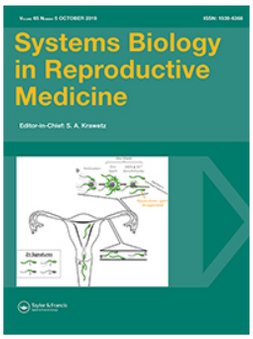

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RESEARCH ARTICLE

Monocyte-derived extracellular trap (MET) formation induces aggregation and affects motility of human spermatozoa *in vitro*

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ABSTRACT

The presence of bacteria and/or leukocytes can alter semen quality resulting in low sperm quality and infertility. Inflammation or infection increases the numbers of PMN or macrophages/monocytes in male genital tract. Release of extracellular traps (ETs) by leukocytes has been recognized as a novel mechanism of early host innate immunity, in response to invasive pathogens. This is the first work that evaluated the mechanism of triggered ETs in monocytes co-incubated with spermatozoa or bacteria and the effect on sperm function. Selected spermatozoa and human monocytes isolated from peripheral blood were obtained by healthy donors. Two experimental models were developed, one aseptic (non-infectious) incubating spermatozoa and monocytes, and septic models (infectious) incubating spermatozoa with monocytes and uropathogenic *Escherichia coli* (*E. coli*). ETs of monocytes (METs) (DNA, global histone and citrullinated histones) were visualized by scanning electron microscopy (SEM) and immunofluorescence analyses. Progressive motility was performed at 0, 10, 30, 60, and 180 min after co-incubation with CASA system. SEM- and immunofluorescence-analyses revealed human spermatozoa alone or in the presence of *E. coli* as strong inducers METs. In aseptic model, the motility decreased to 65.2 ± 3.5% at 10 min of incubation and 29.3 ± 3.3% at 30 min ($p < 0.001$). In septic model, motility decreased to 44.5 ± 5.9% (10 min) and 12.7 ± 2.2% (30 min) ($p < 0.001$). MET-derived small spermatozoa aggregations were observed in both models. METs might physically block spermatozoa and decrease motility after a brief contact. This may impair male fertility, especially in patients with genital tract infections or chronic inflammation.

Abbreviations: PMN: polymorphonuclear; ETs: extracellular traps; *E. coli: Escherichia coli*; METs: ETs of monocytes; SEM: scanning electron microscopy; NE: neutrophil elastase; MPO: myeloperoxidase; MAGI: male accessory gland infection; PBMC: peripheral blood mononuclear cells; RT: room temperature; CFU: colony forming units; CASA: computer-aided sperm analysis; H4Cit3: histone H4 citrullinated 3

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Introduction

Release of extracellular traps (ETs) by activated leukocytes has been recognized as a novel mechanism of early host innate immunity, in response to invasive pathogens (Brinkmann et al. 2004). The formation of ETs prevents the dissemination of pathogens in the tissue, in which the plasma membrane breaks down, allowing the release of chromatin after the collapse of the nuclear membrane, in a process called ETosis (Fuchs et al. 2007; Brinkmann and Zychlinsky 2012; Granger et al. 2017). The production of ET is not exclusive to mammals or vertebrates; they can also be produced by leukocytes from invertebrates, insects and plants (Altincicek et al. 2008; Wen et al. 2009; Hawes et al. 2011).

Molecular composition of ETs has intensively been investigated during recent years. Alongside the backbone composed by DNA and citrullinated histones, mammalian-derived ETs comprise a number of molecules which impart antimicrobial effect including neutrophil elastase (NE), myeloperoxidase (MPO), lactoferrin, pentraxin, cathepsin G, bacterial permeability increasing protein and defensins

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(Papayannopoulos et al. 2010; Goldmann and Medina 2013; Silva et al. 2016; Granger et al. 2017).

In recent years, it has become evident that ETs are not exclusively released by PMN but also other leukocytes, including mast cells (von Köckritz-Blickwede et al. 2008), eosinophils (Yousefi et al. 2008; Muñoz-Caro et al. 2015), macrophages (Chow et al. 2010; Wei et al. 2018) and monocytes (Muñoz-Caro et al. 2014; Reichel et al. 2015; Pérez et al. 2016; Yang et al. 2018). Although a vast amount of ETosis data in humans focused on infective agents, a recent study demonstrated the significance of human PMN-derived NETosis against spermatozoa thereby decreasing progressive sperm motility in human spermatozoa exposed to NETs (Zambrano et al. 2016).

The presence of bacteria and/or leukocytes can alter semen quality, i.e., sperm integrity and function (Hahn et al. 2012; Aitken and Baker 2013; Fraczek and Kurpisz 2015) resulting in low sperm quality and infertility (Pelliccione et al. 2009; Tremellen and Tunc 2010; Fathy et al. 2014). Consequently, monocytes/macrophages have an important function in response to different infectious diseases of the female/male reproductive system such as trichomoniasis (Kim et al. 2016) candidiasis (Dominguez-Andrés et al. 2017), urogenital *Escherichia coli* (*E. coli*)- and Chlamydia trachomatis-infections (De Clercq et al. 2014). Whilst monocyte-released ETs (METs) have been recently described to efficiently entrap protozoan parasites, i.e. *Toxoplasma gondii* (Reichel et al. 2015), *Besnoitia besnoiti* (Muñoz-Caro et al. 2014) and *Candida albicans* (Halder et al. 2016) nothing is known on their effects on urogenital pathogens of the human reproductive tract. The increase of macrophages in semen is related mainly to chronic epididymitis (Haidl et al. 2008) and prostatitis (Schuppe et al. 2017). Indeed, urogenital infections and inflammatory disease within the male genital tract are considered significant etiological factors in male infertility, with prevalence rates of 6–15% reported from andrological outpatient clinics (Tüttelmann and Nieschlag 2010; Punab et al. 2017; Olesen et al. 2017). These conditions have been defined as 'male accessory gland infection (MAGI)' (Rowe et al. 2000). As most infertile patients with MAGI present high numbers of PMN and monocytes yet nevertheless asymptomatic and without any signs during clinical examination, the diagnostic approach is entirely based on laboratory study (Haidl et al. 2008; Schuppe et al. 2017).

Sexually transmitted bacteria or urinary tract pathogens represent the most frequent etiology of local ascending infections resulting in MAGI, or even epididymitis and epididymo-orchitis (Pilatz et al. 2015; Calogero et al. 2017). Elimination of a bacterial infection, however, does not always lead to the recovery of semen quality and spermatozoa function, especially in patients suffering acute epididymitis (Rusz et al. 2012). There is suggestive evidence that persistence of an inflammatory response, reflected by increased numbers of PMN or macrophages/monocytes, may exert detrimental effects on spermatozoa by overproduction of reactive oxygen species (ROS), pro-inflammatory cytokines, and other compounds such as NE (Moretti et al. 2005; Aitken and Baker 2013; Hagan et al. 2015).

The putative impact of METosis on human reproduction and its association with the deterioration of sperm function require *ex vivo* and *in vitro* studies. Considering the abundance of PMN in human semen, NETosis has been the focus of attention (Zambrano et al. 2016). ETosis in monocytes/macrophages possibly induced by human spermatozoa have also been considered. Respective underlying mechanisms and consequences on male fertility have not been addressed until now. Therefore, we established an *in vitro*- model, mimicking either 'inflammatory conditions' by co-incubating human monocytes and spermatozoa, or an 'infectious' state, by co-incubating monocytes, spermatozoa and E. coli, to investigate the activation and release of METs.

Results

Human spermatozoa induce METosis

Human spermatozoa induced METosis in aseptic as well as septic conditions was considered. SEM images revealed that exposure of human monocytes to spermatozoa resulted in the formation of extracellular structures derived from DNA of the nucleus of monocytes, both thin and thick, in contact with these gametes. MET formation in the aseptic model entraps spermatozoa mainly through the midpiece and tail in small groups of two to three, forming aggregates. The spermatozoa presented morphological changes mainly in the shape of the tails, which were coiled (Figure 1A–C).

The images of monocytes with spermatozoa plus *E. coli* (septic model) (Figure 1D–F) showed a larger number of monocytes forming METs, with some more elongated fibers and a larger number of spermatozoa being trapped by the head, midpiece and tail. In relation with monocytes morphology, most were activated, with an irregular membrane surface and early formation of fine but short prolongations (Figure 1D–F).

To confirm classical MET components (DNA, global histones and H4Cit3) in spermatozoa-triggered structures, fluorescence-based analyses were performed. Sytox Orange staining (Figure 2A) shows the DNA nature of extracellular MET-like structures being formed by human monocytes after exposure of motile spermatozoa. Additionally, global histone and H4Cit3

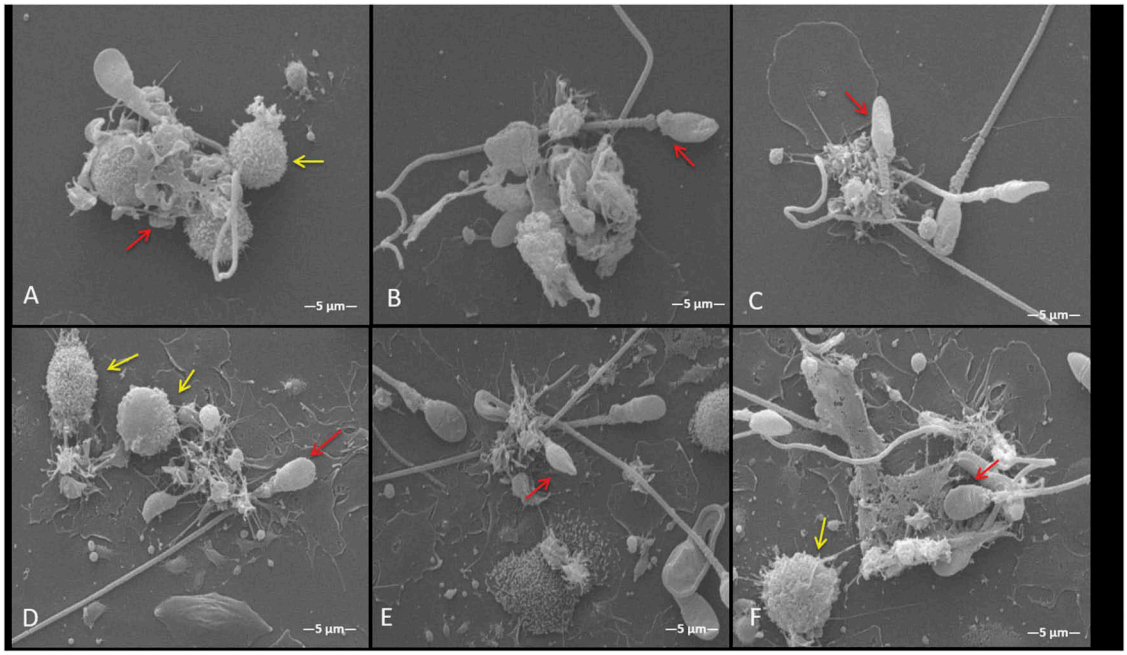

Figure 1. Human spermatozoa-induced monocyte extracellular trap (METs) visualized by scanning electron microscopy (SEM). Co-incubation of human monocytes with spermatozoa (aseptic conditions) (A–C) and spermatozoa-*E. coli* (septic conditions) (D–F) after 180 min exposure. Overview of the physical structure of METs composed of thin and thick nets that adhere and trap the spermatozoa, forming small sperm aggregates, producing alteration of the morphology mainly of the middle piece and spermatic tail (A–C). In the presence of *E. coli* (D–F), monocytes produce greater METs formation. Yellow arrows indicate monocytes and red arrows indicate spermatozoa being trapped by METs.

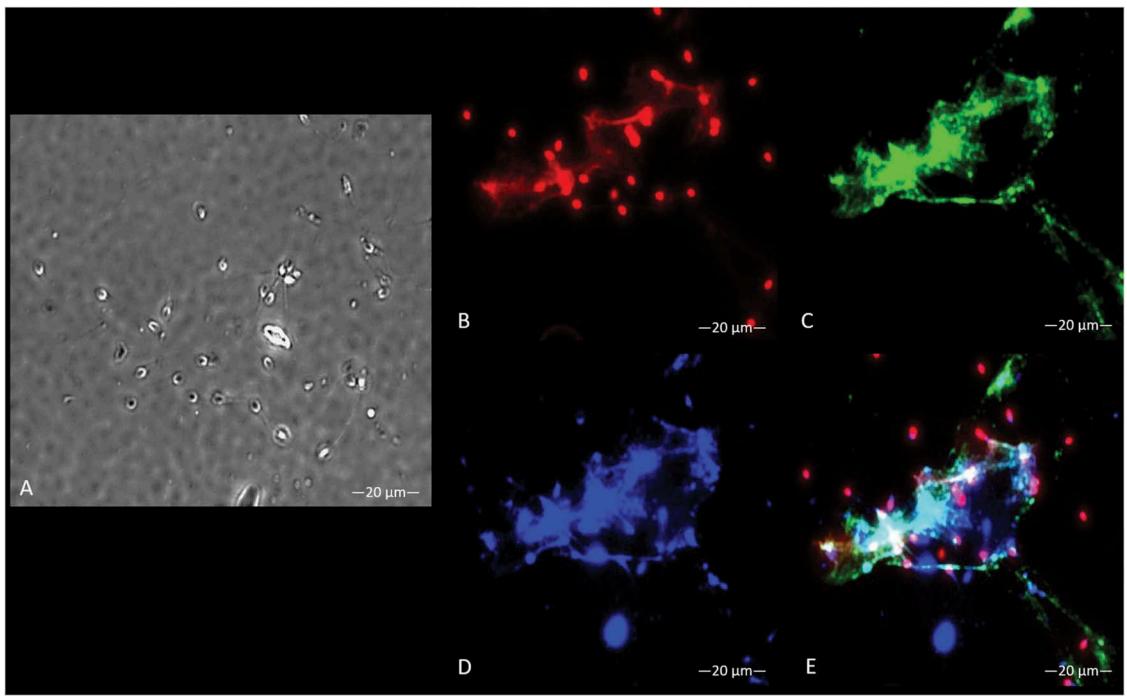

Figure 2. Immunofluorescence analyses on human spermatozoa-induced monocyte extracellular trap (METs) formation. Co-localization experiment of extracellular DNA and histones in spermatozoa-induced METs structures using DNA-marker Sytox Orange and anti-global histones H1, H2A/H2B, H3, H4, and anti-histone H4 citrullinated 3 antibodies. (A) Phase contrast. (B) Extracellular DNA stained with Sytox Orange (red). (C) Anti-global histone (green). (D) Anti-histone H4 citrullinated 3 (blue). (E) Merge. Full color available online.

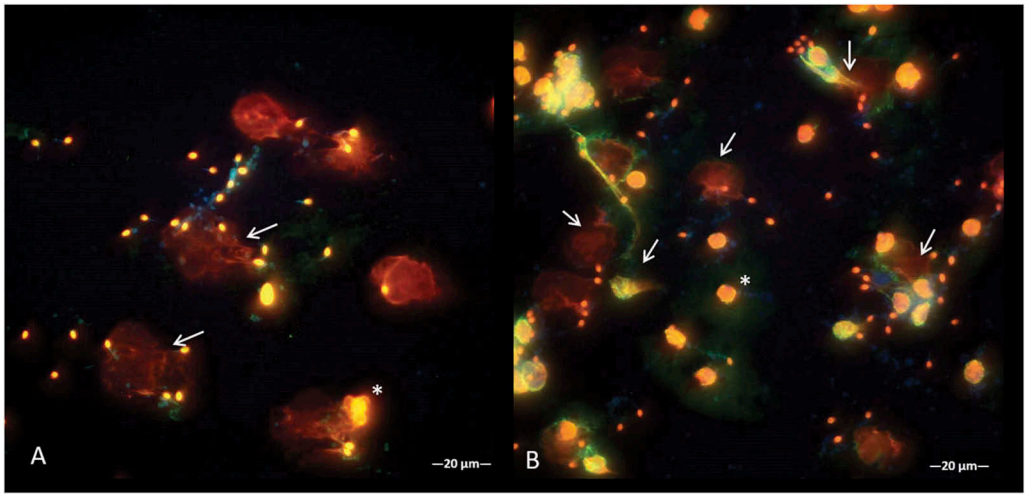

Figure 3. Representative images of the co-localization areas of three immunofluorescence channels (merge) experiment on extracellular DNA and histones in spermatozoa-induced METs structures ('diffuse' METs) (white arrow) (*indicate monocytes). (A) Co-incubations of monocytes + spermatozoa. (B) Co-incubations of monocytes + spermatozoa + *E. coli*.

positive signals were detected in colocalization with DNA-positive MET structures (Figure 3B,C). SEM analysis shows the morphological changes of activated monocytes as well as the formation of METs were exacerbated in co-incubation with spermatozoa and *E. coli*. In both experimental settings METs were mainly of '*diffuse*' MET-types (*diff*METs), composed of a complex of extracellular decondensed chromatin with globular and compact form with a size of 25–28 nm diameter as previously reported (Muñoz-Caro et al. 2015, 2018) (Figure 3A,B white arrow).

MET decreases sperm motility due to entrapment

The sperm preparations tested at baseline presented a progressive motility greater than 91.3 ± 2.6% (motility control) (Table 1). In the aseptic model, there was a decrease to 65.2 ± 3.5% at 10 min and 29.3 ± 3.3% at 30 min of co-incubation of spermatozoa with monocytes ($P< 0.001$). However, in the septic model, the loss of progressive motility was highly significant with values of 44.5 ± 5.9% after 10 min of incubation and 12.7 ± 2.2% after 30 min ($P< 0.001$) when compared to motility control. In addition, MET-derived small aggregations of spermatozoa were observed in both models, those that were evaluated by optical microscopy (40x) at each time point presented characteristics similar to what is described in the WHO (2010) guidelines, such as immobile spermatozoa entrapment to cells, which is different from spermatic agglutination. After 60 min of incubation, in both models, very few motile sperm were observed (3.5% and 2.0%, respectively, $P< 0.001$), and only '*in situ*' movement was observed. After 180 min of incubation, all spermatozoa were found immotile and most formed part of these MET-derived sperm aggregations.

Discussion

The etiology of male infertility is multifactorial and presents differences in terms of prevalence (Agarwal et al. 2015), being higher in countries where health care is inadequate (Ahmed et al. 2010) and with a higher rate of infection of the male genital tract (Weidner et al. 2013; Schuppe et al. 2017). In patients with infection or inflammation of accessory glands, sperm function is not only altered in presence of microorganisms, but also by the effector action of leukocytes such as PMN and macrophages which are associated

Table 1. Progressive motility (%) in selected spermatozoa incubated with monocytes or monocytes and *E. coli* for 180 min at 37°C.

Time (minutes)	Spermatozoa (Control motility)	Spermatozoa/ *E. coli*	Monocytes/spermatozoa (Aseptic model)	Monocytes/ermatozoa/*E.coli*(Septic model)
0	91.3 ± 2.6	-	-	-
10	90.0 ± 1.0	63.0 ± 3.7*	65.2 ± 3.5*	44.5 ± 5.9*
30	87.3 ± 2.1	35.5 ± 4.8*	29.3 ± 3.3*	12.7 ± 2.2*
60	86.7 ± 4.8	12.9 ± 3.9*	3.5 ± 1.3*	2.0 ± 1.6*
180	84.5 ± 6.5	1.0 ± 5.7*	0	0

The results correspond to the mean ± SD of five biological replicas of monocytes and spermatozoa donors by each group. *Indicates significant differences compared with control motility (spermatozoa incubated with medium only). Differences were determined as significant at a level of $P < 0.001$.

with chronicity (Fraczek and Kurpisz 2007). Spermatozoa damage caused by the accumulation of leukocytes is produced mainly by increased ROS and pro-inflammatory cytokine (i.e., IL 6, IL 8, and TNF) secretion which may induce apoptosis (Henkel et al. 2005; Motrich et al. 2006; Perdichizzi et al. 2007; Allam et al. 2008) and/or peroxidative damage of spermatozoa membrane. In addition, the persistence of oxidative stress may produce continuous spermatozoa damage (Fraczek and Kurpisz 2007) even though the infection has been eradicated (Calogero et al. 2017).

In vivo, the relevance of monocytes/macrophages and their diverse reactions in innate immunity during infection or chronic inflammation are related to the production of pro-inflammatory cytokines and antibacterial components (i. e. neopterin, IDO) as well phagocytosis (Murr et al. 2002); however, new evidence, particularly in PMN has recently shown that they also undergo ETosis as a powerful and effective effector mechanism of innate immunity.

Results of this study reveal for the first time that monocytes in contact with spermatozoa are rapidly activated inducing formation of METs. MET-entrapped spermatozoa formed small aggregates, with consequent immobilization after a short incubation period, as motility significantly decreased after 10 min of co-incubation with these professional phagocytes. These 'non-infectious' model results are of importance considering the fact that seminal macrophages are thought to originate mainly from epididymis in human patients and increased monocyte numbers have been associated with chronic inflammation of epididymis (Haidl et al. 2008).

In the 'infectious' model, the motility decreased even more rapidly and SEM analysis demonstrated increased production of fine networks from monocytes which were projected toward the spermatozoa head, midpiece and flagellum. It is important to consider that in this experimental setting monocytes encountered dual stimulation, namely spermatozoa and *E. coli* as well. It is important to consider the direct effect of *E. coli* on the spermatozoa. In previous studies, we have already evaluated the effect of *E. coli* and its soluble products during their interaction with human spermatozoa (Schulz et al. 2010). In the septic model, monocytes not only reacted with spermatozoa but also against bacteria and therefore the increased production of MET and direct effect of E. coli, drastically affected sperm motility.

Human METosis has characteristics similar to previous reports on human NETosis in terms of being generated by a variety of stimuli (Brinkmann et al. 2004; Brinkmann and Zychlinsky 2012). Various infectious agents such as bacteria, fungi and protozoa (Reichel et al. 2015) induce ETosis, as well as products of bacterial degradation and inflammatory stimuli such as IL 8 (Brinkmann et al. 2004), in addition to reacting with spermatozoa (Zambrano et al. 2016). According to our results, extracellular chromatin acts like a sticky and physical barrier not only to trap E. coli but also motile spermatozoa.

Monocytes/macrophages are key cells in many pathological contexts and accumulate quickly in tissues during inflammation, not only by infectious diseases but other pathologies. ETs have several common characteristics, regardless of the type of cells releasing them. The main ET backbone is composed of DNA strand embedded with histones, antimicrobial peptides and proteases (von Köckritz-Blickwede and Nizet 2009; Goldmann and Medina 2013). However, they also show remarkable individual differences, such as the type of cell organelles where the main DNA strand originated, which may be either from nucleus or mitochondria (Yousefi et al. 2008) as well as the molecular signaling pathways involved in ETosis (Goldmann and Medina 2013).

In our experiments, the rapid production of METosis and resulting reduction in sperm motility in the infectious model with *E. coli* could be explained by the presence of lipopolysaccharide (LPS), since the stimulation with LPS can generate ETs in min (Clark et al. 2007), whereas with chemical stimuli such as phorbol-myristate-acetate or biological stimuli such as Staphylococcus aureus or *Candida albicans* the process takes 2–3 h (Fuchs et al. 2007). With respect to the composition of the ETs, initially they were described as being of nuclear origin, but depending on the cell type and stimulus used, the DNA can also stem from mitochondria in NETosis (Keshari et al. 2012; Lood et al. 2016; Granger et al. 2017).

In the case of METosis, the released DNA strands are of mitochondrial and nuclear origin (Granger et al. 2017). This could help to explain the morphology of METs, which mainly show a 'diffuse' appearance, comparable to network phenotypes produced by PMN (*diff*NETs) and being mainly released from cell nucleus. These are composed of a spherical and compact extracellular decondensed chromatin mesh, trapping more spermatozoa at a short distance than 'extended' and fine networks (*spr*NETs) (Muñoz-Caro et al. 2015, 2018).

Based on presented results NETs or METs might physically block spermatozoa, inhibiting their motility after a brief contact. If this early host innate effectors mechanism develops in an infectious environment, the negative/detrimental effects on spermatozoa functions might be even higher. Recruitment of leukocytes, such as PMN, monocytes, macrophages, into the male genital tract either after inflammation or after infection

might result in ETosis reacting against own spermatozoa as if these gametes were recognized as foreign agents.

Thus, better knowledge on sperm-triggered METosis might contribute to lending greater attention to the handling of human ejaculates, from the time of patient producing the semen sample to its arrival in the laboratory, the 15–30 min which it takes to liquefy the sample before further processing. Our results suggest that if there is an increase in seminal inflammatory cells such as monocytes/macrophages, this could significantly affect sperm function, particularly motility. In addition, in sperm selection techniques, the spermatozoa remains in permanent contact with these leukocytes for at least 20 min, more than sufficient time to generate METs, exerting physical trapping and chemical effects on MET-exposed spermatozoa.

METs might physically block spermatozoa, inhibiting their motility after a brief contact. If this early host innate effectors mechanism develops in an infectious environment, the negative/detrimental effects on spermatozoa functions might be even higher. It will be necessary to investigate the factors which induce the release of ETs by seminal monocytes and PMN in response to human spermatozoa, and if these two seminal fluid phagocytes perform exclusively phagocytosis or degranulation or these two processes together. Furthermore, the exact mechanisms and molecular pathways of different phenotypes of METosis have to be deciphered in order to develop therapeutic strategies that will help to prevent or reduce negative effects on spermatozoa function and male fertility.

Materials and methods

Human semen samples

Semen samples were obtained from donors with normal semen parameters (WHO 2010), aged 22–28 years. All donors gave their written informed consent according to the ethical protocols of ongoing cohort studies, approved by the Ethics Committees of the Medical Faculty at Justus Liebig University Giessen, Germany (Ref. Nos. 32/11 and 53/13).

The semen (n = 5) samples were obtained by masturbation after two days of sexual abstinence. After 30 min liquefaction at room temperature (RT), routine semen analysis and sperm selection by swim up were performed using gamete buffer medium (Cook Medical). Motile spermatozoa (>90% progressive motility post swim up) were suspended in gamete buffer medium adjusted to 20×10^6 and maintained at 37°C until use.

Isolation of human monocytes from peripheral blood

Monocytes were isolated by density gradient separation from human blood according to the manufacturer's instructions with own modifications. For experiments, 30 mL of venous heparinized blood was obtained from the cephalic vein from healthy volunteer donors (n = 5, women and men) between 22 and 40 years of age. A double gradient was prepared with 3 mL histopaque 1.119 (g/mL) (Sigma-Aldrich) and 3 mL histopaque 1.077 (g/mL) (Sigma-Aldrich). Then, 6 mL of blood diluted 1:1 with phosphate-buffered saline (PBS) 1X (Sigma-Aldrich) were carefully deposited on gradients and centrifuged for 30 min without break at 700 x g and at RT. As a result of this double separation, two distinct cellular layers were observed: the upper one with peripheral blood mononuclear cells (PBMC) and the lower one with PMN and erythrocytes. The PBMC layer was carefully aspirated and washed with RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (5 min, 300 x g, RT). The supernatant fraction was discarded, and cells were resuspended in 5 mL of RPMI-10% FCS. To isolate monocytes, a 46% Percoll (Amersham) gradient was used. Firstly, to prepare 25 mL of this gradient solution 11.6 mL of 100% Percoll (Amersham) were taken and 0.935 mL of 10X PBS were added. Then, 11.5 mL of this mixture wastaken and 13.5 mL of RPMI-10% FCS were added to obtain an iso-osmotic solution of 46% Percoll. In conical tubes with 3 mL of 46% Percoll, 1 mL of PBMC suspension was placed and centrifuged for 30 min without break at 550 x g (RT). Finally, monocytes were washed with 5 mL of RPMI-10% FCS (5 min, 200 x g, RT) and resuspended in 1 mL of RPMI-10% FCS. Monocytes were counted in a Neubauer hemocytometer and viability was assessed with trypan blue (Sigma-Aldrich) staining (>95% in each assay) and kept at RT until further use.

Monocyte identification

Smears from each cell suspension were prepared and fixed as described above. For the identification of monocytes, mouse anti-human monoclonal antibody CD68-FITC was used (RRID: AB_795842; Thermo Fischer Scientific). After incubation (1 h, RT) in dark, sample was washed three times with PBS and mounted face down onto a glass coverslip (Nunc), to which one drop of antifading mounting buffer (Sigma-Aldrich) was added. Visualization was achieved using an Olympus IX81 inverted fluorescence microscope

equipped with an XM10 digital camera (Olympus) determining that 94% of cells were positive for the CD68 marker.

Uropathogenic *escherichia coli* strain

The uropathogenic *E. coli* UTI89 strain was here used, provided by the Institute of Microbiology at the JLU Giessen, Germany. On the day of experiment settings, the strain was re-suspended in trypticase soy broth (Becton Dickinson GmbH) and used at a final concentration of 6 x 10^6colony forming units (CFU) per mL.

Co-incubations of monocytes, spermatozoa and *E.* coli

In order to determine the release of spermatozoa-induced METs (aseptic model), 0.25 × 10^6 monocytes were co-incubated with 1.5 × 10^6 selected motile spermatozoa, at 37°C. For septic model, monocytes and spermatozoa were incubated in the same concentration with 6 × 10^6 CFU of *E. coli*, at 37°C As a positive control of motility, spermatozoa were incubated in absence of monocytes and bacteria, and as a negative control, were incubated with bacteria. As a negative control of METs, monocytes were incubated in the absence of spermatozoa and bacteria. All cell suspensions were adjusted to a final volume of 300 µL with gamete buffer medium and incubated at 37°C. Measurements of motility and sperm aggregation (WHO 2010) have been evaluated by computer-aided sperm analysis (CASA) equipped with the integrated sperm analysis system (ISAS; Proiser, Valencia, Spain) software, and optical microscopy (40 x) have been performed at 0, 10, 30, 60, and 180 min. The extrusion of METs have been visualized by Scanning electron microscopy (SEM)- and fluorescence microscopy-analyses after 180 min of incubation as described elsewhere (Muñoz-Caro et al. 2014).

METs visualization by immunofluorescence analysis

For the identification of METs, 10 µL of each cell suspension was taken to prepare smears, which were air-dried and then fixed with pure acetone at RT for 2 min and stored at 4°C until analysis (24 h). To identify METs, global histones (H1, H2A/H2B, H3, H4) and histone H4 citrullinated 3 (H4Cit3) were identified simultaneously by indirect immunofluorescence analysis. The smears were blocked with bovine serum albumin (BSA) 20% (Sigma-Aldrich) and 0.005% saponin (Sigma-Aldrich) in PBS for 30 min at RT. Then, two washes were performed with 500 µL of PBS. Subsequently, the primary antibodies were added: anti-histones, anti-mouse monoclonal raised against histones (clone H11-4, MAB3422, RRID:AB_2114845, Millipore), and anti-H4Cit3 (anti-rabbit polyclonal, 07–596, RRID:AB_441947, Millipore).

After incubation (1 h, RT) the samples were washed twice with 500 µl of PBS, and the secondary-conjugated antibodies were then added: goat anti-mouse IgG, Alexa Fluor 488 (Thermo Fisher Scientific, A-11,001, RRID:AB_2534069) for detection of global histones (H1, H2A/H2B, H3, H4), and goat anti-rabbit IgG Alexa Fluor 405 (Thermo Fisher Scientific A-31,556, RRID:AB_221605) for the detection of H4Cit3. After incubation for 1 h, RT in the dark, specimens were washed three times with 500 µL of PBS, and the DNA was stained with Sytox Orange® (Invitrogen) for 20 min in the dark. Finally, the samples were washed twice with 500 µL of PBS and mounted face down onto a glass coverslip (Nunc), to which one drop of anti-fading mounting buffer (Fluoromount G®, Invitrogen) was added. Visualization was achieved using an Olympus IX81 inverted fluorescence microscope equipped with an XM10 digital camera (Olympus).

Evaluation of sperm motility

For the evaluation of progressive motility, CASA was used in a phase-contrast microscope (Zeiss) with a slide warmer at 37°C. In total 10 µL of cell, suspension were taken from each group (control; monocytes + spermatozoa; monocytes + spermatozoa + *E. coli*) and covered with a 22 × 22 mm coverslip (Nunc). The evaluations were performed at 0, 10, 30, 60 and 180 min after co-culture. At least 200 spermatozoa were evaluated in five different randomly selected power vision fields, excluding spermatozoa in aggregates of cells and bacteria (i. e. spermatozoa + monocytes ± *E. coli*).

Scanning electron microscopy (SEM) analyses

Human monocytes (n =3 x 10^5) were co-cultured with either spermatozoa alone or spermatozoa plus *E. coli* on poly-$_L$-lysine (0.01%; Sigma-Aldrich) pre-coated coverslips (10 mm diameter, Nunc, 60 min, 37°C). After incubation, samples were then fixed in 4.0% glutaraldehyde (60 min, RT, Merck), post-fixed in 1% osmium tetraoxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2 treatment and spayed with gold. The samples were examined using a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, JLU Giessen, Germany.

Statistical analysis for evaluation of sperm motility

The differences of quantitative data were evaluated using the Kolmogorov–Smirnov test (analysis of normality of the data) and Levene's test (homoscedasticity of the variances). Differences between the groups were analyzed with a one-way ANOVA, followed by Tukey's post-hoc HSD test or Dunnett's T3 test, as applicable. $P< 0.05$ was considered statistically significant (IBM SPSS Statistics, Version 21, IBM Corp., Armonk, NY, USA).

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Disclosure statement

No potential conflict of interest was reported by the authors.

Authors' contributions

Study concept, design, analysis and interpretation of data: MS, RS, AT, CH; acquisition of data: MS, FZ, UG; critical revision of the manuscript for important intellectual content: CH, RS, FW, HS.

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