in physiological growth and tissue remodelling and also in cellular involution and apoptosis. However, their involvement in multiple pathological processes, such as tissue destruction in rheumatoid arthritis, neoplastic growth, metastasis and neoangiogenesis, brings them into the focus of new diagnostic and therapeutic strategies (Chambers and Matrisian, 1997).

To date five subgroups, the collagenases, gelatinases, stromelysins, matrilysins and membrane type MMPs are known. Common substrates of the gelatinases MMP-2 and MMP-9 are type I, IV, V, VII and X collagens, elastin, fibronectin and tumour necrosis factor-α. Their activity is co-regulated and inhibited by the tissue inhibitors of metalloproteinases (TIMP) (Woessner, 1994).

MMPs have been linked extensively with female reproductive function (Hulboy *et al*., 1997). During menstruation, their increased expression in the endometrium of the uterine wall (Salamonsen and Woolley, 1996; Xu *et al*., 2000) is believed to contribute to the sloughing process. MMP-2 expression and activity increase at the site of the rupturing follicle in the ovary (Russell *et al*., 1995). Human trophoblasts utilize MMP-2 and MMP-9 in the invasion of the uterine stroma (Hulboy *et al*., 1997).

However, very little is known about the expression and function of MMPs and their inhibitors in the male reproductive tract. MMP-2 as well as TIMP-1 and -2 (Ulisse *et al*., 1994) have been detected in rat Sertoli cell cultures. TIMP-2 extracted from bovine seminal plasma binding to sperm was described and its influence on bull fertility was postulated (McCauley *et al*., 2001). A significant portion of MMPs such as MMP-2 and MMP-9 seem to come from accessory sex glands such as the prostate and the seminal vesicles, as demonstrated by split ejaculate analysis (Yin *et al*., 1990). This is supported by the findings of two other groups who detected MMP-2 gelatinolytic activity in prostatic secretions of benign hyperplastic tissue (Lokeshwar *et al*., 1993; Wilson *et al*., 1993).

Parallel to our investigations, the occurrence of MMP-2 and MMP-9 activity in human seminal plasma has been recently

reported (Shimokawa *et al*., 2002). However, no quantitative data have been described until now using robust assays such as ELISAs.

The purpose of this study was: (i) to quantitate MMP-2 and MMP-9, as well as their inhibitors TIMP-1 and TIMP-2, in human seminal plasma; and (ii) to examine the relationship between their concentrations and the sperm count and other characteristics of sperm.

Material and methods

Study subjects and specimens

Samples of seminal plasma were prepared from ejaculates collected for semen analysis from 138 men who attended the infertility clinic (median age 33 years, range 25–50). This included 12 men with azoospermia (seven idiopathic, five obstructive) and normozoospermia for the initial evaluation of MMP-2, MMP-9, TIMP-1 and TIMP-2. A further 114 patients were then analysed for a possible correlation between MMP-2 and sperm count: 16 patients with azoospermia (10 idiopathic; six obstructive), 20 patients with azoospermia after vasectomy, and 78 patients with increasing sperm counts within the following ranges: 0.3–19$\times10^6$/ml (n = 20), 20–23$\times10^6$/ml (n = 11), 49–57$\times10^6$/ml (n = 12), 96–110$\times10^6$/ml (n = 12), 139–161$\times10^6$/ml (n = 12), 215–346$\times10^6$/ml (n = 11). There were no exclusion criteria such as smoking or alcohol consumption. Ethical approval was obtained from the Ethics Commission at the Charité University Hospital.

Retrieval, analysis and classification of the ejaculates were performed according to World Health Organization recommendations (World Health Organization, 1993). Briefly, samples were obtained by masturbation into a sterile plastic tube after 4–5 days of abstinence. Following physical examination of the ejaculate (pH, volume, consistency, motility, morphology etc.), samples were centrifuged at 3000 *g* and the seminal plasma was stored at –80°C until analysis.

To investigate whether the release of MMPs from sperm was dependent on time after sampling, semen specimens from five patients were divided into five aliquots of 0.3 ml, stored at room temperature (23°C) and centrifuged after 1, 2, 4, 8 and 24 h after sampling. The supernatants were immediately frozen at –80°C for subsequent use.

To characterize the MMP-2 and -9 patterns in sperm, pelleted sperm were suspended in 0.25% Triton X-100 solution after the final centrifugation and an ultrasonic disintegration was performed. Prior to this, the pelleted sperm were washed three times with 5 ml phosphate-buffered saline to avoid any influence of seminal plasma remnants. The homogenate was then centrifuged (13 000 *g* for 15 min) and the supernatant was used for further investigations.

Assays

The quantification of MMP-2, MMP-9, TIMP-1 and TIMP-2 was performed using ELISAs (ELISAs for MMP-2 and MMP-9 from Oncogene, Boston, USA; TIMP-1 and TIMP-2 from Amersham Int., Little Chalfont, UK).

All measurements were performed in duplicate according to the manufacturer's instructions. Briefly, seminal plasma in a 1:5 to 1:51 dilution was placed into microwells equipped with biotinylated monoclonal anti-human MMP-2 antibody and incubated for 2 h. After washing, horseradish peroxidase was added to catalyse the conversion of chromogenic tetramethylbenzidine into a blue solution. Following the addition of 2.5 mol/l sulphuric acid as a stopping reagent, the absorbance was determined using the microplate reader Anthos HTIII (Anthos Labtec Instruments, Salzburg, Austria) with the four-parameter method for calculation of concentrations (Mikrowin software, version 3.0; Mikrotek Laborsysteme, Overath, Germany).

Zymography for MMP-2 and -9 was performed in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE; 8%) containing 0.5% gelatin on the Midget Electrophoresis Unit 2050 (LKB, Uppsala, Sweden). Samples of seminal plasma and sperm extracts were mixed with non-reducing sample buffer and subjected to electrophoretic analysis without boiling. Purified human proMMP-2 and proMMP-9 (Chemicon Inc., Temecula, USA) were included in each gel run as standards. After electrophoresis, gels were soaked for 2 h in 2.5% Triton-X 100 solution with four washing steps. The gels were then incubated for 18 h at 37°C in buffer containing 50 mmol/l Tris–HCl, pH 7.6, 150 mmol/l NaCl, 10 mmol/l $CaCl_2$, 0.2% Brij-35 and 0.02% NaN_3. After incubation, the gels were stained with 0.2% Coomassie Blue and destained until clear proteolytic bands appeared. Gels were scanned using a flatbed scanner (Scanmaker 4; Microtek Lab, Redondo Beach, USA). Studies with inhibitors in the incubation solution (10 mmol/l EDTA or 1 mmol/l o-phenanthroline) verified the specificity of the method. Preincubation of samples with 1 mmol/l p-amino-phenyl-mercuric acetate (18 h, 37 °C) as an in-vitro MMP activator was used to differentiate between latent and active forms of MMPs (Crabbe *et al*., 1993). Controls were performed without activator but with a corresponding volume of buffer solution instead of p-amino-phenylmercuric acetate.

Statistical analysis

Data were analysed using the statistical softwares SPSS 10.0 for Windows (SPSS, Chicago, USA) and GraphPad Prism 3.02 (GraphPad Software, San Diego, USA). Student's t-test, analysis of variance, linear regressions and correlation analysis using the correlation coefficient according to Pearson (r) were performed. $P < 0.05$ was considered significant.

Results

In a first step, seminal plasma samples of men with normozoospermia (group 1; mean sperm count 52 ± 2.2$\times10^6$/ml) and azoospermia (group 2) were analysed using ELISAs for MMP-2, MMP-9, TIMP-1 and TIMP-2. Only MMP-2 (Figure 1) was significantly different (P = 0.0002) in normozoospermic subjects (mean ± SD, 1677 ± 910.4 ng/ml) compared with azoospermic subjects (650.6 ± 288.9 ng/ml). However, with regard to the aetiology of the azoospermia (idiopathic versus obstructive) there appeared to be no differences for any of the investigated proteins. The ratio of MMP-9 either to TIMP-1 or TIMP-2 did not show any significant differences between the groups. No significant correlation was found between MMP-2 and TIMP-2 in the normozoospermic group (r = –0.46, not significant).

As a consequence of these results, only MMP-2 was investigated in more detail. Samples of a vasectomy group with azoospermia and of subjects with sperm counts of 0.3–19$\times10^6$/ml, 20–23$\times10^6$/ml, 49–57$\times10^6$/ml, 96–110$\times10^6$/ml, 139–161$\times10^6$/ml and 215–346$\times10^6$/ml were analysed (Figure 2). Scatter plots in Figure 2 show that the MMP-2 concentration in seminal plasma increased along with rising number of sperm in the ejaculate. Furthermore, there was no significant difference between the vasectomy and azoospermic groups (759.1 ± 335.3 versus 650.6 ± 288.9 ng/ml, Figure 2). Linear regression analysis utilizing the individual MMP-2 concentrations showed a linear relationship between MMP-2

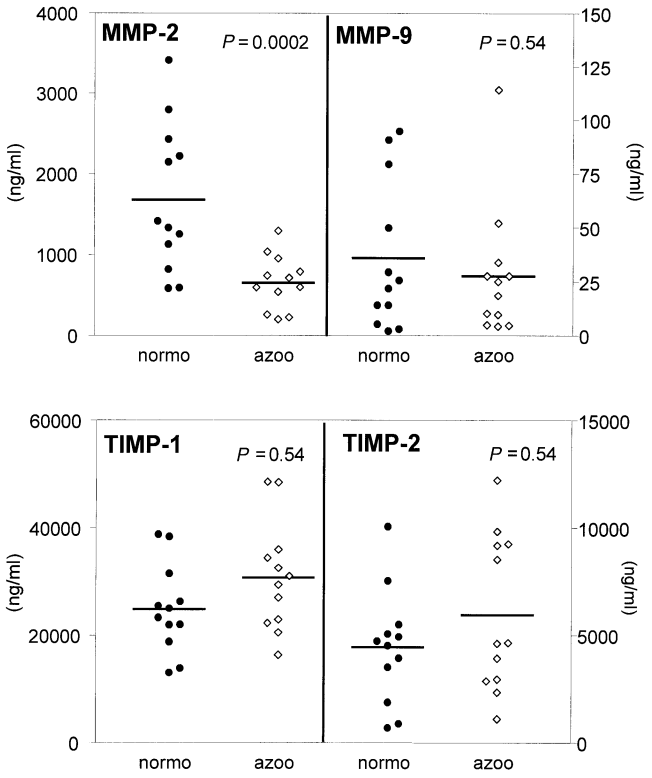

Figure 1. MMP-2, MMP-9, TIMP-1 and TIMP-2 concentrations in the seminal plasma of normozoospermic versus azoospermic individuals. Normo. = normozoospermia; azoo. = azoospermia. Horizontal bars in the scatter plots indicate mean values.

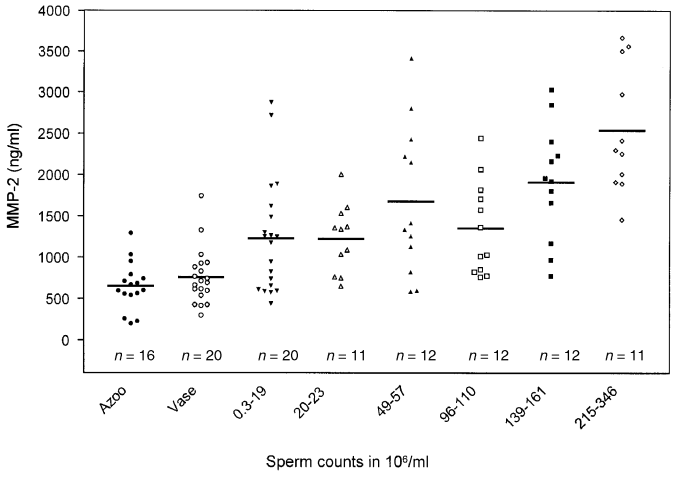

Figure 2. MMP-2 concentrations in seminal plasma prepared from ejaculates with increasing sperm counts. Scatterplots (with mean values as horizontal bars) represent groups within the depicted ranges of sperm counts $\times 10^6$/ml. For further details see Text.

levels and sperm counts ($r = 0.54$, $P < 0.0001$). However, no correlation was found between MMP-2 levels and sperm parameters such as motility or morphology.

To discern any possible influence of leukocytospermia on MMP-2 levels in the experimental groups, a correlation analysis was performed for granulocyte counts and associated MMP-2 concentrations. It showed no significant correlation between these two parameters ($r = 0.007$, $P = 0.96$). Therefore, it can be assumed that the MMP-2 concentrations measured were not a result of a secretion by inflammatory cells.

To further characterize the MMP pattern in seminal plasma, samples were assessed with SDS–PAGE (Figure 3). Figure 3A shows a major band at 72 kDa representing proMMP-2 and a faint band at 92 kDa that is equivalent to proMMP-9. The MMP-2 and MMP-9 patterns are similar in seminal plasma and sperm (Figure 3B). The minor band at 62 kDa corresponds to the active MMP-2 (Figure 3A) confirmed by in-vitro experiments with samples preincubated with 1 mmol/l p-amino-phenylmercuric acetate (Figure 3C). In comparison with control samples without the treatment with p-amino-phenylmercuric acetate, samples preincubated with the in-vitro MMP activator showed the typically changed zymographic pattern: an increased band of the active form at 62 kDa and a decreased band of the proMMP-2 at 72 kDa (Figure 3C). A similar change in the MMP-9 pattern was observed (Figure 3C).

In order to investigate whether the differences of MMP-2 concentration in seminal plasma in normozoospermic versus azoospermic patients resulted from an increased release from the sperm, samples from five patients with differing sperm counts (2.5–247.5$\times 10^6$/ml) were divided into five aliquots of 0.3 ml and centrifuged after liquefaction at 1, 2, 4, 8 and 24 h. MMP-2 concentrations were related to the values measured at the starting point 1 h after liquefaction. They remained stable for the first 2 h and decreased gradually in an almost linear fashion (Figure 4). After 8 h, significant decreases were observed ($P < 0.05$).

Discussion

To date, only scarce knowledge exists about the expression and function of MMPs and TIMPs in the male reproductive system. It is generally believed that the gelatinases MMP-2 and -9 are involved in remodelling processes of structural proteins (Woessner, 1994; Hulboy *et al*., 1997). Gelatinolytic activity coinciding with differentiation stages has been found in maturing prostate and seminal vesicles in a rat model (Wilson *et al*., 1992) and also during prostate involution following castration (Wilson *et al*., 1991). In rat Sertoli cell cultures, MMP-2 was detected and was suspected to contribute to remodelling of the basement membrane during development of the seminiferous tubules and in the release of differentiating germ cells from the basal lamina (Sang *et al*., 1990). Since MMPs and TIMPs are ubiquitary, it seems only logical that they should also be present in seminal plasma.

Utilizing ELISAs for MMP-2, MMP-9, TIMP-1 and TIMP-2 as well as SDS–PAGE, these proteins were identified in all measured specimens. After we had finished our experiments, another group (Shimokawa *et al*., 2002) published similar results. They described several gelatinolytic proteins with molecular weights of 72, 67, 92 and 84 kDa in seminal plasma with gelatin zymography without giving quantitative data. Using Western blot techniques, proMMP-2 (72 kDa) and proMMP-9 (92 kDa), as well as their active forms MMP-2 and -9, were identified. The authors discussed a possible

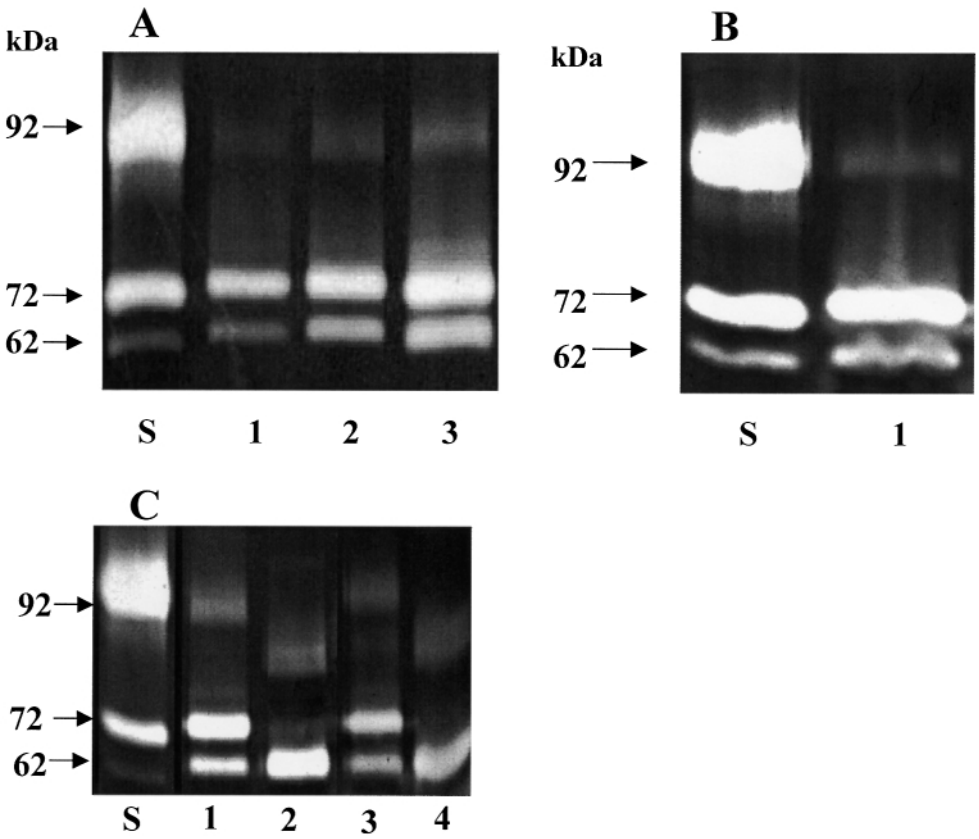

Figure 3. Gelatin zymography of (**A**) seminal plasma samples, (**B**) protein extracts of sperm and (**C**) seminal plasma samples and protein extracts of sperm after preincubation with and without 1 mmol/l p-amino-phenylmercuric acetate. (**A**) Lane S contained standards of MMP-2 (72 kDa) and MMP-9 (92 kDa). Lanes 1–3: typical digestion patterns of seminal plasma of an azoospermic, oligozoospermic and normozoospermic patient respectively. Equal volumes were loaded. (**B**) Lane S contained standards as mentioned; lane 1 presents a digestion pattern of the protein extract of sperm. (**C**) Lane S = standard; lanes 1,3: seminal plasma sample and spermatozoal protein extract after preincubation without 1 mmol/l p-amino- phenylmercuric acetate; lanes 2,4: seminal plasma sample and spermatozoal protein extract after preincubation with 1 mmol/l p-amino-phenylmercuric acetate. For further details see Materials and methods.

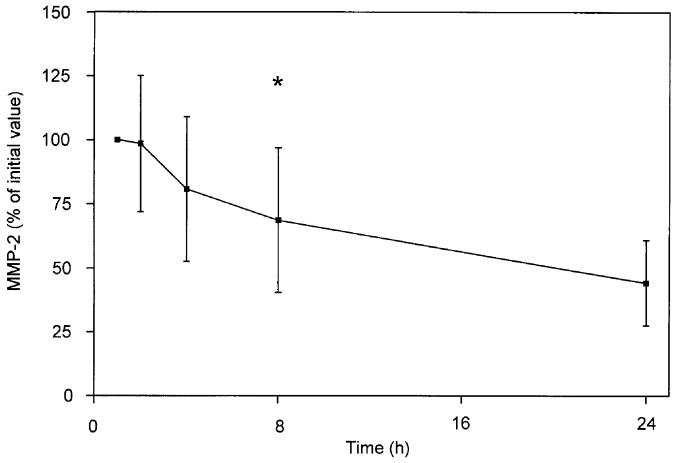

Figure 4. Changes of MMP-2 concentration in seminal plasma with time after ejaculation (ejaculates were stored at room temperature and centrifuged after 1, 2, 4, 8 and 24 h). Percentual changes compared with the initial MMP-2 values 1 h after ejaculation are displayed (percentage means ± SD). Significant decreases were seen from 8 h onwards (*$P < 0.05$).

function of MMPs in liquefaction of the ejaculate. Our results not only confirm the presence of the two MMPs and TIMPs in seminal plasma, but also show quantitative data for the first time. In addition, we could demonstrate a strong correlation between the sperm count and MMP-2, but not MMP-9, TIMP-1 or TIMP-2. There were significantly higher concentrations of MMP-2 in normozoospermic compared with azoospermic plasma. No difference within the azoospermic group with regard to the aetiology, whether obstructive or idiopathic, could be found.

In the present study, the analysis of seminal plasma originating from ejaculates with increasing sperm counts produced evidence of a link between sperm count and MMP-2. This is further supported by the fact that there were no significant differences in MMP-2 levels between azoospermic and vasectomy samples. Since MMP-2 is also secreted by accessory sex glands, the non-linear relationship in the sperm count range of 49–110×10^6/ml could be due to a changed baseline secretion by these glands. However, it remains unclear exactly where MMP-2 is expressed. It has been shown that MMP-2 is secreted by Sertoli cells and seems to be involved in the release of differentiating germ cells from the basal lamina in the seminiferous tubules (Sang *et al.*, 1990). It therefore seems possible that it is also released into the tubular lumen this way.

TIMPs represent natural inhibitors to MMPs (Woessner, 1994). They bind in a 1:1 ratio to the haemopexin domain of

MMPs and interfere with their activation (DeClerck *et al*., 1993). TIMP-1 is specific for MMP-9 and TIMP-2 for MMP-2 (Goldberg *et al*., 1989). In bovine seminal fluid, a 24 kDa heparin-binding protein (HBP-24) was recently described (McCauley *et al*., 2001). This protein bound to sperm membranes was identified as TIMP-2 and was suggested to have a role in bull fertility. The authors suspected an interaction with MMP-2 either expressed in the seminal plasma or directly on sperm (McCauley *et al*., 2001).

However, in somatic cells one way of MMP-2 activation is through formation of a trimolecular complex between MMP-14, TIMP-2 and proMMP-2 on the cell surface (Strongin *et al*., 1995). TIMP-2, which usually acts as an inhibitor to MMP-2, interestingly is also required for its activation (Wang *et al*., 2000) and could be derived from the seminal plasma. Activated MMP-2 is subsequently released into the extracellular matrix. Although not yet reported, the same activation process could theoretically be present on sperm, and was responsible for the detection of MMP-2 in the present study. This would explain the correlation between sperm count and MMP-2 concentration.

MMP-2 concentrations in seminal plasma decreased with time, being significantly lower from 8 h onwards following ejaculation (Figure 4). The decrease was somewhat linear and could be explained by the degradation of MMP-2. This would, however, exclude any prolonged release of MMP-2 by the sperm themselves unless it is outweighed by degradation processes.

In summary, in this work we have reported on the concentrations of MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 in human seminal plasma. MMP-2 was strongly correlated to the sperm count in a linear fashion. Its origin and function remain to be elucidated.

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