SOCE-inhibitor reduced human sperm-induced formation of neutrophil extracellular traps

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Abstract

Human spermatozoa activate neutrophil extracellular traps (NETs) in vitro. NETosis is an efficient mechanism through which polymorphonuclear neutrophils (PMN) capture sperm in vitro. The objective of this study was to establish the role of store-operated Ca^{2+} entry (SOCE) in human sperm-triggered NETs and its impact on sperm integrity and oocyte binding capacity. PMN isolated from donors were exposed to spermatozoa isolated from normozoospermic donors using the swim-up technique and were divided into the following groups: (1) sperm, (2) PMN, (3) PMN + sperm, (4) PMN (pretreated with 2-APB, SOCE inhibitor) + sperm, (5) (PMN + DNase) + sperm, and (6) (PMN + PMA) + sperm (positive control). NETs were quantified using PicoGreen® and visualised by scanning electron microscopy and immunofluorescence of extracellular DNA and neutrophil elastase. Plasma membrane, acrosome, and DNA integrity were analysed by flow cytometry, and oocyte binding was evaluated using the hemizona pellucida assay. Sperm-triggered NETosis negatively affected the sperm membrane and acrosome integrity and decreased the oocyte binding capacity. These effects were negated by an SOCE inhibitor, thus improving sperm function and achieving high oocyte binding capacity. The SOCE inhibitor significantly reduced NET formation compared with that in control PMN/sperm ($P < 0.05$). Collectively, these results advance the knowledge about the role of PMN in reproduction and will allow the development of strategies to block NET formation in situations of reduced fertilisation success.

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Introduction

Polymorphonuclear neutrophils (PMN) play an important role as the first line of innate immune defence in mammals. The formation of neutrophil extracellular traps (NETs) is a novel effector mechanism of PMN (Brinkmann *et al.* 2004). NETs are sticky extracellular fibrous structures that are released from activated PMN in response to pathogenic or non-pathogenic stimuli in both mammals and invertebrates (Brinkmann *et al.* 2004).

The main components of NETs include DNA, histones (H1, H2A/H2B, H3, H4), myeloperoxidase (MPO), neutrophil elastase (NE), pentraxin 3 (PTX3), cathepsin G, matrix metalloproteinase-9 (MMP9), peptidoglycan recognition proteins, and peptidyl arginine deiminase type IV (PAD4) (Brinkmann *et al.* 2004). The signalling cascade triggering NETosis comprises the production of reactive oxygen species (ROS) by neutrophil NADPH

oxidase (NOX), translocation of NE and MPO to the nucleus, association of the citrullinating activity of PAD4 on histones, and induction of chromatin decondensation (Leshner *et al.* 2012). Besides DNA-related analysis, co-localisation experiments have demonstrated the concomitant presence of H3, PTX3, NE, and MPO in bovine sperm-induced NETosis, confirming the classical molecular properties of NETs (Brinkmann *et al.* 2004). Interestingly, PTX3 was previously shown to be expressed in the male genital tract and to be bound to human spermatozoa (Doni *et al.* 2009). In agreement with this report, a recent study found PTX3 in a co-culture of bovine sperm and PMN (Fichtner *et al.* 2020).

Human spermatozoa are known to act as antigens in the female reproductive tract (FRT), and sperm immunisation can even result in infertility (Hahn *et al.* 2012). Human PMN are actively recruited to the FRT following insemination in a process similar to sterile inflammation, and leukocytes act as efficient

phagocytes, since they are involved in the removal of excess spermatozoa in vivo (Strzemiński 1989, Alghamdi *et al.* 2009). As human spermatozoa within the FRT are directly exposed to PMN and are disposable targets of PMN-derived NETs (Alghamdi *et al.* 2009), an interaction might occur with unknown consequences for sperm physiology. Given that NET-related cytotoxicity via direct damage to membrane integrity has been previously described for several invasive pathogens such as bacteria (Marin-Esteban *et al.* 2012), fungi (Urban *et al.* 2006), and protozoan/metazoan parasites (Silva *et al.* 2014), it is plausible that human NETs may also harm spermatozoa via antimicrobial effector molecules (Saffarzadeh *et al.* 2012). Moreover, in vitro, human spermatozoa induce NETosis in PMN, resulting in the formation of different types of NETs (i.e. aggregated NETs (aggNETs), diffuse NETs (diffNETs) and spread NETs (sprNETs)) (Zambrano *et al.* 2016). Recently, similar types of NETs have been demonstrated in the seminal fluid (ex vivo) of infertile patients (Schulz *et al.* 2019b) and in seminal samples from patients with epididymitis (Zambrano *et al.* 2020).

Analysis of sperm/PMN co-cultures have confirmed the essential role of NOX, MPO, and NE in the formation of NETs, which adversely affects sperm function and motility (Zambrano *et al.* 2016); however, very little information is available concerning the role of ROS production, which is known to be dependent on store-operated Ca^{2+} entry (SOCE) and which is a key factor for the adequate release of NETs by PMN (Gupta *et al.* 2014). The aim of this study was to establish the role of SOCE in human sperm-triggered NETosis and its impact on sperm integrity and oocyte binding capacity.

Materials and methods

Ethics statement

This study was approved by the Scientific Ethics Committee of the Universidad de La Frontera, Temuco, Chile, and the Justus Liebig University (JLU) Giessen, Germany. The healthy sperm and PMN donors included in this study were volunteers from these two universities. All donors provided written informed consent according to the ethical protocols of both institutions.

Reagents

All reagents were obtained from Sigma Chemicals Ltd., unless otherwise stated.

Blood and semen samples

In each experiment, blood samples were obtained from healthy women volunteers ($n = 3$), aged 20–35 years. Thirty millilitres of blood were extracted from the cephalic vein of each donor at the blood bank unit of the JLU, and PMN were immediately isolated. We used female PMN to simulate the interaction that would occur during the natural reproductive process, wherein

the sperm interacts with PMN in the FRT. The sperm samples were obtained from healthy male volunteers ($n = 3$) with normal semen parameters (WHO 2010), aged 20–26 years. Samples were collected in sterile plastic tubes (Deltalab®) via masturbation after at least 3 days of sexual abstinence.

In total, semen samples from 12 male donors and peripheral blood samples from 12 healthy female donors were used in this study.

Isolation of PMN

Blood samples were placed in a Vacutainer® containing heparin (10 U/mL). The isolation method employed was as described in a previous report (Oh *et al.* 2008). Briefly, 6 mL of Histopaque® 1119 (Gibco) was placed in a 15 mL Falcon® plastic tube and 7 mL of heparinised blood was carefully layered on top. This mixture was centrifuged at 800 *g* for 20 min. Next, the clear yellowish top layer was aspirated and discarded, and the lower reddish phase containing PMN was transferred into fresh Falcon® plastic tubes. The cells were then washed by filling the Falcon® tubes with sterile PBS and centrifuged for 10 min at 300 *g*. Percoll solution (100%; Amersham) was prepared by mixing 18 mL of Percoll with 2 mL of 10x PBS. Four millilitres each of 85%, 80%, 75%, 70%, and 65% Percoll gradients were then diluted with 1x PBS. Next, two Falcon® tubes (for each blood sample) containing descending Percoll gradients (85%, 80%, 75%, 70%, and 65%, respectively) were layered with blood. After centrifugation (10 min at 300 *g*), the supernatant was discarded, and the pellets were resuspended in 4 mL of PBS. Two millilitres of resuspended pellet from each of the gradients was collected and centrifuged at 800 *g* for 20 min. After centrifugation, the top layer and most of the 65% layer containing peripheral blood mononuclear cells (PBMCs) were discarded, while the remaining white interphase, comprising the 85% layer, was collected in a new Falcon® tube. Cells were then washed by filling up the Falcon® tube with PBS and centrifuging for 10 min at 300 *g*. Finally, the supernatant was removed, and cell pellets were resuspended in 2 mL of RPMI 1640 medium. PMN were counted using a Neubauer haemocytometer.

Spermatozoa and PMN co-culturing assay

Motile spermatozoa were isolated using the swim-up technique. For this, 500 μL of modified human tubal fluid (HTF) medium with gentamicin-HEPES (HTF; Irvine Scientific) was placed in a 1.7 mL polypropylene tube. Then, 150 μL of semen was placed at the bottom of the tube. The tube was tilted to 45° and incubated at 37°C for 60 min. After incubation, 350 μL of the sample was recovered from the upper part of the tube without aspirating the contents at the bottom of the tube. Only samples with $\geq 75\%$ spermatozoa showing progressive motility and suitable sperm counts were included in the experiments. Human PMN and spermatozoa were co-cultured at a ratio of 1:6 (2.5×10^5 PMN: 1.5×10^6 spermatozoa) in serum-free RPMI 1640 cell culture medium (Gibco) without phenol red, as described previously (Munoz-Caro *et al.* 2015b). The co-culture was incubated for 180 min at 37°C with 5% CO_2 and saturated humidity.

Immunofluorescence analysis of sperm-triggered NETosis

After co-incubation of PMN with human spermatozoa on coverslips (22 mm × 60 mm; Nunc, Thermo Fisher Scientific) previously coated with poly-L-lysine (0.01%), 15 min at room temperature (RT), the samples were fixed in 4% paraformaldehyde (15 min, RT). Then, the samples were blocked with 2% BSA (15 min, 37°C), washed twice in PBS, and incubated with the following primary monoclonal antibodies: anti-global histone (H1, H2A/H2B, H3, H4) antibody (anti-mouse, clone H11–4, diluted 1:1000 in PBS) and anti-PTX3 antibody (SAB2104614-50UG, diluted 1:1000 in PBS). The samples were washed twice in PBS and then incubated with the following secondary antibodies: Alexa Fluor® 488 goat anti-mouse IgG (ref. A11001, Molecular Probes) for the detection of histones and Alexa Fluor 488 goat anti-rabbit IgG (ref. A11008, Life Technologies) for the detection of PTX3. All samples were incubated for 1 h at RT in the dark. Finally, the samples were washed twice in PBS and stained for 20 min with Sytox Orange® (Invitrogen), as described previously (Martinelli *et al.* 2004, Lippolis *et al.* 2006). After two washes with PBS, each sample was mounted face-down on a glass coverslip and immediately analysed using an inverted fluorescence microscope equipped with a digital camera (Olympus IX81®).

Quantification of NETs

To quantify NETs induced by spermatozoa, PMN and spermatozoa were co-cultured at a ratio of 1:6 for 180 min. The experimental groups were as follows: (1) sperm, (2) PMN, (3) PMN+sperm, (4) PMN (previously treated with 2 aminoethoxydiphenyl borate (2-APB) for SOCE inhibition (100 µM, Sigma-Aldrich))+sperm and (5) (PMN+PMA 500 nM (Adipogen®))+sperm. Stimulation with PMA was used as positive control, as described in a previous report (Munoz-Caro *et al.* 2015a). All samples were incubated for 180 min at 37°C. After incubation, samples were treated with 0.1 U/µL of micrococcal nuclease (New England Biolabs) for 15 min at 37°C and centrifuged (300 g, 5 min). Then, 100 µL of supernatant of each sample was transferred in duplicate to a 96-well cell culture plate (Greiner). Finally, 50 µL of PicoGreen® (1:200 in 10 mM of Tris base buffered with 1 mM EDTA) was added to the samples, and the samples were incubated for 4 min in total darkness. NET formation was quantified by spectrofluorometric analysis (484 nm excitation wavelength and 520 nm emission wavelength) using an automated plate monochrome reader (Varioskan Flash®; Thermo Scientific) as previously described (Silva *et al.* 2014, Munoz-Caro *et al.* 2015a,b).

Immunofluorescence staining to confirm the formation of NETs

Immunofluorescence staining was performed according to a previously described protocol (Fichtner *et al.* 2020). Monoclonal anti-NE antibody (AB68672, 1:200, Abcam) was used as the primary antibody to detect human NE during 1 h; subsequently, the samples were incubated 1 h with a secondary antibody conjugated with Alexa Fluor 488 (anti-

rabbit ref. A31556, Invitrogen). To view the leucocyte nuclei and the DNA released into the extracellular space, antifade mounting medium with DAPI was used (ProLong®). The samples were analysed using an Olympus IX81® inverted fluorescence microscope equipped with an Olympus XM10® digital camera.

Sperm plasma membrane and acrosome integrity

Sperm plasma membrane integrity was evaluated using the LIVE/DEAD Sperm Viability Kit® (Molecular Probes). The different groups (sperm, PMN/sperm, (PMN+2APB)+sperm and (PMN+DNase (90 U of DNase I (Roche Diagnostics))+sperm), were resuspended in 400 µL of HTF medium and incubated with SYBR-14 at 1 nM concentration for 10 min. Acrosome evaluation assay was performed using PNA/FITC (300 µg/mL) and propidium iodide (PI) (18 mM) for 15 min at 37°C in darkness. PI was added and incubated for 15 min. Finally, the spermatozoa were washed by centrifugation at 500 g for 5 min and resuspended in 300 µL of PBS.

DNA fragmentation

DNA fragmentation was evaluated by the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay, using the *in situ* Cell Death Detection Kit® (Roche Biochemical) according to the manufacturer's instructions. Briefly, spermatozoa were either untreated (controls) or were treated with PMN (180 min), washed with 1% PBS/BSA (w/v) (pH 7.4) (400 g, 5 min), resuspended in 1 mL of PBS/BSA (1%) and fixed in 0.2% paraformaldehyde for 45 min. The samples were centrifuged at 400 g for 5 min and washed with 500 µL of 1% PBS/BSA. Subsequently, the spermatozoa were incubated for 1 h at RT in permeabilisation solution (PBS supplemented with 1% BSA, 0.3% Triton and 0.1% sodium citrate), and then the samples were washed in HTF medium (500 g for 5 min) and resuspended in 500 µL of 1% PBS/BSA. All samples were then incubated with 5 µL of enzyme solution and 45 µL of label solution for 60 min in a humidity chamber at 38.5°C in the darkness. Finally, the samples were centrifuged at 500 g for 5 min and resuspended in 300 µL of PBS for flow cytometry evaluation. To verify successful cell permeabilisation, samples were counterstained with PI (18 mM) for the last 5 min of incubation.

Hemizone binding assay for assessment of spermatozoa function

The hemizona binding assay was carried out according to a previous report (Franken *et al.* 1989), with slight modifications. Only discarded oocytes obtained by *in vitro* fertilisation program, stored in salt storage solution were used (Yanagimachi *et al.* 1979). The oocytes were placed in 100 mm petri dishes in 50 µL drops of modified HTF medium supplemented with 0.5% albumin and bisected into two hemizonae by a scalpel cut, with the blade at right angle (perpendicular to the cutting surface). The spermatozoa of each group were incubated for 180 min with PMN and NETosis inhibitor as described previously. After the completion of this incubation period,

NET-entrapped spermatozoa were released from extracellular structures by treatment with DNase I (addition of 90 U/well of DNase I for 15 min) in order to dissolve the NETs. In each assessment group, 60,000 spermatozoa were added to each hemizona for 4 h and incubated in a humidified chamber at 37°C and 5% CO₂. The hemizonae were then washed three times in 1x PBS and gently placed on a coverslip containing 15 µL of mounting medium with DAPI (sperm nucleus marker). Hemizona imaging analysis was performed using an epifluorescence microscope (NIKON eclipse TS100F®).

Flow cytometry

All fluorescence analyses were performed using a BD FACS Canto II Flow Cytometer (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA). One straw was used for all treatments in each experiment, and each experiment was repeated three times on different days. A minimum of 10,000 spermatozoa were included in each analysis. When evaluating plasma membrane, acrosome, and DNA integrity in the co-cultures, the sperm populations were separated from the PMN based on cell size (Forward Scatter, FSC) and cell complexity (Side Scatter, SSC) to ensure that only fluorescence emitted by the sperm was considered while excluding that emitted by the PMN.

Statistical analysis

The current data were analysed by descriptive statistics and represented as the mean $\pm$ s.d. calculated for each of the variables using the GraphPad Prism software, version 5.0a. Gaussian distribution was analysed by D'Agostino's test. One-way ANOVA was performed to determine statistically significant differences ($P < 0.05$) between groups. In cases where statistically significant differences were observed, Tukey's test was performed for more detailed analysis.

Results

Scanning electron microscopy (SEM) and localisation of histones and PTX3 in human sperm-induced NETs

SEM and immunofluorescence analyses revealed the release of both fine and thick NETs by human PMN and the presence of entrapped spermatozoa after 180 min (Fig. 1). Following a 3 h incubation period, PMN were detected with initial cell membrane damage occurring prior to NET extrusion (Fig. 1A). Further, we observed localisation of histones (Fig. 1C and D) and PTX3 (green fluorescence) (Fig. 1E and F) with DNA-containing structures (red fluorescence (Fig. 1C and E)). In addition, different types of NETs, such as sperm-induced spread NETs (*sprNETs*), were observed in sperm/PMN co-cultures (Fig. 1C and D).

Effect of SOCE inhibitor on human sperm-induced NETosis

The quantification of NET formation confirmed human spermatozoa as potent inducers of NETs (Fig. 2). Thus,

Figure 1 Visualisation and analyses of human sperm-triggered NETs by SEM (A and B) and epifluorescence microscopy (C, D, E and F). Images (A and B) show human PMN entrapping spermatozoa; image (A) shows a human PMN carrying out NETosis, and thereby entrapping a spermatozoon within thin extracellular fibres (B). NET-derived structures showing DNA labelling with Sytox Orange® dye (red fluorescence, C) and global histones (H1, H2A/H2B, H3, H4) labelled with Alexa Fluor 488 (green fluorescence, D). Image (F) shows PTX3 immunofluorescence labelling. Green fluorescence represents PTX3 labelling; in (E) orange fluorescence represents DNA staining (cell nuclei and NETs). Red arrows indicate PMN; white arrows indicate NETs; yellow arrows indicate spermatozoa. Epifluorescence images, magnification 40x (C and D) and 60x (E and F).

a significantly enhanced quantity of extracellular DNA was observed in sperm/PMN co-cultures compared to PMN or sperm alone ($*P < 0.05$). Stimulation of PMN with PMA showed significantly enhanced NET formation compared to all other groups. The relevance of SOCE in sperm-triggered NETosis was examined via functional inhibitor treatment. Pretreatment of PMN with 2-ABP and subsequent co-culturing with spermatozoa led to significantly reduced NET formation compared with that in the control PMN/sperm group ($*P < 0.05$), thus proving the key role of SOCE in sperm-mediated NETosis.

Effect of NETs on sperm plasma membrane, acrosome integrity and DNA integrity

Sperm plasma membrane integrity was significantly reduced after 180 min of co-culture with PMN (Figs 3A and 4A) in comparison to spermatozoa which were

Figure 2 (A) Graph representing quantification of NETs showing a significantly enhanced quantity of extracellular DNA in sperm/PMN co-cultures compared to PMN or sperm alone ($*P < 0.05$) at 180 min. Stimulation of PMN with PMA shows significantly enhanced NET formation compared with all the other groups. Treatment of PMN/sperm with 2-ABP (for SOCE inhibition (100 μ M)) significantly reduced NET formation compared with that in control PMN/sperm ($*P < 0.05$). (B) Epifluorescence microscopy images showing NET structures of different groups: DNA (blue staining, DAPI); NE (green staining, Alexa Fluor 488). Epifluorescence images, magnification 60x.

not exposed to PMN ($P < 0.05$). This effect could be partially prevented by pretreatment of PMN with the SOCE inhibitor (2-APB) ($P < 0.05$).

The integrity of the acrosomal membrane was found to be significantly ($P < 0.05$) reduced after 180 min of PMN/spermatozoa co-culture, indicating the adverse effects of NETs (Figs 3B and 4B). However, in this experimental setting treatment of PMN with 2-APB prior to co-culture with spermatozoa failed to significantly prevent this reaction. Interestingly, DNA integrity was not affected in spermatozoa exposed to PMN (Figs 3C and 4C).

Capacity of sperm to bind to the hemizona after exposure to NETs

Spermatozoa previously exposed to PMN had a significantly lower capacity to bind to zona pellucida than control spermatozoa ($*P < 0.05$; Fig. 5A and B).

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Figure 3 Effects of NETs on membrane integrity, acrosome integrity, and DNA fragmentation in human spermatozoa in base medium (spermatozoa without PMN), in co-cultures of PMN/sperm (1:6) for 180 min, using NETosis inhibitor (SOCE inhibitor (2-APB, 100 μ M)) and DNase control group. The data are represented as the mean percentage $\pm$ s.d.; statistical significance was considered at $*P < 0.05$.

Thus, 48% less spermatozoa (relative to the control) bound in the hemizona assay. This effect could be partially prevented by pretreatment of PMN with the SOCE inhibitor before incubation with spermatozoa. This result indicated the relevance of SOCE in sperm-mediated NETosis. Thus, the mean number of spermatozoa binding to the zona pellucida increased significantly after 2-APB treatment when compared to the untreated PMN/sperm group ($*P < 0.05$). However, the mean number of the PMN + SOCE inhibitor-treated group (prior to exposure with spermatozoa) did not reach that of the control spermatozoa and still showed significantly reduced levels of hemizona binding when compared to unexposed spermatozoa ($*P < 0.05$). PMN group with sperm and DNase maintained sperm function similar to sperm group without PMN.

Discussion

Human spermatozoa induce NETosis in PMN, resulting in the formation of different types of NETs (aggNETs,

Figure 4 Flow cytometry images, (A) percentage of sperm population with intact plasma membrane (viable, SYBR14+/PI-). (B) The viable sperm population with intact acrosome (PI-/PNA/FITC-). (C) The percentage of sperm population with fragmented DNA (TUNEL+).

sprNETs and *diffNETs*), which adversely affect sperm motility (Zambrano *et al.* 2016). NETosis-derived sperm entrapment might be linked to PTX3 activity, since the latter could form molecular complexes with other NET components cooperating as a binding matrix to enhance entrapment (Daigo & Hamakubo 2012). In this study, we documented for the first time, the presence of PTX3 in human sperm-triggered NETosis. PTX3 is a known antimicrobial factor of the mammalian innate immune system, wherein it plays a role in microbe recognition

and facilitates the entrapment of invasive pathogens (Bottazzi *et al.* 2009). This finding is in accordance with the current microscopy evidence, showing effective sperm-entrapping capacities of NETs.

Various studies have described that spermatozoa can interact with their environment and that these interactions have an impact on sperm viability (Taylor *et al.* 2008). Herein, we use a concentration of neutrophils which establishes the point at which NETs have a negative effect on male gamete viability, which is consistent

Figure 5 Hemizona binding ability of sperm. (A) Spermatozoa exposed to PMN had a significantly lower capacity to bind to zona pellucida than control spermatozoa did (* $P < 0.05$). The mean number of spermatozoa binding to the zona pellucida increased significantly after 2-APB treatment compared with that in the untreated PMN/sperm group (all: * $P < 0.05$). However, inhibitor-treated PMN/sperm showed significantly reduced levels of hemizona binding compared with unexposed spermatozoa (* $P < 0.05$). (B) Shows images of sperm binding to the hemizona (sperm cell nuclei stained blue with DAPI); epifluorescence microscopy, magnification 40x. Three biological replicates were included in each assay. The data are represented as mean percentage $\pm$ S.D.; statistical significance was considered at * $P < 0.05$.

with the previously determined decreased progressive motility (Zambrano *et al.* 2016). These results confirm an unfavourable influence of inflammatory cells on the quality of spermatozoa; this may reduce their fertilising potential, given the trapping of sperm on the one hand, and on the other, the damage caused by NETs to spermatozoa that come in contact with them (Zambrano *et al.* 2016). The dynamic nature of the sperm membrane plays a fundamental role in sperm maturation, capacitation and fertilisation. However, exposure to NETs causes loss of membrane integrity, as has been reported in macrophages and dendritic cells. It has been reported that specific damage is mediated principally by the high contents of NETs and the presence of cathepsin G in the extracellular network (Donis-Maturano *et al.* 2015). Furthermore, excessive exposure to ROS may cause oxidative stress with irreparable damage to the sperm membrane (Muller *et al.* 1999). This is an interesting aspect since we think that the production of ROS from NETs could be one of the most critical points affecting sperm physiology. The greatest damage might be caused by membrane lipid peroxidation, which principally impacts long-chain polyunsaturated fatty acids (PUFAs), such as docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Waterhouse *et al.* 2006). This leads to loss of viability and motility and diminished capability of the spermatozoon to fuse with the oocyte (Maldjian *et al.* 2005) and may partly explain the loss of sperm function in this model, where in the presence of NETs caused direct damage to the sperm membrane. Furthermore, the trapping of spermatozoa by NETs may be accentuated by the DNA present in the NETs: the NET surface has a negative charge and contains molecules that may mediate binding, for example, with microorganisms, possibly through electrostatic interactions between the cationic components of the NETs and their anionic surfaces (Brinkmann & Zychlinsky 2007). These interactions also explain the diminished viability of the spermatozoa that causes their agglomeration and/or immobilisation, which are thought to be closely related to unexplained infertility (Schulz *et al.* 2019a,b). These responses may be similar to infection processes in the male genital tract in which sperm parameters are altered, generally due to inflammation, and increased formation of different NET structures, as described previously (Zambrano *et al.* 2020).

Another important event in the formation of NETs is NOX activation and the subsequent production of ROS, both processes dependent on Ca^{2+} influx mediated by SOCE (Brechard & Tschirhart 2008). In our study, sperm-induced NETosis leads to a partial loss of sperm plasma membrane and acrosome integrity in addition to reduced capacity of sperm adhesion and binding to the oocyte. The implications of these effects will most likely include reduced fertilisation success. Interestingly, NET-induced damage to the spermatozoa was significantly

prevented by the SOCE inhibitor 2-APB, underscoring our previous data on the relevance of SOCE-derived Ca^{2+} in sperm-induced NETosis (Conejeros *et al.* 2011, Munoz-Caro *et al.* 2015a). These data demonstrate that reduced contact of sperm with toxic or adverse NET-derived molecules is beneficial to sperm function. As a result, the use of SOCE inhibitor in sperm-exposed human PMN helped these cells to maintain the dynamics and integrity of the sperm membrane, and, therefore, subsequent capacitation and fertilisation (Zhou *et al.* 2010). Despite the positive effects of 2-APB on sperm function, this treatment failed to significantly improve acrosome integrity. This may be due to the very stable and rigid nature of this structure compared to the sperm plasma membrane (Zambrano *et al.* 2016). Using the current experimental conditions, we did not find any impact of NETs on sperm DNA fragmentation. This was reasonable given that high ROS levels are associated with plasma membrane damage, and DNA fragmentation indicates the final stage of cell death (Galluzzi *et al.* 2012).

Conclusion

We demonstrated the key role of SOCE and, for the first time, the presence of PTX3 in human sperm-triggered NETs. NETosis induced damage in the sperm membrane and acrosome, thus negatively impacting sperm function. However, the use of SOCE inhibitor maintains sperm function with a high oocyte binding capacity. This new data will improve the current understanding of the role of PMN in female and male reproductive tracts and may contribute to the development of alternative strategies to block the formation of NETs in situations of reduced fertilisation success.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

F Z, A T, R S and C H involved in conception and design of the study. F Z, L S, P U, U G and M S contributed to acquisition of data; F Z, A T, R S, C H involved in analysis and interpretation of data. F Z and P U contributed drafting the manuscript. F Z, R S and C H revised the manuscript critically for important

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