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Mast cell–sperm interaction: evidence for tryptase and proteinase-activated receptors in the regulation of sperm motility

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BACKGROUND: The detection of significant levels of tryptase in human seminal plasma and follicular fluid and of tryptase-positive mast cells (MCs) in the wall of human Fallopian tubes lead us to hypothesize that tryptase may exert regulatory actions on human spermatozoa. METHODS AND RESULTS: Immunoelectronmicroscopy revealed proteinase-activated receptor 2 (PAR-2) in the membranes of the acrosomal region and midpiece of human spermatozoa. These PAR-2 were functional, as exposure of spermatozoa from healthy men (n = 12) with regular standard semen parameters to human recombinant tryptase significantly decreased motility in a dose- and time-dependent fashion. Motile spermatozoa (WHO a + b) were significantly decreased within 10 min of incubation with 1.000 ng/ml tryptase (P = 0.045). After 30 and 60 min, significant reduction of motility was also observed in the presence of lower tryptase concentrations (100 ng/ml, P = 0.037; 10 ng/ml, P = 0.046). The inhibitory effects of tryptase progressed throughout an observation period of 180 min. Furthermore, tryptase effects were reversible after washing procedures and could be inhibited by pretreatment with anti-tryptase antibody or anti-PAR-2 antiserum. CONCLUSIONS: The observations presented raise the possibility that tryptase directly interacts with human spermatozoa during their migration through the female genital tract. Genital tract MCs and their products may be as yet unrecognized factors involved in human fertility/sterility.

Key words: motility/PAR-2/spermatozoa/tryptase

Introduction

Mast cells (MCs) are commonly found in connective tissue or in mucosa of many organs including skin, airways and digestive tract. They are also present in testis and epididymis (Behrendt *et al*., 1981; Maseki *et al*., 1981; Nistal *et al*., 1984) and the female reproductive tract (Ferenczy *et al*., 1972; Tursi *et al*., 1984; Sandvei *et al*., 1986). Although the physiological role of MCs has not been established, they are known to be involved in inflammatory, hypersensitivity and fibrotic disorders (Yong, 1997; Metcalfe *et al*., 1999). The effects of MCs are thought to be mediated by soluble substances, namely biogenic amines, proteoglycans and prostaglandins, but also by neutral proteases (Schwartz *et al*., 1984; Yong, 1997; Metcalfe *et al*., 1999). In addition, there is growing evidence for a role of MCs in host immune defense and autoimmunity (Benoist *et al*., 2002; Malaviya *et al*., 2002). After antigen uptake and presentation, a variety of receptors and cytokines, including tumour necrosis factor-α, granulocyte–macrophage colony-stimulating factor, transforming growth factor-β, interferon-γ and several interleukins, are expressed and secreted. Therefore, MCs contribute to immunological reactions by affecting lymphocyte growth, recruitment and function (reviewed in Artuc *et al*., 1999; Bradding *et al*., 1999; Henz *et al*., 2001). Recent studies have shown that MCs also play a crucial role in innate immunity (Galli *et al*., 1999; Mekori *et al*., 2000).

In humans, the content of the proteases tryptase and chymase allows one to distinguish tryptase-containing MC_T cells from tryptase- and chymase-containing MC_{TC} cells (Welle, 1997; Yong, 1997). MC_T and MC_{TC} cells in human testes are increased in number in patients with spermatogenic arrest and Sertoli-cell-only syndrome (Jezek *et al*., 1999; Meineke *et al*., 2000; Yamanaka *et al*., 2000), suggesting MC involvement in the pathogenesis of infertility.

Importantly, tryptase is a serine protease, which can specifically regulate functions of cells expressing proteinase-activated-receptor 2 (PAR-2) (Nystedt *et al*., 1996; Molino *et al*., 1997; Frungieri *et al*., 2002), whereas other PARs are not activated by tryptase (Mirza *et al*., 1997). These G-protein-coupled receptors are expressed on the surface of keratinocytes, fibroblasts, enterocytes, endothelial cells, smooth

muscles, T-cell lines and certain tumour cell lines (Nystedt *et al*., 1996; Corvera *et al*., 1997; Frungieri *et al*., 2002). Recently, the presence of PAR-2 could be demonstrated on cells of the testicular germinal epithelium (D'Andrea *et al*., 1998). Whether this result indicates a role of tryptase in germ cell development is currently not known. Likewise, it has not been explored whether PAR-2 is present in mature ejaculated spermatozoa or whether tryptase may affect its functions. In this case, tryptase would be required to be present in seminal plasma and/or fluids of the female reproductive tract. The purpose of the present study was to examine the possibility that sperm function is regulated by MCs/MC products. Therefore, we determined tryptase concentrations in seminal plasma, located PAR-2 on human spermatozoa and studied effects of tryptase and chymase on sperm motility.

Materials and methods

Chemicals

Recombinant human tryptase (activity >1200 U/mg protein as measured by the thiobenzyl/DTNB) was obtained from Promega (Mannheim, Germany). Buffers and chemicals were purchased from Sigma (Deisenhofen, Germany) unless otherwise mentioned. Heparine was obtained from Merck (Darmstadt, Germany) and mouse-anti-tryptase-IgG monoclonal antibody from Pharmacia & Upjohn (Dübendorf, Germany). Immunoelectronmicroscopy of PAR-2 was performed with affinity-purified goat anti-PAR2 polyclonal antiserum raised against a peptide mapping near the carboxyterminus of human PAR-2 that is not cross-reactive with PAR-1, PAR-3 or PAR-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Human tubular fluid medium (HTFM) was obtained from Stefan Gück GmbH (Berlin, Germany). Recombinant human chymase was supplied by Norman Schechter (University of Pennsylvania, Philadelphia, PA, USA; Wang *et al*., 1998).

Sperm preparation

Fresh semen was collected from healthy students ($n = 12$) who had no history of diseases related to infertility, and who had given informed consent. The ejaculates were analysed for standard semen parameters according to the World Health Organization (WHO, 1999). Motility was determined by computer-assisted semen analysis CASA (Strömberg-Mika). Only ejaculates with $<1 \times 10^6$ white blood cells/ml and motility (WHO a + b) >50% or progressive motility (WHO a) >25% were used for the experiments. In all experiments samples were washed (500 *g*, 10 min) and resuspended in HTFM.

Localization of MC in human Fallopian tubes

Tissue samples of human Fallopian tubes from autopsies of women <40 years old with no known pathology of their genital tract were examined by immunohistochemistry for the presence of tryptase-positive MCs according to previously described procedures (Meineke *et al*., 2000).

Localization of PAR-2 on spermatozoa

Immunoelectronmicroscopy for PAR-2 was performed in ejaculated spermatoza [fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.05 mol/l phosphate-buffered saline (PBS), pH 7.4] using Lowicryl (K4M; Polysciences, Eppenheim, Germany) with goat anti-PAR-2 polyclonal antibodies and a secondary gold-labelled antiserum (1:20; 10 nm; Aurion, Wageningen, The Netherlands). Electron microscopy was performed with a Zeiss Electron microscope EM10 using a previously described method (Mayerhofer *et al*., 2001). For control purposes, the antiserum was omitted or replaced with normal rabbit serum.

Measurement of tryptase in human seminal plasma and human follicular fluid

Fresh semen was collected from male patients ($n = 110$, aged 18–65 years) attending the andrological outpatient department and from 25 healthy donors (aged 18–55 years) with no history of diseases related to infertility, all of whom gave informed consent. Semen analysis was performed according to the WHO (1999) and included determination of pH, volume, total sperm count, sperm density, motility, morphology (Shorr stain), vitality (eosin stain), peroxidase-positive leukocytes, granulocyte elastase and α-glucosidase. Seminal plasma was obtained by repeated centrifugation (1000 *g*, three times for 15 min).

Follicular fluid was obtained from women ($n = 8$, aged 32–39 years) undergoing IVF. Tryptase levels were determined by a solid-phase commercial radioimmunoassay (Pharmacia tryptase RIACT; Castells and Schwartz, 1988). The detection limit of this assay was 1 µg/l.

Effects of tryptase/chymase on human spermatozoa

Stock solutions of 100 µg/ml tryptase in a buffered solution (pH 6.1) containing 10 mmol/l MES (morpholinoethansulfonic acid), 200 mmol/l NaCl, 10% glycerole, 0.5 mg/ml heparine and 100 µg/ml chymase in 2.0 mol/l NaCl and 1 mmol/l MOPS [3-(*N*-morpholino)-2-hydroxypropanesulfonic acid] (pH 6.8) were prepared and kept frozen at –20°C. Prior to the beginning of experiments, stock solutions were diluted in PBS (pH 7.4) to achieve final concentrations of 10, 100 and 1.000 ng/ml. Ejaculates were washed twice (500 *g*, 10 min) and resuspended in HTFM. Sperm concentration was adjusted to $10–20 \times 10^6$/ml prior to incubation with different concentrations of tryptase or chymase. Samples incubated with buffer or PBS in the absence of tryptase/chymase were measured as controls. After 30, 60 and 180 min, various sperm motility parameters were determined by CASA: motility (% WHO a/b/c), curvilinear velocity (VCL), average path velocity (VAP) and straight-line velocity (VSL). Since incubation with 1.000 ng/ml tryptase demonstrated the most rapid results, and this concentration was used for further studies.

In order to test for reversibility of tryptase/chymase effects, spermatozoa were washed twice with HTFM to remove the protease after 60 min of incubation. Sperm motility was determined again after 10 min.

To examine specific mediation of tryptase effects by PAR-2, pretreatment with either anti-tryptase antibodies or anti-PAR-2 antibodies was performed. Therefore, stock solutions of 100 µg/ml mouse anti-tryptase antibodies (in 100% PBS, pH 7.2) were prepared and kept frozen at –20°C. For experiments, stock solutions were diluted in PBS containing 0.1% sodium acide and 0.2% gelatin to achieve final concentrations of 10, 100 and 1.000 ng/ml. After 5 min of incubation with different concentrations of antibodies tryptase was added, and sperm motility was determined after 30, 60 and 180 min. Untreated spermatozoa or samples that were treated with antibodies or tryptase only were measured as controls.

Statistical analysis

Data are presented as mean ± SD. For graphical visualization only upper limits are drawn to indicate the limits of uncertainty. Statistical comparisons were made by ANOVA followed by Scheffe test. All tests were performed two-sided. Analysis was made using SPSS 11.0 software. P values ≤ 0.05 were considered statistically significant.

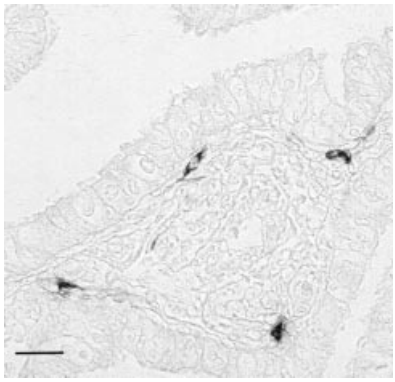

Figure 1. Immunohistochemical localization of tryptase-immunoreactive MCs in the human Fallopian tubes. Four MCs can be seen directly under the epithelium. Bar = 40 μm.

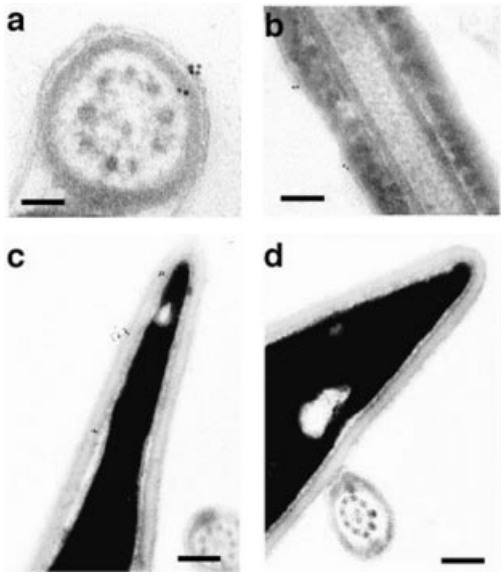

Figure 2. Immunoelectronmicroscopy of PAR-2 on human spermatozoa. Receptors can be found at the plasma membranes of the acrosomal region and the flagellum. Control (in which antibody was omitted) is shown in (**d**). Scale bars: **a** = 250 nm; **b** = 100 nm; **c** = 200 nm; **d** = 150 nm.

Results

Tryptase in human seminal fluid and human follicular fluid

Tryptase could be detected in 14.4% of 110 seminal plasma samples from andrological patients with a mean of 4.18 ± 1.95 ng/ml tryptase and values ranging from 1 ng/ml (detection limit) to 18.7 ng/ml. Twenty percent of 25 seminal plasma samples from healthy donors exhibited detectable levels of tryptase with a mean of 1.66 ± 0.65 ng/ml. Levels of tryptase were not correlated to semen parameters (motility, morphology, peroxidase-positive cells, granulocyte elastase).

Tryptase concentrations in human follicular fluid ranged from 1.60 to 3.73 ng/ml.

Immunohistochemical localization of MC in human Fallopian tubes

Tryptase-immunoreactive MC were localized in the muscle layer and subepithelial layer of the Fallopian tubes (Figure 1). All controls performed were negative (not shown).

Detection of PAR-2 on spermatozoa by immunoelectronmicroscopy

PAR-2 was localized at the plasma membrane and outer acrosomal membrane of human spermatozoa. In addition, it was found at the plasma membrane of the flagellum. All controls were negative (Figure 2).

Effects of tryptase/chymase on human sperm motility

Tryptase significantly reduced sperm motility in a dose- and time-dependent fashion. Motile spermatozoa (WHO a + b) were significantly decreased after 10 min of incubation with 1.000 ng/ml tryptase (P = 0.045), an effect that progressed significantly throughout the observation period (180 min). Lower concentrations also reduced motility, and these effects became statistically significant within 30 min (100 ng/ml tryptase; P = 0.037) or 60 min (10 ng/ml; P = 0.046; Figures 3 and 4). No significant effects of tryptase were observed concerning VCL, VAP or VSL.

The inhibitory effect of tryptase (60 min treatment) was reversible after washing twice with HTFM; impaired motility improved significantly (P = 0.014), returning to the levels of untreated controls.

Pre-incubation with monoclonal mouse anti-tryptase IgG prevented tryptase action, resulting in significantly increased motility after 30 (P = 0.006), 60 (P = 0.003) and 180 min (P = 0.004), i.e. levels similar to those of untreated controls. Furthermore, tryptase effects were abolished by pretreatment with goat anti-PAR2 antiserum. Compared with controls treated with tryptase only, samples with 1.000 ng/ml antibodies showed a significantly higher motility after 30 (P = 0.013), 60 (P = 0.004) and 180 min (P = 0.001), which was not different from untreated spermatozoa (Figure 3).

Sperm motility was not significantly influenced after incubation with 10 and 100 ng/ml chymase. However, a significant (P = 0.042) decrease of sperm motility was observed 60 min after incubation with 1000 ng/ml chymase. Incubation for 180 min had no further effects. Reduction of sperm motility by chymase was not reversible. After two washing procedures, motility parameters did not recover (Figure 5).

Discussion

The results of this study demonstrate that the MC product tryptase, via PAR-2, directly reduces human sperm motility in a dose- and time-dependent manner. These results could be of physiological and/or pathophysiological relevance, since the activator of PAR-2, tryptase, is present in seminal plasma, and probably in tubular fluid, as suggested by tryptase-containing MCs in the wall of human Fallopian tubes.

Our results showing tryptase-positive MCs under the lining epithelium of human Fallopian tubes are in concordance with and extend previous studies, which showed MCs in the female genital tract (Sivridis *et al*., 2001; Hunt *et al*., 2002). MCs were

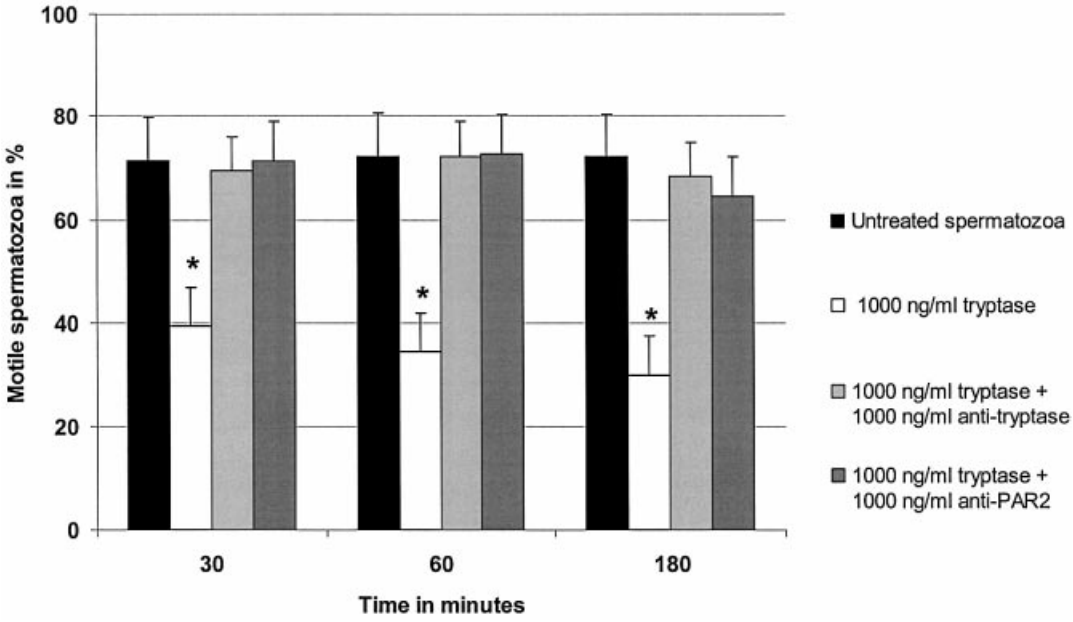

Figure 3. Inhibitory effects of tryptase on sperm motility and their inhibition by anti-tryptase and anti-PAR-2 antibodies (n = 12/group). An asterisk indicates statistically significant difference from control ($P < 0.05$).

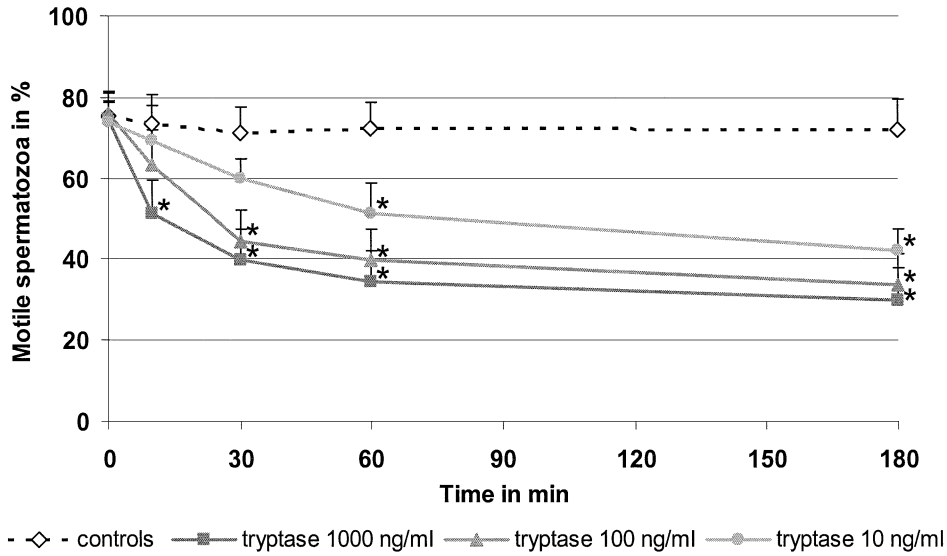

Figure 4. Time dependency of tryptase effects on sperm motility. Impairment of motility progressed throughout the observation period. Compared with baseline, reduction of motility became statistically significant after 10 min in samples treated with 1000 ng/ml tryptase, after 30 min of incubation with 100 ng/ml and after 60 min treatment with 10 ng/ml. An asterisk indicates statistically significant difference from baseline ($P < 0.05$).

demonstrated in the oviduct wall (Hunt *et al*., 2002), where they were found to be increased in women using intrauterine contraceptive device (Tursi *et al*., 1984; Sandvei *et al*., 1986). MCs are also normal constituents of the human myometrium and endometrium, where they are considerably reduced and depleted of metachromatic granules during the immediate premenstrual phase (Sivridis *et al*., 2001), implying cycle-related regulation. It should be noted that histological sections of the human oviducts for the present study were obtained from autopsies of women <40 years old without any known pathology of their genital tract. While clearly showing MC_Ts, it remains unclear whether tryptase from the tubal or uterine MCs can reach the lumina of these organs. To our knowledge, neither concentrations of tryptase in the uterus nor in Fallopian tubal fluid have been reported. Since we were not able to obtain human tubular fluid, tryptase was measured in follicular fluid from women undergoing IVF. Follicular fluid upon ovulation may also reach the tubal lumen and may thus be of relevance. Tryptase concentrations in this fluid ranged from 1.60 to 3.73 ng/ml (Tryptase CAP-FEIA; Pharmacia; n = 8; 2.71 ±

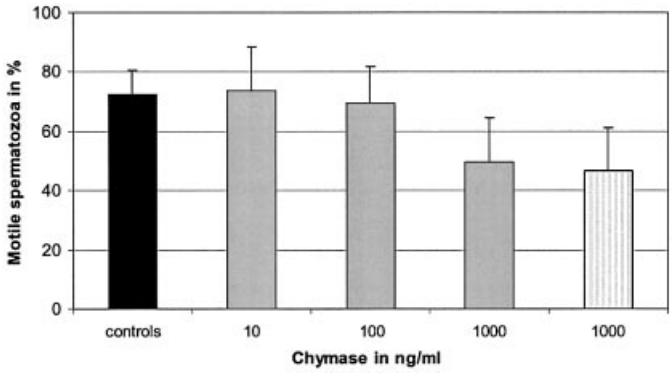

Figure 5. Impairment of sperm motility after incubation with chymase for 60 min. Reduction of sperm motility by 1000 ng/ml chymase is not reversible after two washing procedures. An asterisk indicates statistically significant difference from control ($P < 0.05$).

0.66 ng/ml). We have, of course, to take into consideration the hormone treatment of these patients and the unknown effects of stimulation therapy on genital tract tryptase levels. Nevertheless, our results provide evidence for the presence of MCs and tryptase in the female genital tract and thus imply that spermatozoa are likely to come into contact with tryptase during their migration through the female genital tract under physiological conditions.

After ejaculation, spermatozoa on their way through the female genital tract may encounter tryptase released from MCs not only of female origin, but also from the male, since seminal plasma contains significant levels of tryptase. This was found in 14.4% of ejaculates examined from 110 andrological patients and 20% of seminal plasma samples examined from healthy donors. The effects of tryptase as observed in the present study were caused by concentrations of as low as 10 ng/ml (12 mU/ml). Thus, in at least a certain percentage, the levels of tryptase in seminal plasma were sufficiently high. The effective concentrations were thus comparable to the ones shown to induce a contractile response in dog airways or potentiate human isolated bronchi contractility (Sekizawa *et al*., 1989; Berger *et al*., 1999).

At the present time it is not clear whether tryptase in the seminal plasma is produced in the testes, deferent ducts, epididymes or accessory genital glands. Further experiments with ejaculates from vasectomized men and immunohistochemical examinations of biopsies from these organs may allow to clarify this aspect. Although we have not found any obvious correlation between seminal plasma concentrations and semen quality or seminal plasma markers for genital tract infection (granulocyte elastase, peroxidase positive cells) so far, further studies including male patients with different classifications of infertility are in progress in order to study this point in more detail.

The present results provide clear evidence for the ability of tryptase to impair sperm motility. These observed inhibitory effects of tryptase are mediated by PAR-2, which we located in the acrosomal region and midpiece of human spermatozoa. They were proven to be reversible and specific, since they could be abolished by anti-tryptase antibody and anti-PAR-2 antiserum. PAR-2 are G-protein-coupled receptors that are expressed in the germinal epithelium (D'Andrea *et al*., 1998). Activation of PAR-2, which in some cell types has been shown to induce transient rises in intracellular Ca^{2+} (Kunzelmann *et al*., 2002; Miki *et al*., 2003). Whether this is also the case in human spermatozoa is currently unknown. However, preliminary data reveals no significant effects of tryptase on acrosomal reaction.

In conclusion, we provide evidence for a direct, specific, non-proteolytic and receptor-mediated effect of tryptase on human spermatozoa. The MC product chymase, a protease not interacting with cell-specific receptors, also inhibited sperm motility. However, these effects were irreversible, indicating that they are due to proteolytic activity.

Our data may also give some clues concerning a mechanisms underlying the reported improvement of pregnancy rates after treatment of infertile men with MC blockers like ketotifen (Schill *et al*., 1986; Yamamoto *et al*., 1995). It appears conceivable that by inhibiting the release of (male) MC products, including tryptase, into the seminal fluid, the inhibitory effect of tryptase on sperm motility was reduced, resulting in enhanced fertility potential.

Although testis and epididymis are known sources of MC products (Maseki *et al*., 1981; Nistal *et al*., 1984; Meineke *et al*., 2000; Yamanaka *et al*., 2000), details of these locations as sources of tryptase in the male efferent duct system and female genital tract remain to be revealed. Tryptase in the female genital tract indicates the interesting possibility that tryptase released from MCs of the female affects spermatozoa.

Further elucidation of these points will be necessary to clarify whether pharmacological inhibition of tryptase release or interference with the tryptase/PAR-2 signalling system may be of benefit for couples suffering from reduced fertility. Thus, our study raises the question whether MCs/MC products may qualify as targets for therapeutical interventions in both female and male infertility.

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