Nitrosative stress in human spermatozoa causes cell death characterized by induction of mitochondrial permeability transition-driven necrosis

Pamela Uribe1,2,3, María E Cabrillana4,5, Miguel W Fornés4,5, Favián Treulen1, Rodrigo Boguen1, Vladimir Isachenko6, Evgenia Isachenko6, Raúl Sánchez2,7, Juana V Villegas1,3

Peroxynitrite is a highly reactive nitrogen species and a potent inducer of apoptosis and necrosis in somatic cells. Peroxynitrite-induced nitrosative stress has emerged as a major cause of impaired sperm function; however, its ability to trigger cell death has not been described in human spermatozoa. The objective here was to characterize biochemical and morphological features of cell death induced by peroxynitrite-mediated nitrosative stress in human spermatozoa. For this, spermatozoa were incubated with and without (untreated control) 3-morpholinosydnonimine (SIN-1), in order to generate peroxynitrite. Sperm viability, mitochondrial permeability transition (MPT), externalization of phosphatidylserine, DNA oxidation and fragmentation, caspase activation, tyrosine nitration, and sperm ultrastructure were analyzed. The results showed that at 24 h of incubation with SIN-1, the sperm viability was significantly reduced compared to untreated control (P < 0.001). Furthermore, the MPT was induced (P < 0.01) and increment in DNA oxidation (P < 0.01), DNA fragmentation (P < 0.01), tyrosine nitration (P < 0.0001) and ultrastructural damage were observed when compared to untreated control. Caspase activation was not evidenced, and although phosphatidylserine externalization increased compared to untreated control (P < 0.001), this process was observed in <10% of the cells and the gradual loss of viability was not characterized by an important increase in this parameter. In conclusion, peroxynitrite-mediated nitrosative stress induces the regulated variant of cell death known as MPT-driven necrosis in human spermatozoa. This study provides a new insight into the pathophysiology of nitrosative stress in human spermatozoa and opens up a new focus for developing specific therapeutic strategies to better preserve sperm viability or to avoid cell death.

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INTRODUCTION

It is currently recognized that eukaryotic cells react to potentially dangerous perturbations of the intracellular or extracellular microenvironment by activating mechanisms that attempt to restore homeostasis; however, if these adaptive responses are not successful, they may actively engage in programmed cell death.1 The mechanism by which a cell dies depends on various exogenous factors as well as the cell’s ability to handle the stress to which it is exposed.2 Thus, a cell can die by different mechanisms including apoptosis, regulated necrosis, and autophagy;2 Programmed cell death manifests with various characteristics, including a series of complex pathways that lead to the elimination of the cells in a regulated manner [for extensive reviews see 1,3,4].

The process of cell death in spermatozoa is also of great biological importance because almost all the millions of spermatozoa that are permanently produced are destined to die in the testes or in the female genital tract, with the exceptions being those that fertilize an oocyte.3 Moreover, it is very striking that in a normal ejaculate from men with verified fertility, more than 40% of spermatozoa are dead.6

Given that spermatozoa are transcriptionally silent cells with a unique nuclear constitution and a highly specialized architecture, they cannot undergo a programmed death in the conventional sense as has been described for somatic cells.7 Thus, the mechanisms that underlie cell death in human spermatozoa remain incompletely understood, and although this subject has been studied, the information available is mainly centered on a type of cell death with partially apoptotic features.7–10 It is currently accepted that spermatozoa exposed to oxidative stress default to an intrinsic apoptotic pathway after the start of a lipid peroxidation cascade.11

However, in addition to oxidative stress, nitrosative stress caused by the excessive levels of reactive nitrogen species (RNS) also leads to impaired sperm function,12–17 Nitrosative stress in sperm cells can be induced by the RNS produced by the spermatozoa themselves15,18
and by other cells in the male reproductive tract. Overproduction of RNS in the male reproductive system is observed in lifestyles and pathophysiological conditions including diabetes mellitus, idiopathic asthenozoospermia, varicocele, smoking, and prolonged time periods. Thus, approaches to properly mimic the pathophysiological generation of peroxynitrite include infusion of compounds such as 3-morpholinosydnonimine (SIN-1) or other generation systems. Furthermore, in vitro studies have demonstrated that peroxynitrite induces lipid peroxidation of the plasma membrane, impairs motility and mitochondrial membrane potential, decreases the ATP production affecting both glycolysis and oxidative phosphorylation, and causes thiol oxidation in human spermatozoa. In somatic cells, peroxynitrite is a potent inducer of apoptosis and necrosis; however, neither the ability of peroxynitrite to induce cell death nor the morphological and biochemical correlates of peroxynitrite-mediated cytotoxicity in human spermatozoa have been described to date. Thus, the aim of this study was to characterize the biochemical and morphological features of cell death induced in vitro by peroxynitrite-mediated nitrosative stress in human spermatozoa.

MATERIALS AND METHODS

Donors of semen samples and ethical approval

Semen samples from healthy donors who were students at the University of La Frontera, Temuco, Chile, and the National University of Cuyo, Mendoza, Argentina, were used. The study was approved by the Scientific Ethics Committee at the University of La Frontera. Furthermore, normozoospermic semen samples from male members of couples consulting for infertility at the Department of Obstetrics and Gynecology at the University of Cologne, Cologne, Germany, were used and the use of these samples for research was approved by the Ethics Committee of the Faculty of Medicine at the University of Cologne. These semen samples were obtained from 29 men. All participants in this study signed written informed consent.

Semen preparation

Semen samples were obtained by masturbation, after at least 3 days of sexual abstinence, collected in sterile containers, and delivered to the laboratory in <60 min. Standard semen analysis was performed according to the World Health Organization (WHO) guidelines, and only normal semen samples according to the WHO criteria were used.

The swim-up technique with modified human tubular fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) was used to select the motile fraction from the semen samples.

In vitro generation of peroxynitrite-mediated nitrosative stress

In vivo peroxynitrite is usually produced at lower rates, but for prolonged time periods. Thus, approaches to properly mimic the pathophysiological generation of peroxynitrite include infusion of peroxynitrite to biological systems, or the simultaneous generation of its precursors nitric oxide (NO) and superoxide anion (O2−), either using compounds such as 3-morpholinosydnonimine (SIN-1) or independent NO and O2− generation systems. According to this, the compound SIN-1 (Enzo Life Science Inc., Farmingdale, NY, USA) was used in this study to generate nitrosative stress in human spermatozoa. This compound in solution and in the presence of molecular oxygen releases NO in a process associated with the generation of O2−, both of them react together and form peroxynitrite. A previous report of our work group demonstrated the proper generation of peroxynitrite in suspensions of human spermatozoa using SIN-1. SIN-1 solution was freshly prepared at 100 mmol L−1 as previously described.

Experimental protocol of exposure to peroxynitrite

Aliquots of sperm suspension in HTF medium were exposed to 0.8 mmol L−1 of SIN-1 for 4 h and for 24 h at 37°C. The generation of peroxynitrite in human sperm suspensions by this SIN-1 concentration was previously demonstrated by our work group. Furthermore, the induction of nitrosative stress by SIN-1 which led to impairment of sperm parameters was previously reported. Thus, the SIN-1 concentration selected for this study was based on these previous reports. The incubation times were selected in order to obtain an early and a late incubation times. For each experimental condition, aliquots of untreated sperm were included as controls. After incubation, the spermatozoa, including controls, were washed twice with HTF by centrifugation (Biofuge Fresco, Heraeus, Hayward, CA, USA) at 500 g for 5 min and resuspended with HTF medium for subsequent analysis.

Analysis of sperm viability

The sperm viability was evaluated by incorporating propidium iodide (PI; Sigma-Aldrich Inc., St. Louis, MO, USA). For this, 1 ml of sperm aliquots (1 × 106 spermatozoa per ml) previously exposed to SIN-1 and controls was incubated with 1 μmol L−1 of PI, incubated for 5 min at room temperature, washed once with Dulbecco’s phosphate-buffered saline (DPBS; IrvineScientific), and resuspended with 300 μl of DPBS for flow cytometry analysis. The sperm viability was determined as the percentage of PI-negative cells.

Analysis of mitochondrial permeability transition

The mitochondrial permeability transition (MPT) was evaluated by the MitoProbe™ Transition Pore Assay kit (Molecular Probes, Invitrogen, Eugene, OR, USA), which has been previously used to assess the mitochondrial permeability transition pore (mPTP) opening. This method uses calcein-acetoxyethyl (calcium-AM) staining, which passively diffuses into cells and accumulates in the cytosolic compartments, including the mitochondria. Cytosolic calcium fluorescence is quenched by the addition of cobalt chloride (CoCl2) while mitochondrial calcium fluorescence is maintained, since the CoCl2 is not able to pass through intact inner mitochondrial membrane. The mPTP opening alters the permeability of the mitochondria, allowing either the release of calcium or the entry of CoCl2 and the complete quenching of calcium fluorescence. Thus, MPT is evidenced by a drastic decrease in calcium fluorescence.

For the experiments, a previously described procedure was followed. Briefly, 1 ml of sperm aliquot (1 × 106 spermatozoa per ml) exposed to SIN-1 and its respective untreated controls was incubated with 0.01 μmol L−1 of calcium-AM and 0.4 μmol L−1 of CoCl2. Three technique controls were also included by incubating sperm aliquots with (i) calcium-AM; (ii) calcium-AM and CoCl2; and (iii) calcium-AM, CoCl2, and 0.5 μmol L−1 ionomycin of which the latter induces the mPTP opening. The cells were incubated at 37°C for 15 min in darkness, washed once, and resuspended in 300 μl of DPBS for flow cytometry analysis. The results were expressed as mean fluorescence intensity (MFI) of calcine.

Analysis of phosphatidylserine (PtdSer) externalization

The PtdSer externalization was evaluated using the Annexin V-Alexa Fluor® 488 conjugate (Thermo Fisher Scientific Inc., Waltham, MA, USA). For experiments, 1 ml of sperm aliquots (1 × 106 spermatozoa per ml) previously exposed to SIN-1 and controls was washed twice and resuspended with 100 μl of binding buffer (MACS
ART Binding Buffer; Millenyi Biotec, Cologne, Germany). Then, 5 μl of Annexin V-Alexa Fluor® 488 conjugate was added and the cells were incubated in the dark for 15 min at room temperature. After the incubation period, 400 μl of binding buffer and 1 μmol l⁻¹ of PI were added and the fluorescence intensity was analyzed by flow cytometry. The externalization of PtdSer was considered as the percentage of Annexin V positive and PI negative cells.

**Analysis of caspase activation**

Caspase activation was evaluated using the FAM-FLICA® in vitro Caspase-3/7 detection kit (ImmunoChemistry Technologies, Bloomington, MN, USA). The fluorescent-labeled inhibitors of caspase (FLICA) probes covalently link to the active caspase, being retained within the cell, allowing fluorescence detection by flow cytometry.

For the experiments, 1 ml of sperm aliquots (1 × 10⁶ spermatozoa per ml) previously exposed to SIN-1 and controls was washed twice, resuspended with 300 μl of DPBS, and incubated with 10 μl of 30 × FLICA solution for 60 min at 37°C. After that, the cells were washed twice with 1 × apoptosis wash buffer and 1 μmol l⁻¹ of PI was added. Fluorescence intensity was analyzed by flow cytometry and the results were expressed as the MFI of FLICA.

**Analysis of DNA oxidation**

The DNA oxidation was evaluated by the OxyDNA Assay kit (Merck KGaA, Darmstadt, Germany), which is based on direct binding of a fluorescein isothiocyanate (FITC)-conjugated probe to the 8-oxoguanine, a product of DNA oxidation induced by free radicals.

For the experiments, 1 ml of sperm aliquot (4 × 10⁶ spermatozoa per ml) previously exposed to SIN-1 and its untreated controls was fixed in 2% paraformaldehyde for 15 min at 4°C and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Subsequently, sperm were incubated with FITC conjugate for 1 h at room temperature in the dark. Finally, the cells were washed twice and resuspended in DPBS. Fluorescence intensity was analyzed by flow cytometry and the results were expressed as the percentage of FITC-positive cells.

**Analysis of DNA fragmentation**

The In Situ Cell Death Detection kit (Roche, Mannheim, Germany), which is based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique, was used to analyze the sperm DNA fragmentation. For the experiments, 1 ml of sperm aliquot (4 × 10⁶ spermatozoa per ml) previously exposed to SIN-1 and its untreated controls was fixed in 2% paraformaldehyde for 15 min at 4°C and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Then, the sperm were incubated with the TUNEL reaction solution and incubated for 1 h at 37°C. Finally, the cells were washed twice, resuspended in DPBS, and analyzed by flow cytometry. The results were expressed as the percentage of FITC-positive cells.

**Analysis of tyrosine nitration**

The analysis of the tyrosine nitration was performed by immunofluorescence using an antibody against 3-nitrotyrosine (3-NT). For the experiments, sperm aliquots (1 × 10⁶ spermatozoa per ml) previously exposed to SIN-1 and its untreated control were attached to coverslips, fixed in 2% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. After that, the cells were covered for 30 min with blocking solution (PBS supplemented with 0.5% bovine serum albumin [BSA]). The spermatozoa were incubated with an anti-3-NT antibody (sc-55256; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted 1:100 in blocking solution overnight at 4°C. Then, the spermatozoa were washed 3 times for 5 min with PBS and incubated for 30 min at room temperature with an anti-rabbit IgG biotin conjugate (Sigma-Aldrich Inc.) diluted 1:800 with blocking solution. Once the incubation time ended, the sperm were washed 3 times with PBS and incubated for 15 min at room temperature with ExtrAvidin®-FITC (Sigma-Aldrich Inc.) diluted 1:600 with blocking solution. The absence of nonspecific staining was assessed by processing a control without primary antibody. Subsequently, the cells were washed 3 times with PBS, 1 μmol l⁻¹ of PI was added, incubated for 5 min at room temperature, and washed twice with PBS. Finally, the coverslips were mounted on slides using Fluoroshield™ Mounting Medium (Sigma-Aldrich Inc.) for subsequent analysis. Images of sperm cells were acquired with a confocal microscope (FV10-ASW; Olympus, New York, NY, USA). The relative fluorescence intensity (RFI) in the principal piece and in the entire flagellum (middle piece plus principal piece) of the sperm was analyzed using ImageJ software (Image J 1.32), National Institutes of Health, Bethesda, MD, USA).

**Analysis of sperm ultrastructure**

The analysis of sperm ultrastructure was performed by transmission electron microscopy (TEM) using a Zeiss EM 900 transmission electron microscope (Zeiss, Oberkochen, Germany). For the experiments, sperm aliquots (2 × 10⁶ spermatozoa per ml) from three different donors previously exposed to SIN-1 and controls were fixed for 2 h at 0°C–4°C with fixative solution consisting of 4% paraformaldehyde (w/v), 4% glutaraldehyde (w/v), and 20% picric acid (v/v) saturated in PBS. Fixed sperm were washed twice with PBS for 10 min at 600 g. Then, the pellet containing the spermatozoa was postfixed by adding 30 ml of 1% osmium (VIII) oxide (OsO₄; w/v) and incubated overnight at 4°C. Osmified samples were dehydrated in ethanol-acetone up to absolute acetone and embedded in Epon 812 epoxy resin (Ted Pella Inc., Redding, CA, USA). Ultrathin sections were obtained by Ultracut R microtome (Leica Biosystems, Vienna, Austria) and stained with classical uranyl acetate and lead citrate TEM stain. The ultrathin sections were examined with a TEM at 80 kV. The results were presented as percentage of alterations on sperm ultrastructure. Representative images of each condition were also obtained.

**Analysis by flow cytometry**

The analyses of viability, MPT, PtdSer externalization, DNA oxidation, and DNA fragmentation were made using a flow cytometer FACScalibur (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA). Samples were acquired and analyzed with the software Cell Quest Pro (Becton, Dickinson and Company). The fluorescence analysis of caspase activation and the analysis of the single sample over time for PtdSer externalization were performed in a FACSCanto II flow cytometer, and data were analyzed with the software FACSDiva™ version 6.1.3 (Becton, Dickinson and Company). The fluorescence from FITC, calcein, and FLICA were detected using the band pass filter of 530/30 nm, and the fluorescence from PI was detected with a band pass filter of 585/42 nm. All analyses by flow cytometry were done on logarithmic scales and data from 10 000 sperm events were analyzed in each experiment.

**Statistical analyses**

The treatment of spermatozoa with SIN-1 and its controls was carried...
Results

Sperm viability

First, experimental conditions in which peroxynitrite causes cell death of human spermatozoa were established. The current recommendations suggest considering as dead cells those that either exhibit irreversible plasma membrane permeabilization or have undergone complete fragmentation. Thus, sperm viability, measured as the integrity of plasma membrane after exposure to SIN-1 for 4 h and for 24 h, was analyzed. No significant differences between the untreated control and spermatozoa exposed to peroxynitrite for 4 h were observed (P > 0.05), whereas a significant decrease (P < 0.001) in sperm viability was observed at 24 h of incubation with 0.8 mmol l⁻¹ of SIN-1 (Table 1). Thus, these experimental conditions were used to analyze the biochemical markers and morphological alterations associated with cell death induced by peroxynitrite in human spermatozoa.

MPT

The MPT in human spermatozoa exposed to 0.8 mmol l⁻¹ of SIN-1 for 4 h and 24 h was analyzed by a technique that uses three controls, which are spermatozoa incubated with: (i) calcein (Cal), (ii) calcein and cobalt chloride (Cal-Co), and (iii) calcein, cobalt chloride, and ionomycin (Cal-Co-Io). The results in Figure 1 show that in the Cal group, the fluorescence was the highest, consistent with the calcein distribution in all cellular compartments. In Cal-Co spermatozoa, the fluorescence of calcein decreased, due to calcein was limited to mitochondria, where cobalt chloride cannot pass indicating the intactness of the inner mitochondrial membrane. In the control group with Cal-Co-Io, the calcein fluorescence was negligible, indicating that MPT process had occurred allowing access of cobalt chloride to the mitochondrial matrix. In the experimental group treated with SIN-1 (Cal-Co-SIN) for 4 h, the MFI of calcein did not differ significantly compared with the Cal-Co spermatozoa (P > 0.05), indicating that at this incubation time, the MPT did not occur (Figure 1a). However, in spermatozoa exposed for 24 h to SIN-1, the MFI of calcein was significantly decreased compared to Cal-Co spermatozoa (P < 0.01), indicating that the MPT process had occurred (Figure 1b).

PtdSer externalization

The percentage of living cells displaying translocation of PtdSer to the outer leaflet of the plasma membrane (Annexin V-positive and PI-negative cells), after 4 h of incubation with peroxynitrite, was not statistically significant compared to the untreated control (P > 0.05); and although was significantly increased after 24 h of incubation (P < 0.001), the percentage of cells with this characteristic was <10% (Table 1). Considering that at 24 h of incubation, the sperm viability was near to 20% and in order to test whether externalization of PtdSer to the outer leaflet of plasma membrane occurs before 24 h of incubation, when the integrity of plasma membrane was still preserved, a single semen sample was tracked from 4 h to 20 h of incubation with 0.8 mmol l⁻¹ of SIN-1. Figure 2 shows that from 12 h of incubation with peroxynitrite, the sperm cells gradually lost their viability, but no important increase in the percentage of PtdSer externalization (Annexin V-positive and PI-negative cells, lower-right quadrant in each dot plot) was observed (Figure 2), indicating that peroxynitrite induces sperm cell death but did not trigger a process of PtdSer translocation.

Caspase activation

The results of caspase activation analyzed in spermatozoa incubated with SIN-1 showed that at 4 h or 24 h, the MFI of FLICA did not differ significantly compared to the untreated control (P > 0.05, Table 1), indicating that under the experimental conditions tested, peroxynitrite did not induce caspase activation in human spermatozoa.

DNA oxidation and fragmentation

DNA integrity was analyzed in terms of DNA oxidation and fragmentation in spermatozoa exposed to SIN-1 for 4 h and 24 h. The results showed that at 4 h of incubation, the percentage of sperm displaying oxidized DNA was similar to the untreated control (P > 0.05). However, after 24 h of incubation, the DNA oxidation was increased in spermatozoa exposed to SIN-1 compared to the untreated control (P < 0.01, Table 1). In the same way, DNA fragmentation increased in spermatozoa exposed to SIN-1 only after 24 h of incubation compared to the untreated control (P < 0.01, Table 1).

Tyrosine nitration

Tyrosine nitration analyzed in untreated human spermatozoa demonstrated positivity for this posttranslational protein modification mainly in the acrosomal region and middle piece (Figure 3a–3c). However, in spermatozoa treated with 0.8 mmol l⁻¹ of SIN-1, the signal increased, and a strongly positive signal for 3-NT was observed in the acrosome and flagellum, which was already evident after 4 h of incubation with SIN-1 (Figure 3d–3f). The sperm incubated without the first antibody showed only a weak signal in the acrosome and middle piece region (Figure 3g–3i). When the relative fluorescence intensity (RFI) was analyzed in the sperm, a significant difference in spermatozoa treated with SIN-1 compared to the untreated control was observed both in the principal piece (11.1±2.3 vs 8.7±1.7, respectively; P < 0.0001) and in the entire flagellum (middle piece plus principal piece; 11.8±2.3 vs 9.6±1.9, respectively; P < 0.0001). These results indicate that peroxynitrite caused an increase in tyrosine nitration in human spermatozoa which was evident after 4 h of incubation.
Ultrastructure

The ultrastructure of spermatozoa was analyzed under the same experimental conditions. Figure 4 shows representative images obtained by transmission electron microscopy analysis from untreated spermatozoa and from spermatozoa exposed to SIN-1. It is noted that the untreated control at 4 h and 24 h of incubation showed a conserved ultrastructure in the acrosomal region, head and middle piece (Figure 4a–4d). The majority of sperm showed nuclei with an appropriate condensation (asterisk in Figure 4c) and normal mitochondria (arrows in Figure 4d), corresponding to selected spermatozoa from normozoospermic donors.

However, compared to the untreated control, in the spermatozoa treated with SIN-1, changes attributable to nitrosative stress were observed in the ultrastructure already at 4 h (Figure 4e and 4f). These changes included swelling in the plasma membrane, evident in 70% of sperm cells ($P < 0.05$); acrosome undulation and detaching of the acrosomal vesicles from the nuclear membrane (arrow head in Figure 4f) were observed in 26% of spermatozoa ($P < 0.05$). Some of sperm mitochondria (19%) displayed mitochondrial swelling ($P > 0.05$). The sperm tails show normal ultrastructural features. Moreover, these alterations were accentuated in the cells exposed to SIN-1 for 24 h (Figure 4g and 4h). The nuclei were found to be less electron-dense and displayed a granulated appearance, evident in 20% of sperm head ($P < 0.05$; asterisk in Figure 4g). Ninety percent of spermatozoa with broken plasma membrane ($P < 0.05$) and 80% of sperm with reacted or absent acrosome ($P < 0.05$) were observed (arrow head in Figure 4h). The cells showing mitochondrial swelling, characterized by electron-lucid areas (arrow in Figure 4h), increased to 60% ($P < 0.05$).

DISCUSSION

Peroxynitrite is a highly reactive nitrogen species which causes nitrosative stress and is a potent inducer of apoptosis and necrosis in somatic cells. Our study demonstrated for the first time that cell death induced by peroxynitrite in human spermatozoa is characterized by induction of the MPT process accompanied by DNA oxidation and fragmentation, tyrosine nitration, and damage to the ultrastructure; however, it is not associated with PtdSer externalization or caspase activation. These results suggest that nitrosative stress induces the regulated variant of cell death.

Table 1: Analysis of biochemical markers of cell death on human spermatozoa at 4 h and 24 h of incubation with 0.8 mmol l$^{-1}$ of 3-morpholinosydnonimine

| Parameter                      | Untreated control | SIN-1 (0.8 mmol l$^{-1}$) | Untreated control | SIN-1 (0.8 mmol l$^{-1}$) |
|--------------------------------|-------------------|---------------------------|-------------------|---------------------------|
| Viability (%)                  | 72.6±7.1          | 75.8±4.5                  | 86.4±4.4          | 23.9±19.3                |
| PtdSer exposure (%)            | 2.9±1.8           | 4.6±3.4                   | 1.8±0.7           | 8.7±4.0                  |
| Caspase activation (MFI)       | 564.2±78.6        | 440.2±174.8               | 544.0±69.9        | 650.1±160.2              |
| DNA oxidation (%)              | 7.6±3.6           | 5.3±2.9                   | 12.2±4.6          | 21.6±8.4                 |
| DNA fragmentation (%)          | 3.2±2.7           | 3.2±2.3                   | 7.6±5.3           | 16.2±4.8                 |

Values correspond to mean±s.d. of 4 (viability, caspase activation) and 5 (PtdSer exposure, DNA oxidation and fragmentation) different experiments. *$P < 0.01$, **$P < 0.001$ compared to the untreated control at the same incubation time. SIN-1: 3-morpholinosydnonimine; PtdSer: phosphatidylserine; MFI: mean fluorescence intensity, s.d.: standard deviation.
Peroxy nitrite-induced sperm cell death

P Uribe et al

Figure 4: Analysis of ultrastructure of human spermatozoa exposed to peroxynitrite. Transmission-electron micrographs of human sperm incubated for 4 h and 24 h without (untreated control; a-d) and with 0.8 mmol l⁻¹ of SIN-1 (e-h). The asterisk indicates normal nuclei in c but indicates degranulated nuclei in g. Arrows indicate preserved mitochondria in d, and impaired mitochondria in h. Arrow heads in f and h indicate acrosome undulation. Magnification is x7000 in a, c, e and g; x20 000 in d; x30 000 in b, f and h. SIN-1: 3-morpholinosydnonimine.

known as MPT-driven necrosis. MPT is characterized by the assembly and prolonged opening of the mitochondrial permeability transition pore which, in somatic cells, is modulated by calcium, reactive oxygen species (ROS), and RNS. MPT causes a rapid increase in the permeability of the inner mitochondrial membrane, leading to dissipation of the mitochondrial membrane potential (ΔΨm), uncoupling of the respiratory chain, and influx of water and ions, which drives the osmotic swelling of the mitochondrial matrix, causing a mechanical breakdown of the outer mitochondrial membrane. Widespread MPT can cause cell death via regulated necrosis or apoptosis. MPT-driven necrosis is induced when the dissipation of the ΔΨm leads to arrest in ATP synthesis. On the contrary, MPT-driven apoptosis is carried out by mitochondrial intermembrane proteins released into the cytoplasm as consequence of MPT and requires the activation of the proteolytic enzyme caspases in an ATP-dependent manner. Thus, MPT may induce apoptosis or regulated necrosis depending on the intracellular availability of ATP (reviewed by Bonora et al. 📂). We have already reported that peroxynitrite decreases the ATP production in sperm cells, thus, we propose that the ATP over-activation consumes nicotinamide adenine dinucleotide (NAD) leading to ATP depletion, culminating in cell dysfunction and death coinciding with what was observed in this study. There are no previous reports regarding the effect of peroxynitrite on sperm DNA, although a positive correlation between RNS and DNA fragmentation has been reported. Sperm DNA damage has an important clinical impact since it is not only associated with reduced male fertility but also associated with birth defects and several forms of morbidity in the offspring (reviewed by Gharagozloo and Aitken 📂).

The exposure of spermatozoa to peroxynitrite also resulted in an increase in tyrosine nitration, which is a posttranslational protein modification due to the addition of a nitro group adjacent to the hydroxyl group on the aromatic ring of tyrosine residues. Tyrosine nitration has been associated with at least 50 human diseases affecting protein structure and function, being considered an essential feature of peroxynitrite-mediated cytotoxicity. In our experimental settings, the basal level of tyrosine nitration was significantly increased in spermatozoa after 4 h of peroxynitrite exposure. This increase was mainly found in sperm flagella, suggesting the involvement of structural proteins and metabolic enzymes, which can explain, at least in part, the decrease in sperm motility also reported for us under the same conditions. In agreement with our results, it was previously reported that the exposure to peroxynitrite caused wider tyrosine nitration in the flagellum, and the exposure to 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine sodium (diethylamine NONOate), a nitric oxide donor, increased the levels of tyrosine nitration, which were also located at the head and tail regions being associated to impaired sperm capacitation. In this regard, it is noteworthy that in asthenozoospermic men, an increase in tyrosine nitration has been associated with impairment of sperm function, and this may be attributed to negative effects on structure, intracellular...
Based on the above, we propose that

\[ \Delta \Psi \]

Figure 5: Diagram of the observed effects after peroxynitrite-mediated nitrosative stress in human spermatozoa and its possible interrelationships. \( \Delta \Psi \): mitochondrial membrane potential; ATP: adenosine triphosphate; MPT: mitochondrial permeability transition.
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