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RESEARCH ARTICLE

Thiol oxidation by nitrosative stress: Cellular localization in human spermatozoa

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ABSTRACT

Peroxynitrite is a highly reactive nitrogen species and when it is generated at high levels it causes nitrosative stress, an important cause of impaired sperm function. High levels of peroxynitrite have been shown to correlate with decreased semen quality in infertile men. Thiol groups in sperm are mainly found in enzymes, antioxidant molecules, and structural proteins in the axoneme. Peroxynitrite primarily reacts with thiol groups of cysteine-containing proteins. Although it is well known that peroxynitrite oxidizes sulfhydryl groups in sperm, the subcellular localization of this oxidation remains unknown. The main objective of this study was to establish the subcellular localization of peroxynitrite-induced nitrosative stress in thiol groups and its relation to sperm motility in human spermatozoa. For this purpose, spermatozoa from healthy donors were exposed *in vitro* to 3-morpholinosydnonimine (SIN-1), a compound which generates peroxynitrite. In order to detect peroxynitrite and reduced thiol groups, the fluorescent probes, dihydrorhodamine 123 and monobromobimane (mBBr), were used respectively. Sperm viability was analyzed by propidium iodide staining. Peroxynitrite generation and thiol redox state were monitored by confocal microscopy whereas sperm viability was evaluated by flow cytometry. Sperm motility was analyzed by CASA using the ISAS® system. The results showed that exposure of human spermatozoa to peroxynitrite results in increased thiol oxidation which is mainly localized in the sperm head and principal piece regions. Thiol oxidation was associated with motility loss. The high susceptibility of thiol groups to peroxynitrite-induced oxidation could explain, at least in part, the negative effect of reactive nitrogen species on sperm motility.

Abbreviations: DHR: dihydrorhodamine 123; mBBr: monobromobimane $ONOO^-$: peroxynitrite RNS: reactive nitrogen species RFI: relative fluorescence intensity SIN-1: 3-morpholinosydnonimine CASA: Computer-Aided Sperm Analysis PARP: poli ADP ribose polimerasa VCL: curvilinear velocity VSL: straight-line velocity VAP: average path velocity PRDXs: peroxiredoxins ODF: outer dense fiber ODF1: outer dense fiber 1 PI: propidium iodide DMSO: dimethyl sulfoxide SD: standard deviation ANOVA: analysis of variance

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Introduction

Nitrosative stress is the result of biomolecular modifications caused by reactive nitrogen species (RNS). Nitrosative stress levels are mainly determined by the concentration and exposure time to RNS and by the ability of cellular antioxidant defense systems to remove them [Calcerrada et al. 2011]. The RNS include nitric oxide (NO), nitrogen dioxide (NO_2), and peroxynitrite ($ONOO^-$), with NO_2 and $ONOO^-$ being the most highly reactive [Pacher et al. 2007; Massari et al. 2011; Nash et al. 2012; Li et al. 2015].

Peroxynitrite is a RNS produced by the diffusion-controlled reaction between nitric oxide and superoxide anion and is considered a highly toxic molecule due to its adverse effects on cell viability and function [Pacher et al. 2007; Szabo et al. 2007; Speckmann et al. 2016]. The high stability and diffusion rate of peroxynitrite contributes to its high toxicity, allowing it to exert its toxic effect far away from its production site [Beckman et al. 1994]. Given its nature, peroxynitrite triggers several cytotoxic processes: lipid peroxidation, inhibition and depletion of antioxidant enzymes,

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matrix metalloproteinases activation, disturbances in signal transduction pathways, mitochondrial dysfunction, inhibition of cytosolic enzymes and membrane pumps, Ca^{2+} dysregulation, DNA damage, PARP activation, and inhibition of NAD-dependent enzymes. These processes are mainly a consequence of oxidation and nitration of cell components that finally lead to cell death by apoptosis or necrosis [Pacher et al. 2005; Tao et al. 2012].

Although RNS at low levels plays an important role in physiological sperm function, when produced at high levels they may induce sperm damage, loss of sperm function, and decreased fertilizing ability [Doshi et al. 2012]. Mammalian spermatozoa are particularly susceptible to RNS-induced damage because of their high content of membrane-bound polyunsaturated fatty acids and their low levels of cytosolic antioxidant enzymes [Agarwal et al. 2003]. Previous studies have found an association between high levels of peroxynitrite and nitration of tyrosine residues [Salvolini et al. 2012], poor morphology [Khosravi et al. 2012], and motility loss [Vignini et al. 2006] in infertile men. In addition, the *in vitro* exposure to high levels of peroxynitrite causes loss of motility, decrease of mitochondrial membrane potential [Uribe et al. 2015], and decrease of total amount of sulfhydryl groups [Oztezcan et al. 1999] on human spermatozoa.

Sulfhydryl group-rich proteins are mainly localized in the head [Seligman et al. 1994; Chabory et al. 2009] and principal piece of the sperm [Calvin and Bedford 1971; Oko 1988; Cabrillana et al. 2011], and many of these proteins are oxidized during sperm transit through the epididymis [Bedford and Calvin 1974; Pellicciari et al. 1983; Cornwall and Chang 1990; Seligman et al. 2004; Chabory et al. 2009; Cabrillana et al. 2011]. Coincident with sulfhydryl group oxidation, spermatozoa undergo a maturational process in the epididymis which results in the acquisition of progressive sperm motility [Bedford et al. 1973; Cornwall and Chang 1990]. However, imbalances in sulfhydryl group oxidation and overoxidation in thiol groups in mature spermatozoa modify progressive motility in rats [Cabrillana et al. 2011] and humans [Seligman et al. 1994; Ramos et al. 2008].

One of the main targets of peroxynitrite in somatic cells are cysteine residues. When peroxynitrite reacts with thiol groups of cysteine residues it forms sulfenic acid (RSOH), which in turn reacts with another thiol group to form disulfide groups (RSSR). Thiol groups may also be oxidized by peroxynitrite-derived radicals, generating thiyl radicals (RS•), and once these react with oxygen they can increase free radical reactions leading to oxidative stress [Pacher et al. 2007].

The oxidative effect of peroxynitrite on total sulfhydryl group content has been previously reported [Oztezcan et al. 1999; Vignini et al. 2009], however, the subcellular localization of thiol oxidation remains unknown. Therefore, the objective of this study was to localize the effect of peroxynitrite on thiol oxidation and its association with sperm motility in human spermatozoa.

Results

Sperm viability

Sperm viability was checked in spermatozoa selected by swim-up following exposure to 3-morpholinosydnonimine (SIN-1) which was used for the peroxynitrite generation. After incubation with 1 mmol/L of SIN-1 for 4 hours at 37°C, the percentage of sperm viability did not significantly decrease compared to the untreated control with the same incubation time (96.5 ± 0.6 and 95.9 ± 1.8, respectively, $p > 0.05$). Considering this result, for the following experiments we decided to work with a maximum concentration of 0.8 mmol/L of SIN-1 to ensure that SIN-1 concentrations were not toxic to spermatozoa.

Detection of peroxynitrite generation

The generation of peroxynitrite following exposure to SIN-1 was assessed using the fluorescent probe dihydrorhodamine 123 (DHR), which becomes highly fluorescent after oxidation by peroxynitrite. After a 2 hour incubation at 37°C, untreated sperm showed low fluorescence intensity and in only a few cells (Figure 1A and B). Exposure of sperm to 0.2 mmol/L of SIN-1 resulted in a slight increase in peroxynitrite as measured by DHR fluorescence (Figure 1C and D) which was highest after incubation with 0.8 mmol/L of SIN-1 (Figure 1E and F). This increase in peroxynitrite was not statistically significant in terms of relative fluorescence intensity (RFI) measured in the sperm flagellum (Figure 1M); however, after 4 hours of incubation at both concentrations of SIN-1, a high fluorescent signal was detected (Figure 1I - L), which also showed a significant increase in terms of RFI (Figure 1M). These results indicate that peroxynitrite generation following exposure to SIN-1 was adequate under our experimental conditions.

Similarly, the percentage of spermatozoa displaying DHR fluorescence at both concentrations of SIN-1 showed a more progressive increase than the untreated control. At 4 hours of incubation with 0.8 mmol/L of SIN-1, almost 80% of the sperm cells showed DHR

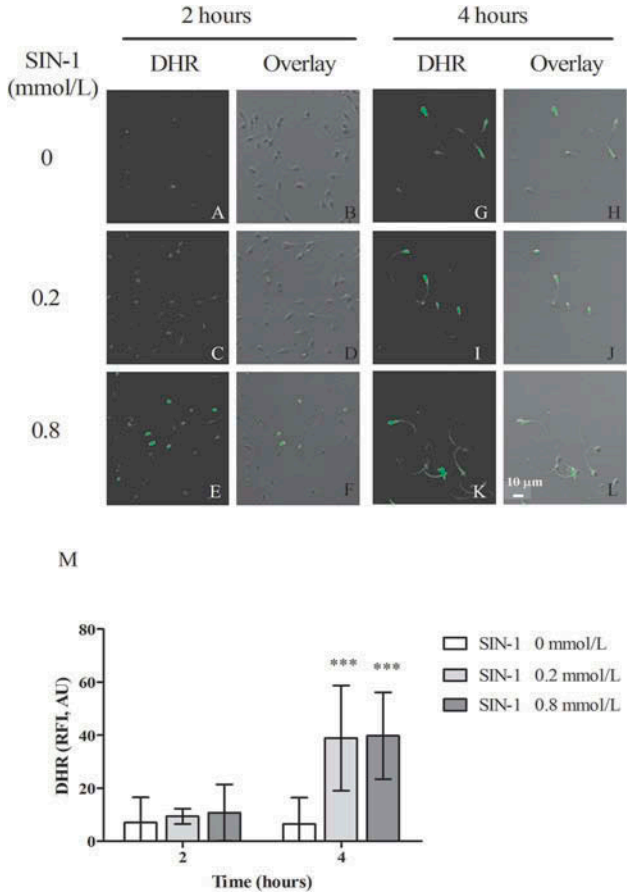

Figure 1. Detection of peroxynitrite generation. Human spermatozoa were exposed for 2 and 4 hours to 0 (A, B, G, and H), 0.2 (C, D, I, and J), and 0.8 (E, F, K, and L) mmol/L of SIN-1. Left column inside panels show sperm images of DHR fluorescence due to exposure to peroxynitrite and right columns correspond to the overlay with negative phase-contrast (PhC). (M) Representative images from one experiment. The relative fluorescence intensity (RFI-AU see text for explanation) of DHR was tabulated at 2 and 4 hours, under 0 (untreated control), 0.2, and 0.8 mmol/L of SIN-1. Values represent the mean ± SD. *** corresponds to $p < 0.05$ compared to the untreated control at the same incubation time. SIN-1: 3-morpholinosydnonimine; DHR: dihydrorhodamine 123.

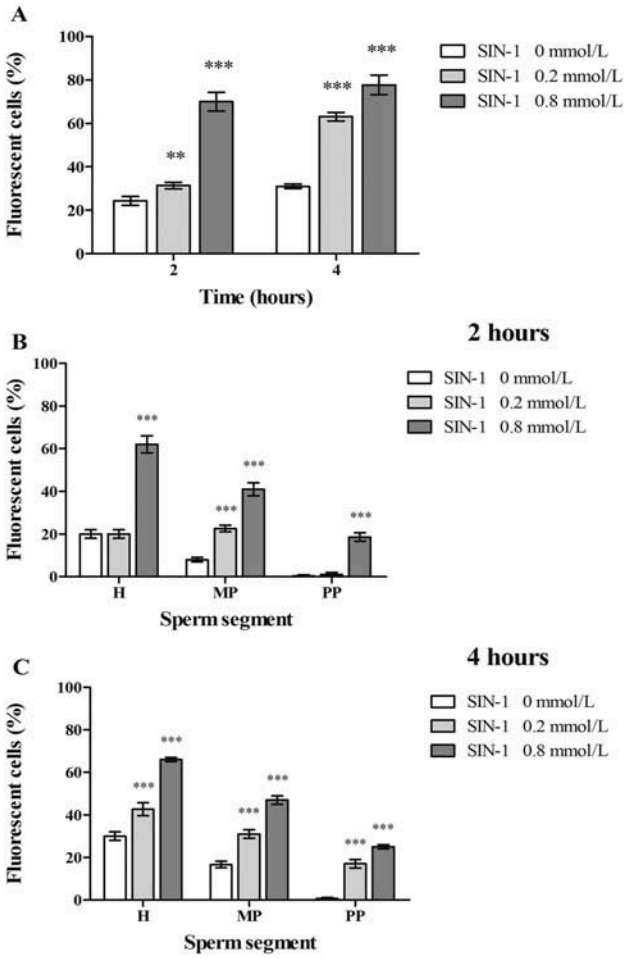

Figure 2. Percentage of spermatozoa showing positive DHR staining. Human spermatozoa were incubated with 0 (untreated control), 0.2, and 0.8 mmol/L of SIN-1 for 2 and 4 hours at 37°C. (A) Percentage of sperm cells displaying DHR fluorescence. (**) $p < 0.001$; (***) $p < 0.0001$ compared with the untreated control at the same incubation time. (B) and (C) percentage of sperm cells displaying DHR fluorescence in the head (H), midpiece (MP), and principal piece (PP) regions at 2 and 4 hours of incubation, respectively. Values represent the mean ± SD. (***) $p < 0.0001$ compared with the untreated control within the same sperm segment analyzed. SIN-1: 3-morpholinosydnonimine; DHR: dihydrorhodamine 123.

fluorescence (Figure 2A). When we analyzed the sperm regions that displayed DHR fluorescence, at 2 hours of incubation the head was the only region showing fluorescence in the untreated sperm. However, this fluorescence was detected in only 20% of the sperm cells (Figure 2B). Figure 2B also shows that at 2 hours of incubation the midpiece displayed a significant increase in DHR fluorescence at a concentration of 0.2 mmol/L of SIN-1 while at 0.8 mmol/L all the sperm regions showed DHR fluorescence. Finally, at 4 hours of incubation, the percentage of spermatozoa with DHR fluorescence in all sperm regions increased at both SN-1 concentrations compared to the untreated control (Figure 2C).

Effect of peroxynitrite on thiol groups

Once adequate peroxynitrite generation was confirmed following exposure to SIN-1, we proceeded to evaluate its effect on sulfhydryl group oxidation. The monobromobimane (mBBr) fluorescence was used to this end. After 2 and 4 hours of incubation at 37°C the untreated control showed spermatozoa displaying strong and homogeneous mBBr fluorescence consistent with sulfhydryl groups in the reduced state (Figures 3A, B, G, H). When spermatozoa were exposed to 0.2 mmol/L of SIN-1, no differences were found compared to the untreated

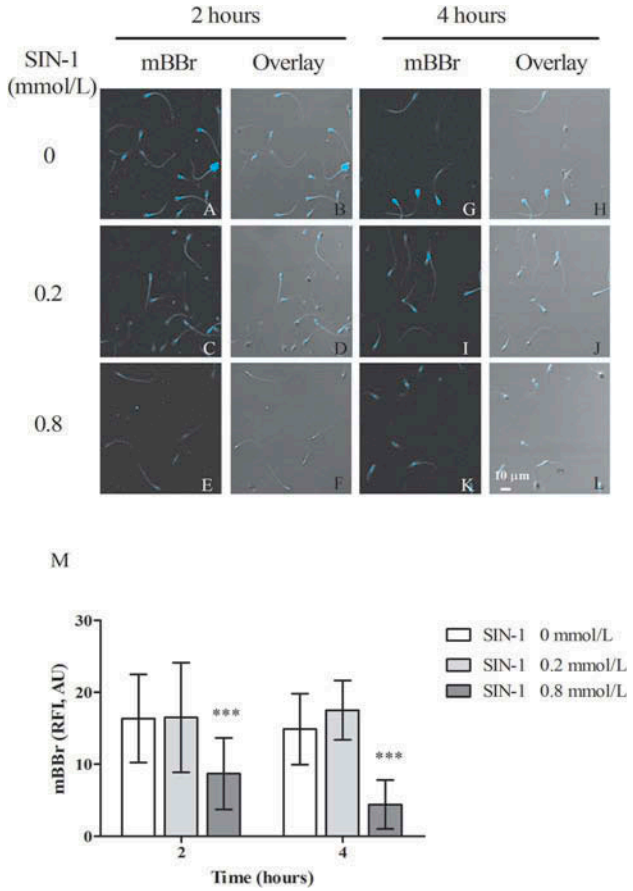

Figure 3. Effect of peroxynitrite on thiol group. Human spermatozoa were exposed for 2 and 4 hours at 0 (A, B, G, and H), 0.2 (C, D, I and J), and 0.8 (E, F, K, and L) mmol/L of SIN-1. Left column inside panels showed mBBr fluorescence sperm pictures due to thiol groups and right columns correspond to the overlay with phase contrast (PhC). (M) Representative images from one experiment. The relative fluorescence intensity (RFI-AU, see text for explanation) of mBBr was tabulated at 2 and 4 hours, under 0 (untreated control), 0.2, and 0.8 mmol/L of SIN-1. Values represent the mean ± SD. (***) $p < 0.05$ compared to the untreated control in the same incubation time. SIN-1: 3-morpholinosydnonimine; mBBr: monobromobimane.

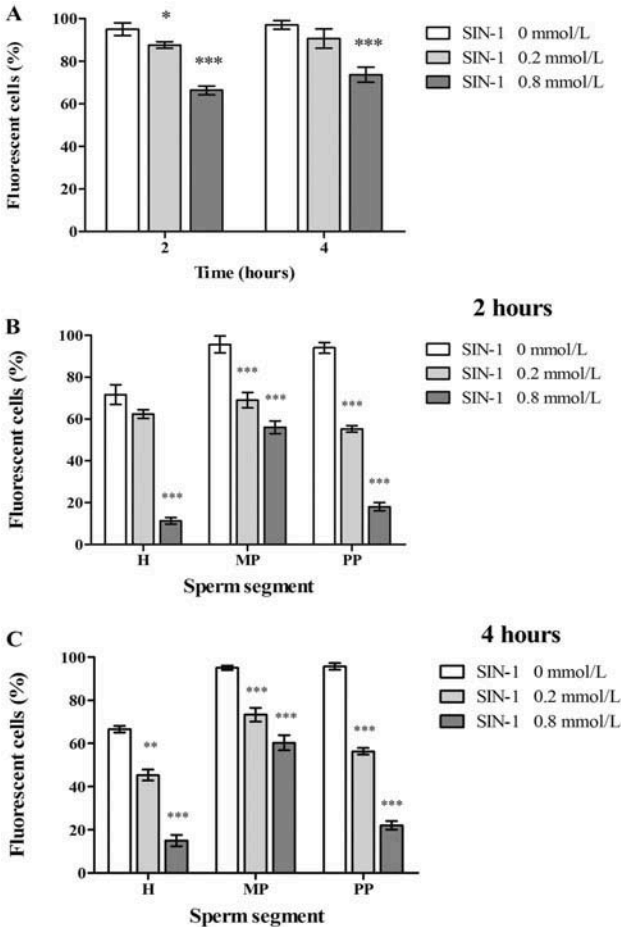

Figure 4. Percentage of spermatozoa showing positive mBBr staining. Human spermatozoa were incubated with 0 (untreated control), 0.2, and 0.8 mmol/L of SIN-1 for 2 and 4 hours at 37°C. (A) Percentage of mBBr fluorescent cells after 2 and 4 hours of incubation with different SIN-1 concentrations. Values represent the mean ± SD. (*) $p < 0.05$; (***) $p < 0.0001$ compared with the untreated control within the same time point. (B) and (C) Percentage of sperm displaying mBBr fluorescence in the head (H), midpiece (MP), and principal piece (PP) regions after 2 and 4 hours of incubation, respectively. Values represent the mean ± SD. (**) $p < 0.001$; (***) $p < 0.0001$ compared with the untreated control within the same sperm segment analyzed. SIN-1: 3-morpholinosydnonimine; mBBr: monobromobimane.

control after 2 and 4 hours of incubation (Figures 3C, D, I, J), which was confirmed when we analyzed the RFI in the sperm flagellum (Figure 3M). However, with 0.8 mmol/L of SIN-1, a significant drop in the fluorescence signal and RFI was observed after 2 and 4 hours of incubation, which was more pronounced at 4 hours (Figures 3E, F; Figures 3K, L quantification in M). This decrease in the RFI of mBBr is consistent with peroxynitrite-induced thiol oxidation.

When the percentage of sperm cells showing mBBr fluorescence after incubation for 2 and 4 hours with SIN-1 was analyzed, a more pronounced decrease in mBBr fluorescence was observed at 0.8 mmol/L of SIN-1 compared to the untreated control (Figure 4A). A progressive decrease in mBBr fluorescence was observed in the different sperm regions when spermatozoa were exposed to SIN-1 compared to the untreated control (Figures 4B, C). This effect was more pronounced in the sperm head and principal piece regions where, following exposure to 0.8 mmol/L of SIN-1 at both incubation times, only about 20% of the sperm cells displayed mBBr fluorescence (Figures 4B, C).

Effect of peroxynitrite on sperm motility

The effect of exposure to SIN-1 on sperm motility was also evaluated during incubation at 37°C for up to 4 hours.

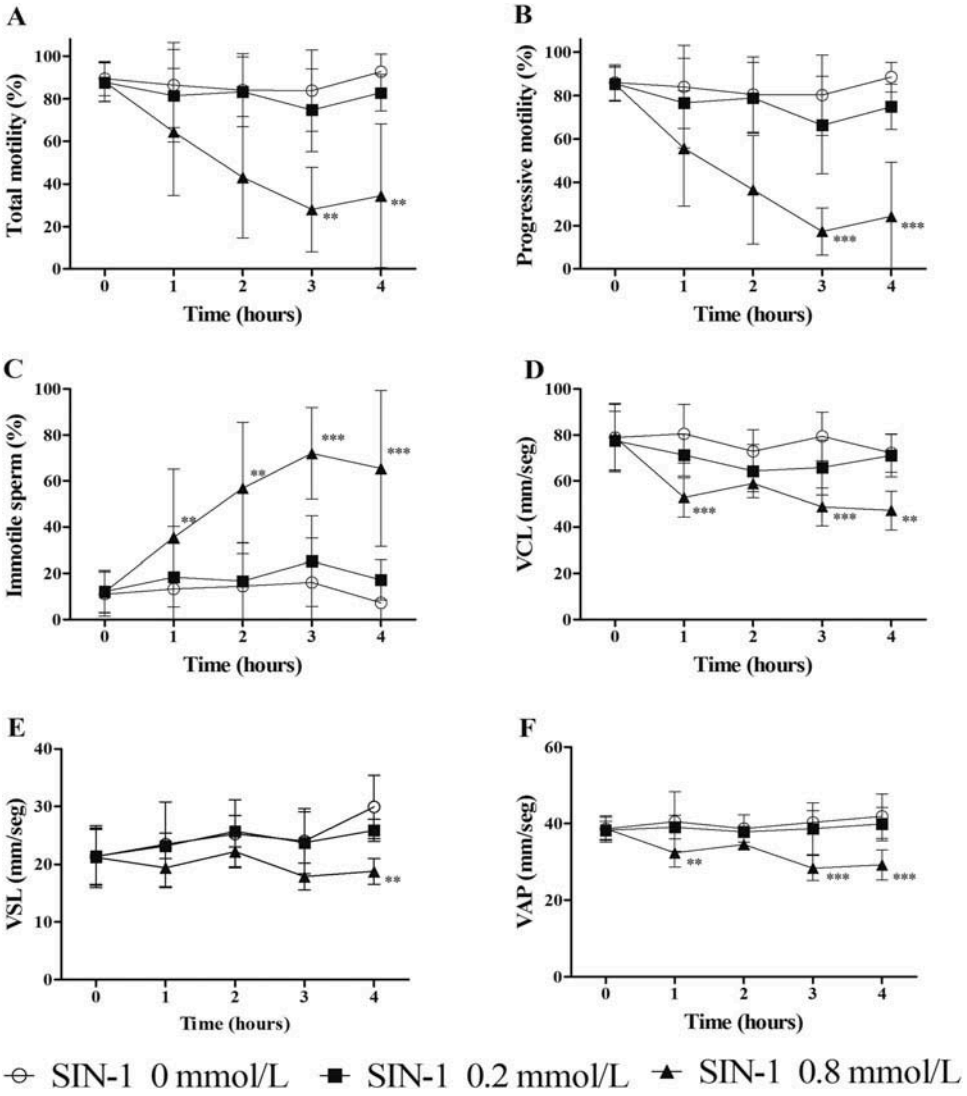

Figure 5. Effect of peroxynitrite on sperm motility. Human spermatozoa were incubated with 0 (untreated control), 0.2 (■), and 0.8 (▲) mmol/L of SIN-1 for up to 4 hours at 37°C. Progressive motility (A), non-progressive motility (B), immotile sperm (C), and kinetic parameters: VCL (curvilinear velocity, D), VSL (straight-line velocity, E), and VAP (average path velocity, F) were analyzed every hour for 4 hours. Values represent the mean ± SD. (**) $p < 0.01$; (***) $p < 0.001$ compared with the untreated control within the same time point. SIN-1: 3-morpholinosydnonimine.

There was a gradual decrease in total and progressive motility in terms of incubation time and SIN-1 concentration (Figures 5A, B). An increase in the percentage of immotile spermatozoa was observed, reaching 65.6 ± 33% at 4 hours of incubation with 0.8 mmol/L of SIN-1 (Figure 5C). In addition, significant differences in curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) were observed following exposure to SIN-1 (Figures 5D - F, respectively).

Discussion

Using an *in vitro* approach, the effect of peroxynitrite on thiol group oxidation and motility in human spermatozoa was evaluated. The results of this study clearly show that exposure of spermatozoa to peroxynitrite resulted in an increase in thiol group oxidation, mainly in the head and principal piece, which was associated with a decrease in sperm motility. It has been previously shown that exposure of somatic cells to high levels of peroxynitrite can alter both protein structure and cellular function when it reacts with thiol-containing amino acids. The reaction of peroxynitrite with the amino acid cysteine is the most prevalent, resulting in thiol oxidation (reviewed by [Pacher et al. 2007]). Thiol groups in mature spermatozoa are found in key components of enzymes [Vignini et al. 2009], antioxidant molecules [Gong et al. 2012], and structural proteins [Shalgi et al. 1989; Cabrillana et al. 2011]; therefore, thiol oxidation may occur at different levels.

An association between sulfhydryl group content and sperm function has been previously reported. Exposure of human spermatozoa to peroxynitrite results in significantly decreased sperm sulfhydryl content, decreased sperm motility, and increased lipid peroxidation [Oztezcan et al. 1999]. A negative correlation between peroxynitrite exposure and total sulfhydryl group content and enzymatic activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase was found, suggesting that peroxynitrite may decrease the activity of these enzymes through the depletion of thiol content [Vignini et al. 2009].

In line with our findings, previous studies have found an association between the oxidation of thiol groups and loss of sperm function [Aitken et al. 2012; Gong et al. 2012; Piomboni et al. 2012]. When human spermatozoa are subjected to prolonged incubation *in vitro*, there is a decrease in sperm motility coupled to a loss of protein thiol content, increase in mitochondrial reactive oxygen species production, and lipid peroxidation [Aitken et al. 2012]. Similarly, it has been reported that seminal plasma from azoospermic and leukocytospermic men has higher levels of oxidized thiol groups than normozoospermic men [Piomboni et al. 2012]. Similarly, the total amount of peroxiredoxins (PRDXs), which are antioxidant enzymes present at high levels in human semen, is lower in seminal plasma and spermatozoa in infertile men and these PRDXs show higher levels of thiol oxidation than in normal donors. Thiol oxidation of PRDXs is correlated with sperm DNA damage, lipid peroxidation, and decreased sperm motility [Gong et al. 2012, Tao et al. 2014]. Since peroxynitrite oxidizes thiol groups, antioxidant enzymes such as PRDXs may be a potential target for peroxynitrite and thiol oxidation leading to the decrease in sperm motility observed in our study and reported by other authors [Gong et al. 2012].

An association between thiol group residues in the outer dense fibers (ODF), particularly ODF1, and sperm motility has also been described. ODF1 has been previously identified as one of the flagellar proteins with the highest thiol content. When thiol groups in ODF1 are oxidized, there is a significant decrease in sperm motility [Cabrillana et al. 2011]. Our results show that peroxynitrite caused thiol oxidation mainly in the head and in the principal piece. Therefore, it could be hypothesized that the decrease in sperm motility may be partially due to thiol oxidation in ODF proteins, which are essential for adequate sperm motility [Yang et al. 2012].

An unexpected finding emerging from this study was that the peroxynitrite-induced loss of motility, was due to damage to the axoneme rather than damage to the plasma membrane. This finding was observed following exposure to 0.8 mmol/L of SIN-1. As indicated above, peroxynitrite-induced damage of the axoneme could be related to structural damage of the dense fibers, i.e., ODF1 and/or microtubules, or to functional damage of flagellar ATPases. These findings are consistent with the fact that the percentage of nonviable spermatozoa in ejaculated spermatozoa, as reported in the standard semen analysis, is consistently lower than the percentage of immotile sperm [Eliasson 1977], suggesting that peroxynitrite-induced damage of the axoneme may play a role in loss of motility during sperm transit through the epididymis.

The sustained decline of motility at 2 and 4 hours of incubation is only observed in the presence of 0.8 mmol/L of SIN-1. It is highly unlikely that this phenomenon may be related to superoxide anion, H_2O_2 or hydroxyl radical-induced sperm damage, as previously reported [Alvarez et al. 1987; Aitken et al. 1989]. In contrast to this high increase in the loss of motility observed at 2 and 4 hours of incubation in the presence of 0.8 mmol/L of SIN-1, oxygen radical-induced membrane damage is a process that occurs after prolonged aerobic incubation [Alvarez et al. 1987]. In addition, the fact that sperm motility at 2 and 4 hours of incubation with 0.2 mmol/L of SIN-1 was not significantly different from the control supports this hypothesis.

In conclusion, using an *in vitro* approach, we showed that peroxynitrite-mediated nitrosative stress increases thiol oxidation in human sperm. The thiol oxidation mainly affects the sperm axoneme and might be one way by which peroxynitrite reduces sperm motility in human spermatozoa. The results of this study may contribute to improve our knowledge concerning the mechanism by which nitrosative stress impairs sperm function. Therefore, the development of new strategies aimed at reducing peroxynitrite-induced damage in human spermatozoa may have significant applications to treat male infertility as well as improve the reproductive outcome of infertile couples.

Materials and methods

Semen collection and analysis

All protocols involving human subjects participating in the study were performed under full compliance with the Scientific Ethics Committee at the Universidad de La Frontera, Temuco, Chile. After the study procedures were explained and all questions answered, subjects signed informed consent forms. The semen samples were obtained from healthy donors by masturbation

after at least 3 d of sexual abstinence. Samples were collected at home in sterile containers and transported to the laboratory within 60 min. Standard semen analysis was performed according to the WHO [2010] criteria.

Isolation of motile sperm

Motile spermatozoa were isolated using the direct swim-up procedure [Mortimer 1994]. For that purpose, 1 mL of liquefied semen was layered under 1 mL of culture medium. The medium used was human tubular fluid (HTF) [Quinn et al. 1985] without albumin. The tube was incubated at an angle close to 45° for 1 h at 37°C, after that it was gently returned to the upright position and the upper 1 mL layer of HTF medium removed. Sperm concentration was adjusted to 10 x 10^6 cells/mL using HTF medium.

Peroxynitrite generation by 3-morpholinosydnonimine

The compound 3-morpholinosydnonimine (SIN-1) (Enzo Life Science Inc., Farmingdale, NY, USA) was used, since it has been shown to be useful for the *in vitro* generation of peroxynitrite in sperm suspensions and other cell types [Blanco Garcia et al. 2009; Rodriguez and Beconi 2009; Agbani et al. 2011; Uribe et al. 2015]. Stock solutions of SIN-1 were prepared at 100 mmol/L in dimethyl sulfoxide (DMSO) and stored at -20°C as was previously described [Uribe et al. 2015].

Sperm viability

It was previously reported that sperm viability is not decreased in human spermatozoa obtained from native semen samples after exposure to 1 mmol/L of SIN-1 for 4 h [Uribe et al. 2015]. Considering that in this study we worked with spermatozoa selected by swim-up, we decided to check sperm viability prior to the experiments. For this, the fluorescent dye propidium iodide (PI; Sigma-Aldrich Inc., St Louis, MO, USA) was used. Aliquots of sperm suspension at 2 x10^6 cells/mL were exposed to 1 mmol/L of SIN-1 for 4 h at 37°C. An untreated control was included. After incubation, the spermatozoa were stained with PI as previously described [Uribe et al. 2015]. The fluorescence intensity of PI was analyzed by flow cytometry (see analysis by flow cytometry below) and the sperm viability was evaluated considering the mean percentage of PI-negative sperm.

Detection of peroxynitrite generation

To verify peroxynitrite generation by SIN-1, dihydrorhodamine 123 (DHR) (Enzo Life Science Inc., Farmingdale, NY, USA) was used as the fluorescent probe. DHR is oxidized by peroxynitrite but not by hydrogen peroxide, superoxide anion, or nitric oxide [Crow 1997]. DHR does not emit fluorescence at 536 nm, but its oxidation product, rhodamine, exhibits a linear-dependent increase in fluorescence emission [Crow 1997; Blanco Garcia et al. 2009]. Stock solutions of DHR at 20 mmol/L in DMSO were prepared and stored at -20°C. Working solutions were freshly prepared by diluting the stock solution 20-fold with DMSO to obtain a 1 mmol/L solution [Aziz et al. 2010].

For experiments, aliquots of 250 µL containing sperm suspension (10 x 10^6 cells/mL) were incubated at 37°C for up to 4 h in the presence of SIN-1 at final concentrations of 0.2 and 0.8 mmol/L. An untreated control was also included. After 2 and 4 h of incubation, an aliquot of 1 µL of the DHR working solution was added (final concentration of 1 µmol/L) to both the experimental and control sperm suspensions. After 20 min of incubation at 37°C, DHR fluorescence was evaluated by confocal microscopy (see analysis by confocal microscopy below).

Detection of thiol oxidation

To analyze the effect of peroxynitrite on the thiol redox state, the fluorescent probe monobromobimane (mBBr) was used. mBBr is a highly sensitive probe used to monitor the thiol redox state under physiological conditions given its lipophilic character. The reaction of mBBr with sulfhydryl groups of cysteine forms highly fluorescent derivatives. In contrast, when thiol groups are oxidized they lack affinity for mBBr, resulting in decreased fluorescence intensity [Kosower et al. 1979; Kosower and Kosower 1987]. Stock solutions of mBBr were prepared at 150 mmol/L in acetonitrile and stored at -20°C.

In the present experimental study we worked with the same conditions described in Detection of peroxynitrite generation above. After 2 and 4 h of incubation, 250 µL aliquots of the sperm suspension (10 x 10^6 cells/mL) were incubated with 0.3 µmol/L of mBBr. After 20 min of incubation at 37°C the sperm cells were washed twice and re-suspended in Dulbecco's phosphate buffered saline (DPBS). mBBr fluorescence was monitored by confocal microscopy (see analysis by confocal microscopy below).

Evaluation of sperm motility

The Computer-Aided Sperm Analysis (CASA) was used through the Integrated Sperm Analysis System

software (ISAS; Proiser, Valencia, Spain) to evaluate the effect of peroxynitrite on sperm motility. A minimum of 200 spermatozoa were examined for each experiment. Negative phase contrast was used and the corresponding settings for the assessment of human sperm motility were selected. Parameter settings were: 25 frames/s, 15–50 μm^2 for head area, VCL > 10 μm/s to classify a spermatozoon as motile. For each experiment the percentage of total and progressive motility and kinetic sperm parameters were assessed.

Spermatozoa selected by swim-up were suspended at a concentration of 10 x 10^6 cells/mL in HTF medium and the suspension incubated at 37°C. Aliquots of 0.5 mL were exposed to 0.2 and 0.8 mmol/L of SIN-1 including an untreated control. The incubation was performed at 37°C for up to 4 h and sperm motility assessed every hour.

Confocal microscopy analysis

In order to monitor DHR and mBBr fluorescence in human spermatozoa, images were taken by confocal microscopy (Olympus FV10-ASW confocal microscope, NY, USA). The excitation/detection wavelengths of the filters used for each fluorescent probes were: DHR, band pass filter of 488/525 ± 25 nm, and for mBBr 405/465 ± 50 nm. From the confocal microscopy images the relative fluorescence intensity (RFI-AU arbitrary units) was quantified using Image J software (Image J 1.32j, http//rsb.info.nih.gov./ij/Java1.3.1_03). For this, a histogram function was applied to a limited area of 200 sperm cells, which included the midpiece and the principal piece regions of the spermatozoa. The percentage of sperm cells that showed DHR and mBBr fluorescence after 2 and 4 hours of incubation was also monitored. Finally, the percentage of head, middle, and principal piece regions that showed fluorescence was also monitored at the same incubation times.

Flow cytometry assay

A flow cytometer FACSCanto II (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA) was used for fluorescence analysis. Samples were acquired and analyzed with the software FACSDiva^{TM} v. 6.1.3 (Becton, Dickinson and Company). Data from 10,000 sperm events were recorded and the sample aspiration speed was 60 mL/min. An argon laser (488 nm) was used for PI excitation and the orange fluorescence was detected using a 585/42 nm bandwidth filter.

Statistical analysis

Each experiment was repeated at least three times on different days and at least 200 spermatozoa were analyzed for experimental and control aliquots. Results are expressed as the mean ± standard deviation (SD). Data were analyzed with the Prism 6 software package (GraphPad, La Jolla, CA, USA). To assess Gaussian distribution D'Agostino's K2 test was used and data were transformed to a logarithmic scale prior to analysis when they did not pass the normality test. To analyze the sperm viability a paired *t*-test was used. A one-way analysis of variance (ANOVA) was used followed by Dunnett's post-test to check the generation of peroxynitrite by SIN-1 and to evaluate the effect of SIN-1 on thiol oxidation. To evaluate the effect of SIN-1 on sperm motility, a two-way ANOVA was used followed by the Bonferroni post-test. Values were considered significantly different with $p < 0.05$.

Declaration of interest

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Study conception and design, data acquisition, analysis and interpretation, drafting of the article: MEC, PU; Study conception and design, drafting of the article, and revising of the article critically for important intellectual content: JVV; Data analysis and interpretation, revising of the article critically for important intellectual content: JA, RS, MWF. All authors have read and approved the final version of the manuscript and agree with the order of presentation of the authors.

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