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# Presence of Matrix Metalloproteinases and Tissue Inhibitor of Matrix Metalloproteinase in Human Sperm

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**ABSTRACT:** The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that degrade protein components of the extracellular matrix. The necessity of breakdown of physical barriers in the fertilization process suggests that MMPs, along with their tissue inhibitors (TIMPs), might be involved in this task. We have examined the presence of MMP and TIMP in normal and abnormal human sperm samples by gel zymography and Western blot analysis. Thirty-five normal sperm samples and 35 abnormal sperm samples were examined in this study. Gel zymography showed 92-, 72-, 62-, and 28-kd molecular-weight bands exhibiting gelatin-degrading activity in both normal and abnormal sperm samples. The 92-, 72-, and 62-kd bands with gelatinolytic activity are consistent with pro-MMP-9, pro-MMP-2, and active MMP-2, respectively (pro-MMP being the zymogen of MMP). Western blot analysis showed the presence of TIMP-1 in both normal and abnormal sperm samples. A higher 28-kd activity and a lower 92-kd MMP activity in normal sperm samples relative to abnormal samples were detected. No marked difference in TIMP-1, 72-kd, and 62-kd release was observed between normal and abnormal sperm samples. In conclusion, this is the first report of MMP activity in normal and abnormal human sperm samples and of TIMP presence in sperm samples. The data indicate a different MMP profile between normal and abnormal sperm samples, with a higher 28-kd activity and a lower 92-kd MMP activity in normal relative to abnormal samples.

Key words: Fertilization, oligo-terato-asthenospermia, infertility.
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For mammalian fertilization to occur, sperm must first penetrate the mass of cumulus cells surrounding the egg to gain access to the egg surface. The ensuing events proceed as a cascade of cell–cell interactions between gametes of opposite sex (ie, sperm and egg) and can be subdivided into the following 5 steps (reviewed in Wasserman, 1995; Snell and White, 1996): 1) sperm binds to the zona pellucida, a thick extracellular matrix comprising the outer layer of the egg; 2) sperm undergoes the acrosome reaction; 3) acrosome-reacted sperm penetrates the zona pellucida; 4) acrosome-reacted sperm binds to the egg plasma membrane; and 5) acrosome-reacted sperm then fuses with the egg plasma membrane, resulting in a fertilized egg.

The above scenario involves sperm penetration of 2 barriers imposed by the egg, the zona pellucida and the egg plasma membrane, the detailed molecular mechanisms of which are still unclear. The necessity of breakdown of physical barriers in the above cascade of events suggests that matrix metalloproteinases (MMPs) might be involved in this task. The MMPs are a family of proteolytic enzymes that degrade protein components of the extracellular matrix and basement membrane (Salamonsen, 1996; Hulboy et al, 1997). These enzymes, together with their tissue inhibitors (TIMPs), are believed to play a key role in a number of physiological processes, among which are ovulation and implantation (Salamonsen, 1996; Hulboy et al, 1997). Most MMPs are secreted from the cell as inactive zymogens (pro-MMP). Activation requires the disruption of a Cys–$Zn^{+2}$ (cysteine switch) interaction and the removal of a propeptide (Hulboy et al, 1997). In this study, we have examined the presence of MMP and TIMP in normal and abnormal human sperm samples to determine if MMPs and TIMP play a role in fertilization.

## Materials and Methods

### Sperm Preparation and Analysis

Semen samples from 70 men (35 normal fertile donors and 35 men of infertile couples undergoing sperm evaluation at the IVF Clinic, Ha'emek Medical Center, Afula, Israel) were examined according to World Health Organization (1992) laboratory guidelines. The ejaculates were collected by masturbation after 72 hours of sexual abstinence into sterile, plastic-capped, glass containers and allowed to liquefy for at least 30 minutes at room temperature. Semen suspensions were examined in a Makler chamber (Sefi Medical Instruments, Haifa, Israel) and divided

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according to their concentration, linear progressive movement (motility), and morphology.

The semen samples were processed by adding an equal volume of human tubal fluid medium (HTF; Irvine Scientific, Santa Ana, Calif) in a conical falcon tube. (HTF serves as the most common medium for sperm treatment in fertility clinics.) The semen samples were then centrifuged (800 × *g*) for 10 minutes. The supernatant was discarded, and the pellet was suspended in 1 mL HTF medium and centrifuged once again (800 × *g*, 10 minutes) to wash seminal plasma. Two hundred microliters of HTF medium was added to the pellet and resuspended. The resuspended sperm was deposited at the bottom of a tube containing HTF medium and incubated for 1 hour at 37°C and 5% $CO_2$. The sperm was collected, and viability (examined by trypan blue) was found to be above 87%. Sperm concentration was adjusted to $1 \times 10^6$ viable sperm/0.1 mL HTF. Sperm suspensions were incubated overnight at room temperature (except for the MMP/TIMP release experiments, in which the sperm was incubated and the medium collected at 2-hour intervals) and then centrifuged (800 × *g*) for 10 minutes. The supernatant was stored at −20°C for gel zymography and Western blot analyses.

### Gel Zymography

Essentially, the same method as that described by Kleiner and Stetler-Stevenson (1994) was used. Samples for analysis (10 μL of stored supernatant) were prepared by dilution into a buffer (4×) consisting of 0.4 M Tris (pH 6.8), 5% sodium dodecyl sulfate (SDS), 20% glycerol, and 0.02% bromophenol blue. The samples were applied onto an 8% polyacrylamide gel (PAGE) containing 0.5% gelatin. After 90 minutes of electrophoresis, the gel was incubated for 30 minutes at room temperature in 30 mL of 2.5% Triton X-100 on a rotary shaker. The Triton X-100 solution was decanted, replaced with 30 mL of enzyme buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35), and incubated again for 30 minutes at room temperature on a rotary shaker. The solution was decanted, replaced with fresh enzyme buffer, and incubated overnight at 37°C. The gel was then stained with 0.5% Coomassie blue G in 30% methanol and 10% acetic acid for 10 minutes at room temperature on a rotary shaker and thereafter washed with water until clean bands were visualized. Finally, the gel was incubated for 30 minutes in 45% methanol and 5% glycerol prior to drying overnight between sheets of cellophane. Areas of proteolytic activity are visualized by the absence of staining in areas of the gel in which the protein substrate (gelatin) has been degraded, and this was quantitated by densitometric analysis of the zymograms with the Bio Imaging gel documentation system (Dinco & Renium, Jerusalem, Israel) and TINA software (Raytest, Staubenhardt, Germany). The percentage of gelatinolytic activity of a sample was defined as the sample's arbitrary optical density value compared to the optical density value of a control sample. No MMP activity was evident on gel zymography when HTF medium was run alone.

### Western Blot Analysis

Western blot analysis was performed as described in our previous study (Goldman et al, 1997). Sperm suspensions (30 μL of stored supernatant) after SDS-PAGE were blotted onto 0.45-μm nitrocellulose membranes (Schleicher & Schuel, Dassel, Germany). Nonspecific binding sites were blocked by incubating the nitrocellulose membranes overnight with 20% nonfat milk and Tris-buffered saline containing 0.01% Tween-20. The membranes were then washed twice with Tris-buffered saline containing 0.5% Tween-20 and incubated for 1 hour with mouse anti-human TIMP-1 (1:100) or MMP-2 (1:100) and with rabbit anti-human MMP-9 (1:2500) monoclonal antibodies (Oncogene Science, Cambridge, Mass) in 10% nonfat milk and Tris-buffered saline containing 0.01% Tween-20. The membranes were subsequently washed with Tris-buffered saline containing 0.5% Tween-20 and incubated for 1 hour with horseradish peroxidase–conjugated anti-rabbit (1:5000) secondary antibody (Jackson ImmunoResearch, West Grove, Pa) in 10% nonfat milk and Tris-buffered saline containing 0.01% Tween-20. The membranes were washed 3 times with Tris-buffered saline containing 0.5% Tween-20 and then detected by enhanced chemiluminescence (Amersham Int, Piscataway, NJ) and quantitated by densitometry as specified above.

### Immunofluorescence

Fresh sperm suspensions were twice poured slowly on the side of a cold glass tube on ice and then washed twice by centrifugation and resuspension with Dulbecco phosphate-buffered saline (DPBS) at 400 × *g* for 10 minutes. The samples were fixed for 10 minutes in 2% paraformaldehyde and washed by centrifugation and resuspension with DPBS at 400 × *g* for 10 minutes. Remaining steps were carried out at ambient temperature. The samples were incubated for 30 minutes in blocking solution (DPBS plus 50 mg/mL bovine serum albumin). Polyclonal rabbit anti-human MMP-9 (1:100) (Oncogene) or monoclonal mouse anti-human MMP-2 (1:50) (R&D Systems, Abingdon, United Kingdom) was added to sperm samples at a concentration of 1.0 μg/mL (which had in preliminary experiments been determined to allow adequate immunostaining of sperm cells). The samples were incubated for 60 minutes, washed twice, and resuspended in 1 mL DPBS. Fluorescein isothiocyanate–conjugated goat anti-rabbit or donkey anti-mouse immunoglobulin G (Jackson) was added as a second antibody to the samples (1:50 dilution in DPBS), which were then incubated for an additional 60 minutes. After secondary antibody incubation, the cell suspensions were washed twice with DPBS. Sperm cell samples were placed on glass microscope slides with coverslips and a fluoroguard antifade reagent (Fluoromount G, Southern Biotechnology, Birmingham, Ala). The localization of MMP-2 and pro-MMP-9 in the sperm was visually demonstrated by a Bio-Rad MRC 1024 Laser Scanning Confocal Microscope (Bio-Rad House, Hertfordshire, United Kingdom) linked to a Nikon Diaphot 300 microscope (Nikon Corp, Tokyo, Japan) at 1000× and interfaced to a Compaq Prosignia 300 (Compaq Computer Corp, Houston, Tex).

Unless otherwise stated, all materials were obtained from Sigma Chemical Company (St Louis, Mo).

### Statistical Analysis

Statistical analysis of the data was carried out by the Student's *t* test when comparing 2 groups, and analysis of variance was used when comparing more than 2 groups. A *P* value less than .05 was considered significant.

*Semen characteristics* (*mean ± SE*)

| Semen Characteristic | Normal (n = 35) | Abnormal (n = 35) |
|---|---|---|
| Count (per mL) | $49.3 \pm 4.5 \times 10^6$ | $3.9 \pm 0.5 \times 10^{6}$* |
| Motility (%) | 53.7 ± 4.8 | 18.0 ± 2.4* |
| Abnormal forms (%) | 28.7 ± 2.8 | 61.7 ± 6.9* |

* $P \leq .001$ vs normal semen samples.

## Results

### Semen Characteristics

The characteristics of the semen samples obtained from 70 men were evaluated according to World Health Organization (1992) classification. The data shown in the Table demonstrate normal (n = 35) and abnormal (n = 35) semen characteristics (designated ''normal'' and ''abnormal'' hereafter). The low sperm count (oligospermia), decreased linear progressive movement/motility (asthenospermia), and high morphologically abnormal forms (teratospermia) of the abnormal semen group fit the classification of oligo-terato-asthenospermia syndrome. No significant difference was found between the viability (as tested by trypan blue) after overnight incubation at room temperature of normal and abnormal sperm samples (92% ± 5% and 87% ± 4%, mean ± SE, respectively).

### MMP and TIMP in Normal and Abnormal Sperm Samples

Four major bands of gelatin-degrading activity, corresponding to the molecular weights of 92, 72, 62, and 28 kd, were evident on gel zymography of normal sperm samples (Figure 1, lanes 2, 4, and 6). No gelating-degrading activity was evident on gel zymography when HTF medium was run alone. Inhibition of the bands was obtained by incubation with 1,10-phenanthroline (data not shown), as expected for metalloproteinases. The molecular weights of 92, 72, and 62 kd are consistent with those of pro-MMP-9, pro-MMP-2, and MMP-2, respectively. The presence of MMP-2 and pro-MMP-9 was confirmed by Western blot analysis (Figure 2A and B). It is to be noted that an additional band of 28 kd was observed with the MMP-2 monoclonal antibody in the Western blot (Figure 2A). The 28-kd band was not MMP-7 or fertilin, as tested by Western blot (data not shown).

The bands obtained by gel zymography were quantified by densitometric scanning. The results, described in Figure 3A, showed that the dominant MMP in normal sperm samples was the 72-kd band with relatively lower band intensities at 92, 62, and 28 kd ($P < .05$, $P < .05$, and $P < .01$, respectively). Western blot analysis demonstrated the presence of TIMP-1 in normal sperm samples (Figure 4).

Three major bands of gelatin-degrading activity, corresponding to the molecular weights of 92, 72, and 62 kd, were evident on gel zymography of abnormal sperm samples (Figure 1, lanes 3, 5, and 7). A very faint band of 28 kd could also be seen. The molecular weights of 92, 72, and 62 kd are consistent with those of pro-MMP-9, pro-MMP-2, and MMP-2, respectively. The presence of pro-MMP-9 and MMP-2 was confirmed by Western blot analysis (Figure 2A and B). The bands obtained by gel zymography were quantified by densitometric scanning. The results, described in Figure 3A, showed no significant differences between the band intensities at 92, 72, and 62 kd; however, a significantly less intense band at 28 kd was shown ($P < .001$). Western blot analysis demonstrated the presence of TIMP-1 in abnormal sperm samples (Figure 4).

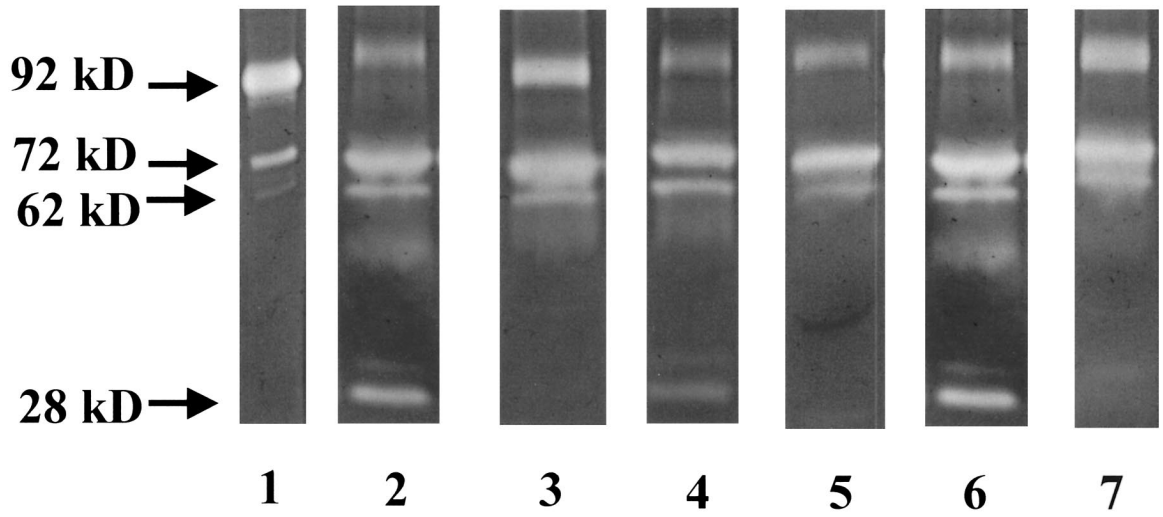


Figure 1. Gel zymography of representative normal and abnormal sperm samples. Molecular-weight markers were run in parallel. A commercial positive control of 92-, 72-, and 62-kd matrix metalloproteinases (MMPs; Chemicon Int, Temecula, Calif) was run in lane 1; 3 normal sperm samples were run in lanes 2, 4, and 6; and 3 abnormal sperm samples were run in lanes 3, 5, and 7.

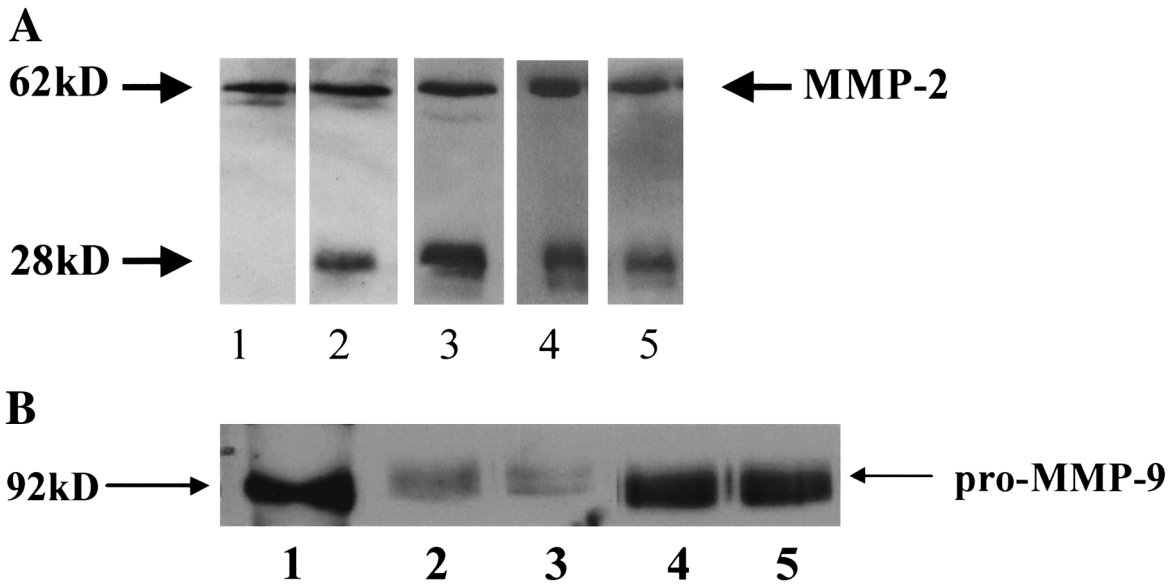


Figure 2. Western blot autoradiographs of matrix metalloproteinase (MMP-2) and pro-MMP-9 (pro-MMP being the zymogen of MMP) samples of normal and abnormal sperm samples. **(A)** Two normal sperm samples were run in lanes 2 and 3, and 2 abnormal sperm samples were run in lanes 4 and 5. MMP-2 standard was run in lane 1. The autoradiograph shown is that of a representative experiment repeated on 20 normal and 20 abnormal sperm samples. **(B)** Two normal sperm samples were run in lanes 2 and 3, and 2 abnormal sperm samples were run in lanes 4 and 5. Pro-MMP-9 standard was run in lane 1. The autoradiograph shown is that of a representative experiment repeated on 20 normal and 20 abnormal sperm samples.

### *MMP and TIMP Expression in Normal vs Abnormal Sperm Samples*

A comparison of normal and abnormal sperm samples revealed that in normal samples, 28-kd activity was 4.5-fold higher ($P < .001$) and that 92-kd activity was significantly lower ($P < .05$) than in abnormal samples (Figure 3B). No significant difference was found when bands between 72 and 62 kd (Figure 3A) and the TIMP-1 band (Figure 4) were compared with normal and abnormal sperm samples using either the gel zymography or Western blot technique (Western blot data not shown).

### *Time Course of MMP and TIMP Release*

The time course of MMP and TIMP-1 release is shown in Figure 5. Pro-MMP-9, pro-MMP-2, MMP-2, and TIMP-1 reached maximal values at 2 hours and then dropped sharply to reach very low (pro-MMP-9) or even undetectable values after a 10-hour incubation. On the other hand, the 28-kd band was maximally active at 4 hours and kept 40% of this activity after 10 hours of incubation.

### *MMP-2 and MMP-9 Localization*

MMP-2 was detected by immunofluorescence in normal (Figure 6A and B) and abnormal sperm samples (Figure 6C and D) in the acrosome region and in the midpiece.

Weak pro-MMP-9 labeling was found mostly in the midpiece and flagella but was almost undetectable in the acrosome region in normal sperm samples (Figure 6E and F). In sharp contrast, abnormal sperm samples showed strong MMP-9 labeling in the acrosome region, midpiece, and flagella (Figure 6G and H).

## *Discussion*

Although many proteases, such as ADAM (a disintegrin and metalloprotease) (Wolfsberg et al, 1995) and endoproteases (Diaz-Perez et al, 1988; Diaz-Perez and Meizel, 1992; Chesneau et al, 1996), have been identified in sperm, this is the first report of MMP and TIMP presence in normal and abnormal human sperm samples.

Gel zymography showed 92-, 72-, 62-, and 28-kd molecular-weight bands exhibiting gelatin-degrading activity. The 92-, 72-, and 62-kd bands with gelatinolytic activity are consistent with pro-MMP-9, pro-MMP-2, and MMP-2, respectively.

Since Western blot analysis with a monoclonal antibody against MMP-2 showed—apart from the expected MMP-2 band—a lower-molecular-weight 28-kd band, as was also observed in gel zymography, this latter band could be a degradation product of MMP-2. Proteolytic processing of MMPs generating lower-molecular-weight products has been frequently reported (see, for example, Okada et al, 1988; Bjarnson and Fox, 1995; Shipley et al, 1996; Stanton et al, 1998). Moreover, unlike normal sperm samples in which a 28-kd band with gelatinolytic activity was clearly seen on gel zymography as well as Western blot analysis, in abnormal sperm samples, an enzymatically active 28-kd band was barely detected in gel

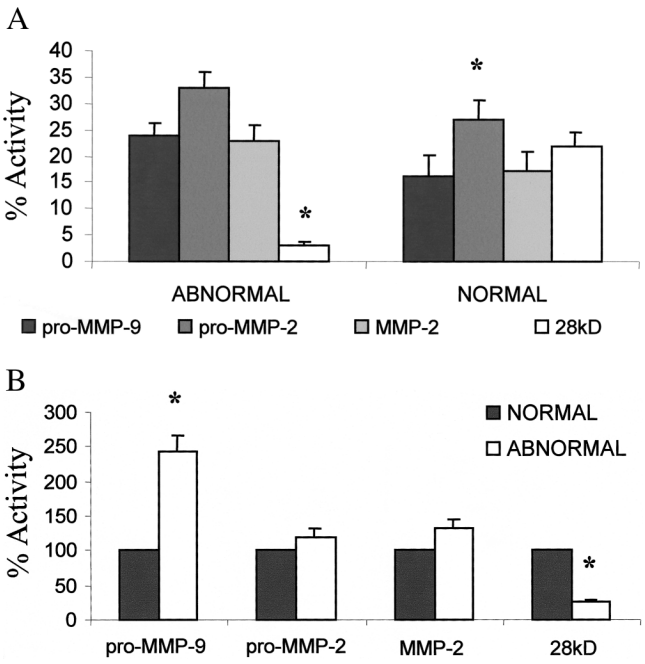


Figure 3. Densitometry of pro-MMP-9, pro-MMP-2, MMP-2 (pro-MMP being the zymogen of matrix metalloproteinase [MMP]), and 28-kd bands obtained by gel zymography (see Figure 1) of the 35 normal and 35 abnormal sperm samples (mean ± SE). **(A)** Histograms representing gelatinolytic activity in normal and abnormal sperm samples (each band expressed as the percentage of total [100%] gelatinolytic activity). * $P <$ .05. **(B)** Comparison of normal and abnormal sperm samples, expressed as the percentage of gelatinolytic activity of abnormal relative to normal sperm samples (normal sperm regarded as 100%). * $P <$ .05.

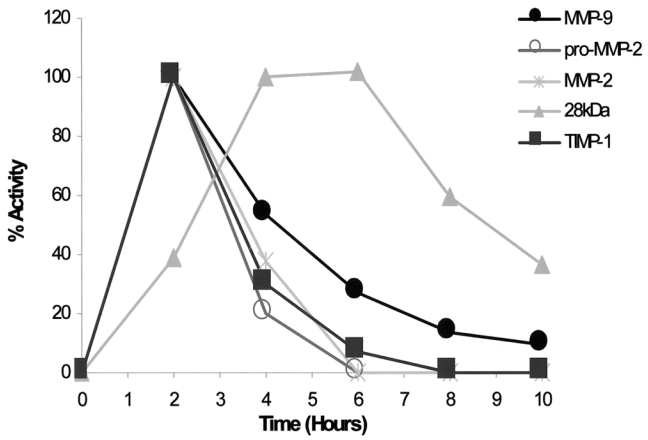


Figure 5. The time course of pro-MMP-9, pro-MMP-2, MMP-2 (pro-MMP being the zymogen of matrix metalloproteinase [MMP]), 28-kd band, and tissue inhibitor of MMP (TIMP-1) release. Sperm samples were washed twice with human tubal fluid (HTF) medium, (0 time), and then aliquots were collected at 2-hour intervals and analyzed by gel zymography or, in the case of TIMP-1, Western blot. Each point is expressed as the percentage of maximal (100%) activity.

zymography but was clearly observed by Western blot. This may indicate that such a 28-kd protein is found in both normal and abnormal sperm samples but is active only in normal sperm. It may therefore be speculated that a lack of 28-kd activation may be characteristic of certain types of male infertility. Of course, experimental verification is necessary to test the possibility of MMP-2 cleavage generating an active/inactive 28-kd product.

Comparison analysis showed no significant difference in 72-kd MMP, 62-kd MMP, and TIMP-1 between normal and abnormal human sperm samples. In contrast, a higher 28-kd MMP activity and a lower 92-kd MMP activity in normal relative to abnormal sperm samples were detected. The relevance to male infertility, if any, of MMP and TIMP found in sperm samples is presently unknown.

The time-course experiments showed that pro-MMP-9, pro-MMP-2, MMP-2, and TIMP-1 reached maximal release after 2 hours, while the 28-kd protein reached maximal activity after 4 hours. It may therefore be possible, that regarding the 28-kd protein, release is being stimulated by one of the MMPs or TIMP-1 or that, as mentioned earlier, the 28-kd product is an MMP degradation product. MMP-9 and the 28-kd protein, in contrast to pro-MMP-2 and MMP-2, remained visible on gel zymography in the time-course experiment even after 10 hours. This may be the result of a gradual release of pro-MMP-9 and 28-kd protein. A possible explanation for the quick release of MMPs into the medium is that MMPs are

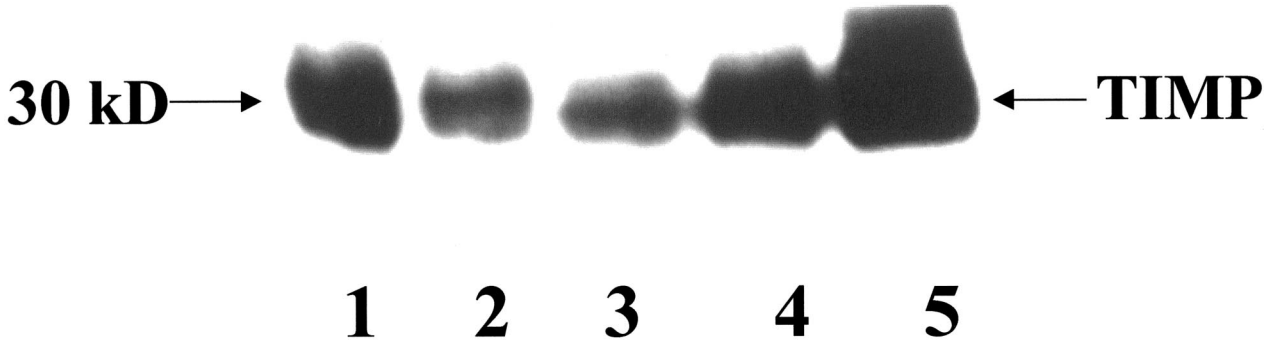


Figure 4. Western blot autoradiograph of tissue inhibitors of matrix metalloproteinases (TIMP-1) in normal and abnormal sperm samples. Two normal sperm samples were run in lanes 1 and 3, and 2 abnormal sperm samples were run in lanes 2 and 4. TIMP-1 standard (kindly donated by Dr H. Nagase) was run in lane 5. The autoradiograph shown is that of a representative experiment repeated on 23 normal and 23 abnormal sperm samples.

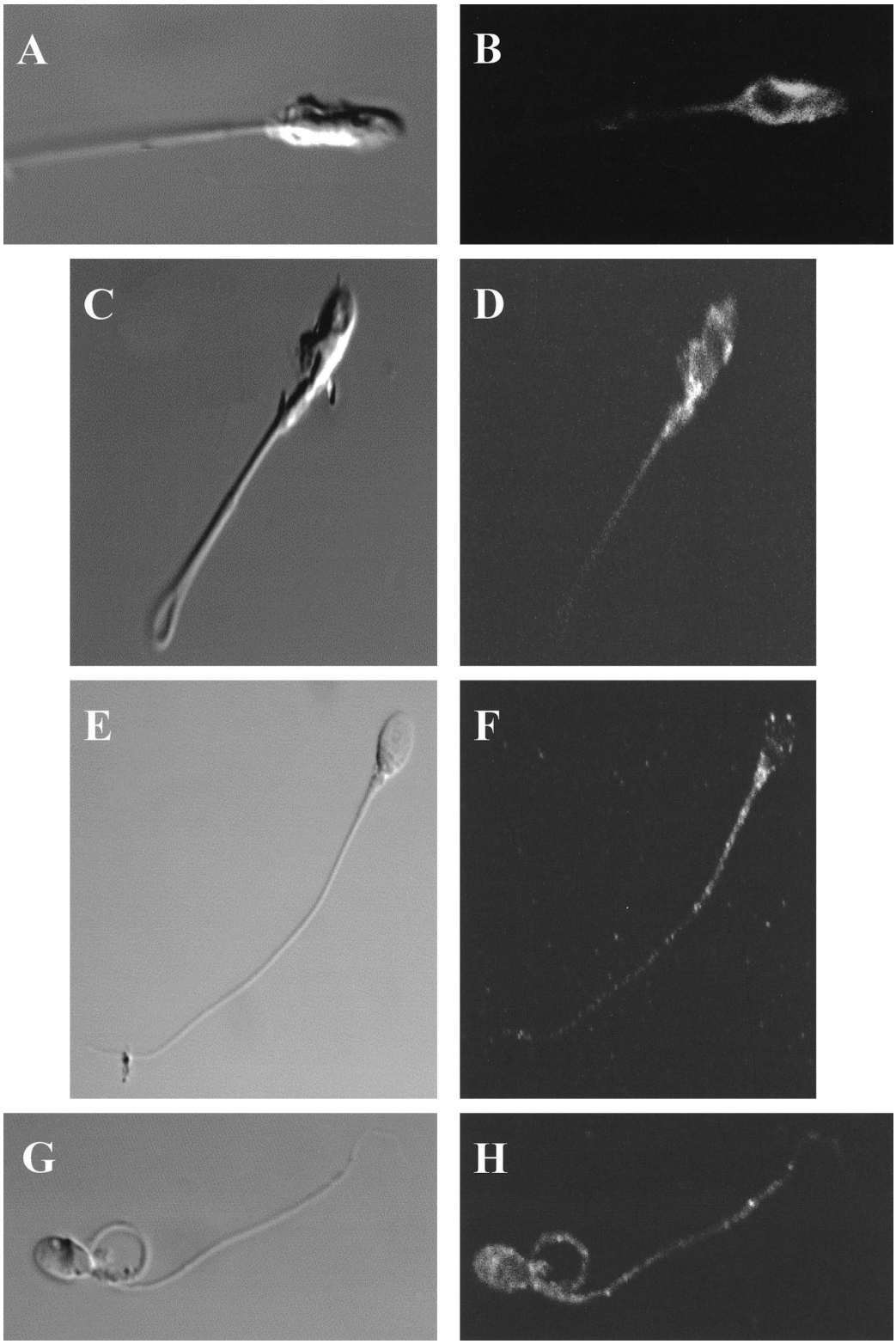


Figure 6. Localization of matrix metalloproteinase (MMP-2) and pro-MMP-9 (pro-MMP being the zymogen of MMP) in human sperm. Phase contrast of MMP-2 (**(A)** normal, **(C)** abnormal) and MMP-9 (**(E)** normal, **(G)** abnormal) and indirect immunofluorescence staining of MMP-2 (**(B)** normal, **(D)** abnormal) and MMP-9 (**(F)** normal, **(H)** abnormal) in human sperm samples.

bound to the sperm from accessory sex gland secretions upon ejaculation. This process is analogous to the situation in mice-ejaculated spermatozoa, which have urokinase associated on the cell surface (Huarte et al, 1987). The mouse spermatozoa pick up urokinase from the vas deferens and seminal vesicle secretion. However, the repeated washing steps of the ejaculates, together with the immunofluorescence experiments demonstrating the presence of MMPs in different regions of the sperm, indicate that the above explanation is a remote one.

The time-course experiments together with the immunofluorescence assays suggest that most of the MMPs were secreted within a short time after sperm was incubated with the capacitation medium (HTF). A fast protein release from the sperm, as observed with MMP-2, can take place if the MMP is localized in the acrosome region. The immunofluorescence assays support this hypothesis, since MMP-2 in normal and abnormal sperm samples and pro-MMP-9 mostly in abnormal sperm samples were found localized in the acrosome region.

In conclusion, the necessity of breakdown of physical barriers in the fertilization process suggests that MMPs are involved in this task. This is the first report of MMP activity in normal and abnormal human sperm samples and of TIMP presence in sperm samples. The data indicate a different MMP profile between normal and abnormal sperm samples, with a higher 28-kd MMP activity and a lower 92-kd MMP activity in normal relative to abnormal samples.

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