Molecular Human Reproduction, Vol.21, No.3 pp. 237–243, 2015
Advanced Access publication on November 25, 2014 doi:10.1093/molehr/gau107

molecular human reproduction

ORIGINAL RESEARCH

Peroxynitrite-mediated nitrosative stress decreases motility and mitochondrial membrane potential in human spermatozoa

P. Uribe[1], R. Boguen[1], F. Treulen[1], R. Sánchez[1], and J.V. Villegas[1,2,*]

[1]Center of Reproductive Biotechnology – Scientific and Technological Bioresource Nucleus (CEBIOR – BIOREN), Universidad de La Frontera, Temuco 4811230, Chile [2]Department of Internal Medicine, Faculty of Medicine, Universidad de La Frontera, Temuco 4781218, Chile

*Correspondence address. E-mail: juana.villegas@ufrontera.cl

Submitted on August 20, 2014; resubmitted on November 11, 2014; accepted on November 20, 2014

ABSTRACT: Nitrosative stress is produced by high levels of reactive nitrogen species (RNS). The RNS include peroxynitrite, a highly reactive free radical produced from a diffusion-controlled reaction between nitric oxide and superoxide anion. Peroxynitrite causes nitration and oxidation of lipids, proteins and DNA, and is thus considered an important pathogenic mechanism in various diseases. Although high levels of peroxynitrite are associated with astenozoospermia, few reports exist regarding the *in vitro* effect of high levels of this RNS on human sperm. The aim of this study was to evaluate the *in vitro* effect of nitrosative stress caused by peroxynitrite on the viability, motility and mitochondrial membrane potential of human spermatozoa. To do this, human spermatozoa from healthy donors were exposed *in vitro* to 3-morpholinosydnonimine (SIN-1), a molecule that generates peroxynitrite. Incubations were done at 37°C for up to 4 h with SIN-1 concentrations between 0.2 and 1.0 mmol/l. Generation of peroxynitrite was confirmed using dihydrorhodamine 123 (DHR) by spectrophotometry and flow cytometry. Sperm viability was assessed by propidium iodide staining; sperm motility was analyzed by CASA, and the state of mitochondrial membrane potential ($\Delta\Psi$m) by JC-1 staining. Viability and $\Delta\Psi$m were measured by flow cytometry. The results showed an increase in DHR oxidation, demonstrating the generation of peroxynitrite through SIN-1. Peroxynitrite decreased progressive and total motility, as well as some sperm kinetic parameters. Mitochondrial membrane potential also decreased. These alterations occurred with no decrease in sperm viability. In conclusion, peroxynitrite-induced nitrosative stress impairs vital functions in the male gamete, possibly contributing to male infertility.

Key words: infertility / mitochondrial membrane potential / sperm motility / peroxynitrite / reactive nitrogen species

Introduction

Oxidative stress caused by an excess of reactive oxygen species (ROS) damages proteins, lipids and DNA in human spermatozoa and is considered a major cause of impaired sperm function (Makker *et al.*, 2009; Gharagozloo and Aitken, 2011). Like ROS, reactive nitrogen species (RNS) when produced at high levels during so-called nitrosative stress, cause modifications to several biomolecules (Nash *et al.*, 2012). RNS include nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$) and nitric oxide (NO) (Nash *et al.*, 2012). NO, a diffusible free radical acting as intracellular messenger, has been implicated in numerous physiological and pathological conditions (Pacher *et al.*, 2007). NO production in several compartments of the male reproductive system (Ehren *et al.*, 1994; Zini *et al.*, 1996; Uckert *et al.*, 2003) and in spermatozoa (Herrero *et al.*, 1996; Lewis *et al.*, 1996; O'Bryan *et al.*, 1998) has been reported, supporting several sperm functions at physiological levels (Zini *et al.*, 1995; Lewis *et al.*, 1996; Sengoku *et al.*, 1998; Revelli *et al.*, 1999; Belen Herrero *et al.*, 2000; Miraglia *et al.*, 2011). In contrast, high levels of NO are associated with alterations in sperm function, particularly with decreased motility (Rosselli *et al.*, 1995; Weinberg *et al.*, 1995; Nobunaga *et al.*, 1996), inhibition of cellular respiration (Weinberg *et al.*, 1995) and DNA damage (Amiri *et al.*, 2007; Santiso *et al.*, 2012).

However, it has been suggested that most detrimental effects attributed to NO are the result of its reaction with superoxide (O_2^-), which produces peroxynitrite (Pacher *et al.*, 2007). Peroxynitrite covalently interacts with most biomolecules and its cytotoxicity is mediated through lipid peroxidation, protein oxidation and nitration, activation of matrix metalloproteinases and inactivation of several enzymes, particularly mitochondrial enzymes, and also DNA oxidation and fragmentation. Peroxynitrite adversely affects viability and cell function and can also induce cell death by apoptosis or necrosis, and has been implicated in several diseases [reviewed in Pacher *et al.* (2007) and Szabo *et al.* (2007)].

High levels of seminal $ONOO^-$ have been associated with male infertility. In asthenozoospermic infertile men, higher levels of $ONOO^-$ and nitration of the tyrosine residues have been found than in normospermic fertile men (Vignini *et al.*, 2006; Salvolini *et al.*, 2012). A negative correlation has been reported between $ONOO^-$ concentration and sperm morphology and motility (Vignini *et al.*, 2006; Khosravi *et al.*, 2012). Also, negative associations were found between $ONOO^-$ levels and Na^+/K^+-ATPase and Ca^{2+}-ATPase activity, and total thiol content (Vignini *et al.*, 2009). A positive correlation has also been reported between RNS and sperm DNA fragmentation (Khosravi *et al.*, 2012). It has been suggested that $ONOO^-$ generation is the result of excessive activity of nitric oxide synthase and nitric oxide formation, which would cause the decreased sperm function observed in patients with varicocele (Mitropoulos *et al.*, 1996). Oztezcan *et al.* reported that upon *in vitro* exposure to $ONOO^-$, human spermatozoa showed reduced motility and a decrease in the total sulfhydryl group as well as increased lipid peroxidation (Oztezcan *et al.*, 1999).

Although many studies have been published regarding the negative effect of peroxynitrite on somatic cells [for an extensive review, see Pacher *et al.* (2007)], few studies have reported the *in vitro* effect of peroxynitrite in human sperm. Therefore, the aim of this study was to evaluate the *in vitro* effect of peroxynitrite on human spermatozoa. To do this, human spermatozoa from healthy donors were exposed to a molecule that generates peroxynitrite and then sperm viability, motility and mitochondrial membrane potential were evaluated.

Materials and Methods

Semen preparation and analysis

The semen samples were obtained from four healthy donors during the study. Donors were informed and signed a written consent form. The samples obtained from the same donor were requested with at least a 15-day interval. The study was approved by the Scientific Ethics Committee of the Universidad de La Frontera.

Semen samples were obtained by masturbation, with at least 3 days of sexual abstinence. Semen was collected in sterile vessels and maintained at room temperature for 30 to 60 min until liquefaction. Standard semen analysis was performed according to the WHO Manual (World Health Organization, 2010). Only normal samples were used in the experiments as per WHO 2010 guidelines.

Generation of peroxynitrite

3-Morpholinosydnonimine (SIN-1; Enzo Life Science Inc., Farmingdale, NY, USA) was used for generation of peroxynitrite. SIN-1 in solution and in the presence of oxygen decomposes spontaneously releasing NO and O_2^-, which react together forming peroxynitrite (Blanco Garcia *et al.*, 2009). A stock solution of SIN-1 was prepared at 100 mmol/l in dimethyl sulfoxide (DMSO), before being aliquoted and stored at −20°C.

Detection of peroxynitrite production

In order to check peroxynitrite generation by SIN-1, we used dihydrorhodamine 123 (DHR; Enzo Life Science Inc.), which is oxidized to the product rhodamine by peroxynitrite but not by other oxidants such as hydrogen peroxide, superoxide anion or nitric oxide (Crow, 1997).

A stock solution of DHR was prepared in DMSO at 20 mmol/l and stored at −20°C. Working solutions were prepared freshly by dilution in DMSO to obtain 1 mmol/l solutions (Aziz *et al.*, 2010). Rhodamine generation was evaluated by flow cytometry and spectrophotometry. DHR does not absorb light or emit fluorescence, but its oxidized product, rhodamine, exhibits a linear oxidant-dependent increase in light absorbance at 500 nm and fluorescence emission at 536 nm (Crow, 1997; Blanco Garcia *et al.*, 2009).

To demonstrate peroxynitrite generation by SIN-1, 1 ml aliquots of 100 μmol/l of DHR in Dulbecco's phosphate buffered saline (DPBS) at pH 7.3 were supplemented with SIN-1 to final concentrations of 0.05, 0.2, 0.4, 0.6, 0.8 and 1.0 mmol/l. A control without SIN-1 was also included. Rhodamine formation was monitored by increasing absorbance every 1 min for 4 h at 500 nm in a spectrophotometer Optizen 3220 UV (Mecasys Co., Daejeon, Republic of Korea)

For analysis of peroxynitrite permeability through cell membranes, aliquots of 1 ml of sperm suspension at 2×10^6 ml^{-1} were incubated with 0.8 mmol/l of SIN-1 at 37°C for 4 h. An untreated sperm control and a vehicle control were included. The vehicle control consisted of sperm aliquots exposed to 1% v/v of DMSO (the highest concentration used). After the SIN-1 incubation period, the cells were washed once and incubated with 1 μmol/l of DHR at 37°C for 20 min. The mean fluorescence intensity (MFI) of DHR in the cells was determined using flow cytometry.

Sperm viability

The effect of peroxynitrite on sperm viability was evaluated by incorporating propidium iodide (PI; Sigma-Aldrich Inc., St Louis, MO, USA). For this, aliquots of a sperm suspension at 2×10^6 ml^{-1} were incubated separately with 0.5 and 1.0 mmol/l of SIN-1 at 37°C for 4 h. An untreated control and a vehicle control were also included in each experiment. The vehicle control consisted of sperm aliquots exposed to 1% v/v of DMSO. After incubation, the spermatozoa were washed twice with DPBS at 500*g* for 5 min, the supernatant discarded and the cells were resuspended with DPBS to 400 μl. The percentage of living spermatozoa was determined by adding PI to a final concentration of 1 μmol/l and analyzing by flow cytometry. The sperm viability was determined as the mean percentage of PI-negative cells.

Sperm motility

To evaluate the effect of SIN-1 on sperm motility, Computer-Aided Sperm Analysis (CASA) was used through the Integrated Sperm Analysis System software (ISAS; Proiser, Valencia, Spain). Negative contrast was used and a minimum of 200 spermatozoa were examined for each test using the adjustments for assessing human spermatozoa. The parameter settings were: 25 frames/s; 15–50 μm^2 for head area; and curvilinear velocity (VCL) > 10 μm/s to classify a spermatozoon as motile. Neat semen samples were diluted to 20×10^6 spermatozoa/ml with spermatozoa-free seminal plasma and the suspension was kept at 37°C (World Health Organization, 2010). Aliquots of 0.5 ml were exposed separately to 0.5 and 1.0 mmol/l of SIN-1 at 37°C for 4 h. An untreated control was included. Sperm motility was assessed every hour. A vehicle control which consisted of sperm aliquots exposed to 1% v/v of DMSO was also incubated at 37°C for 4 h. Progressive motility and kinetic sperm parameters were assessed.

Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi m$) was assessed using the Mît-E-Ψ^{TM} mitochondrial permeability detection kit (Enzo Life Science Inc.) which uses the reagent JC-1 (5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide). JC-1 is a cationic dye which accumulates in $\Delta\Psi m$ intact mitochondria forming aggregates and emits orange fluorescence. Orange fluorescence levels of JC-1 aggregates are correlated to $\Delta\Psi m$.

The experiments to evaluate the $\Delta\Psi m$ entailed sperm suspensions at 2×10^6 spermatozoa/ml in DPBS being incubated with SIN-1 to 0.2, 0.4, 0.6, 0.8 and 1.0 mmol/l. An untreated control and a vehicle control were also included. The vehicle control consisted of sperm aliquots exposed to 1% v/v of DMSO. The incubation was performed at 37°C for

1 h. After incubation, the sperm suspensions were washed once by centrifugation to 500*g* for 5 min and then the spermatozoa were resuspended in 1 ml of DPBS and 1 μl of reagent JC-1 was added. After 15 min incubation at 37°C, the spermatozoa were washed once, resuspended in 500 μl of DPBS and then 1 μmol/l of PI was added prior to analysis in order to exclude dead cells from the analysis. The $\Delta\Psi m$ was evaluated by flow cytometry as the geometric mean of fluorescence of the JC-1 aggregates (see Flow cytometry analysis below).

Flow cytometry analysis

Fluorescence analysis was performed in a flow cytometer FACSCanto II (Becton, Dickinson and Company, BD Biosciences, San Jose, CA, USA). Samples were acquired and analyzed with the software FACSDiva™ v. 6.1.3 (Becton, Dickinson and Company). Sample aspiration speed was 60 μl/min and data from 10 000 sperm events were recorded. The excitation was realized at 488 nm with an argon laser. The green fluorescent staining (DHR) was detected using a 530/30 nm bandwidth filter; orange fluorescence (PI alone; JC-1 orange aggregates) was detected using a 585/42 nm bandwidth filter; and a band-pass filter >LP670 nm (long-pass, far red) was used to measure the fluorescence of PI when used in combination with JC-1. All were done on logarithmic scales.

Statistical analysis

The treatment of spermatozoa with SIN-1 at each dose was carried out in duplicate and the experiments were repeated at least three times on different days. Results were expressed as mean ± standard deviation (SD). Statistical evaluation was performed with the Prism 6 software package (GraphPad, La Jolla, CA, USA), applying D'Agostino's K2 test to assess Gaussian distribution. To evaluate the effect of concentration of peroxynitrite on sperm viability, mitochondrial membrane potential, kinetic parameters of sperm motility and peroxynitrite permeability through cell membranes, a one-way analysis of variance (ANOVA) was used followed by Dunnett's post-test. To evaluate the effect of time and concentration of peroxynitrite on sperm motility, a two-way ANOVA was used followed by Bonferroni's post-test. A paired *t*- test was used to analyze vehicle control on total, progressive and kinetic parameters of sperm motility. *P*-values <0.05 were considered statistically significant.

Results

Demonstrating the generation of peroxynitrite

First, the proper generation of peroxynitrite with SIN-1 was verified. The generation of peroxynitrite through SIN-1 was demonstrated by oxidation of DHR, which was evidenced by the increase in absorbance at 500 nm. Absorbance of DHR increased exponentially in direct proportion to the concentration of SIN-1. For concentrations of SIN-1 between 0.4 and 1.0 mmol/l, the steady state was reached after about 100 min. These results confirmed the peroxynitrite generation by SIN-1 (Fig. 1).

Subsequently human sperm were exposed to 0.8 mmol/l SIN-1 for 4 h at 37°C and DHR fluorescence intensity was measured in the cells by flow cytometry. A statistically significant increase was observed in the MFI of sperm cells treated with SIN-1 compared with untreated sperm (9780 ± 2472 versus 401 ± 223, respectively, $P < 0.0001$). MFI of vehicle control (403 ± 192) did not show a significant increase compared with the untreated control ($P > 0.05$). The increase in MFI shows that peroxynitrite, generated by SIN-1 under the conditions used in this study, was able to cross cell membranes and enter the cells.

Effect of peroxynitrite on sperm viability and motility

The percentage of sperm viability was not affected by 0.5 and 1.0 mmol/l of SIN-1 (76.5 ± 10.4 and 73.0 ± 10.1, respectively) or vehicle (75.4 ± 10.0) when compared with the untreated control (75.8 ± 7.5) after 4 h of incubation at 37°C ($P > 0.05$).

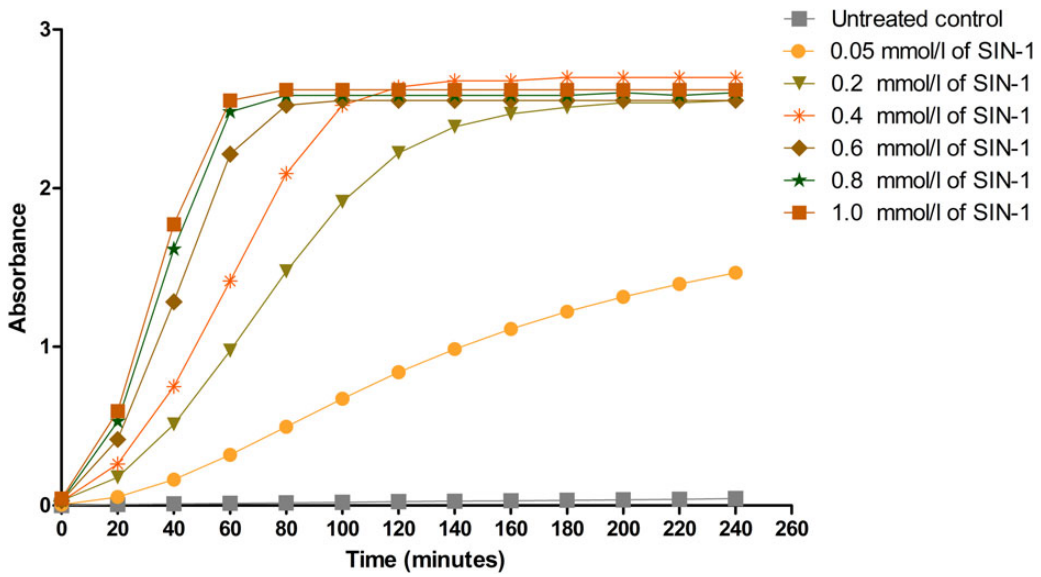

Figure 1 Generation of peroxynitrite by SIN-1 overtime. Spectrophotometric measurement of the increase in absorbance due to oxidation of DHR by peroxynitrite in incubation medium (DPBS) without spermatozoa. Curves correspond to different concentrations of SIN-1, indicated to the right of the graph. Representative example of one experiment. SIN-1, 3-morpholinosydnonimine; DHR, dihydrorhodamine 123.

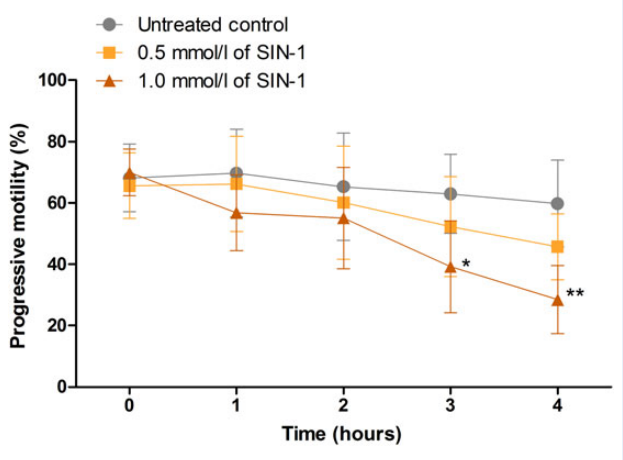

Figure 2 Effect of peroxynitrite on progressive sperm motility over time. Results are presented as mean ± SD. The experiment was repeated six times on different days using semen samples from four healthy donors. Two-way ANOVA indicated statistically significant differences ($P < 0.0001$) for both time and dose factors. (*) $P < 0.01$; (**) $P < 0.001$ compared with the untreated control within the same time point. SIN-1, 3-morpholinosydnonimine.

Then, the effect of peroxynitrite on sperm motility was assessed. For this, the spermatozoa were incubated with SIN-1 for 4 h at 37°C and sperm motility was measured every 1 h from the onset of incubation. A decrease in progressive sperm motility, which was both time- ($P < 0.0001$) and dose- ($P < 0.0001$) dependent, was observed after exposure to peroxynitrite. The decrease in progressive motility was significant compared with the untreated control from 3 h of incubation with 1.0 mmol/l of SIN-1 (Fig. 2). Total motility was also affected, decreasing significantly with 1.0 mmol/l of SIN-1 at 4 h in a time- and dose-dependent manner (data not shown). The vehicle control showed no differences in progressive sperm motility compared with the untreated control after 4 h of incubation (52.0 ± 5.4 versus 53.2 ± 8.1, respectively, $P > 0.05$). In the same way, the vehicle control did not decrease total sperm motility compared with the untreated control (63.6 ± 4.9 versus 61.8 ± 8.9, respectively, $P > 0.05$).

Table I presents kinetic motility data of spermatozoa exposed to peroxynitrite for 4 h, showing that VCL, straight-line velocity (VSL) and average path velocity (VAP) were also affected. For all kinetic motility parameters, the vehicle control did not show any statistically significant differences compared with the untreated control (data not shown).

Effect of peroxynitrite on sperm mitochondrial membrane potential

After exposure of spermatozoa to concentrations between 0.2 and 1.0 mmol/l SIN-1 for 1 h, there was a statistically significant decrease in the sperm $\Delta\Psi$m compared with the untreated cells. The vehicle of SIN-1 did not induce a significant change. Figure 3 illustrates that the sperm $\Delta\Psi$m decrease was SIN-1 concentration-dependent.

Discussion

Although oxidative stress has been widely associated with impaired sperm function and male infertility (Gharagozloo and Aitken, 2011), nitrosative stress caused by excessive RNS production may also contribute to impaired sperm function (Khosravi *et al.*, 2012; Salvolini *et al.*, 2012).

We have demonstrated here the association between peroxynitrite and decreased motility and mitochondrial membrane potential in human spermatozoa. To our knowledge, this is the first study reporting the influence of peroxynitrite on the $\Delta\Psi$m of human spermatozoa.

To mimic pathophysiological states *in vitro*, the peroxynitrite precursors NO and O_2^- can be simultaneously generated using compounds

Table I Effect of peroxynitrite on sperm kinetic parameters at 4 h of incubation with SIN-1 at 37°C.

Parameter	Untreated control	0.5 mmol/l of SIN-1	1.0 mmol/l of SIN-1
VCL (μm/s)	49.5 ± 3.9	45.4 ± 2.2	39.3 ± 5.4[a]
VSL (μm/s)	24.4 ± 1.3	22.8 ± 0.9	18.5 ± 1.6[b]
VAP (μm/s)	34.4 ± 1.9	31.8 ± 1.3	27.3 ± 2.8[a]
LIN (%)	49.0 ± 6.0	50.4 ± 3.5	47.6 ± 6.3
STR (%)	71.0 ± 6.1	71.9 ± 5.2	68.0 ± 6.3
WOB (%)	69.8 ± 3.5	70.1 ± 2.6	69.5 ± 4.0
ALH (μm)	2.1 ± 0.2	2.0 ± 0.2	1.9 ± 0.2
BCF (Hz)	8.0 ± 0.2	7.8 ± 0.4	6.9 ± 1.2

Values correspond to mean ± SD.
VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency.
[a]$P < 0.01$.
[b]$P < 0.0001$, both compared with untreated control.

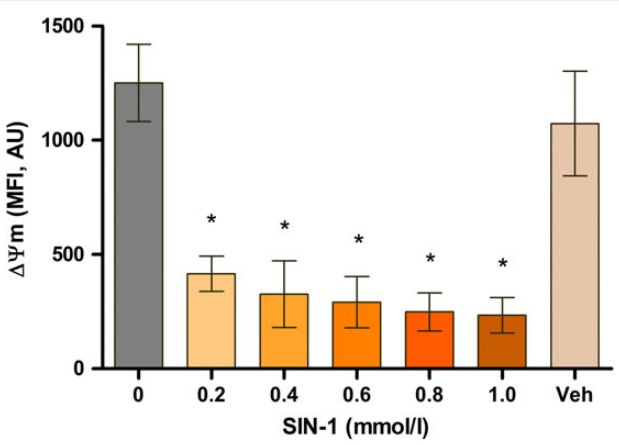

Figure 3 Effect of peroxynitrite on sperm mitochondrial membrane potential. Sperm were incubated for 1 h at 37°C with different concentrations of SIN-1; vehicle corresponds to 10 μl of DMSO; 0 mmol/l of SIN-1 corresponds to the untreated control. Results are presented as mean ± SD. The experiment was repeated three times on different days using semen samples from three healthy donors. (*) $P < 0.001$ compared with the untreated control. $\Delta\Psi$m, mitochondrial membrane potential; MFI, mean fluorescence intensity; AU, arbitrary units; SIN-1, 3-morpholinosydnonimine; Veh, vehicle.

such as SIN-1 (Szabo *et al.*, 2007). SIN-1 has been used in studies with several cell types, including human and bovine spermatozoa (Weinberg *et al.*, 1995; Blanco Garcia *et al.*, 2009; Rodriguez and Beconi, 2009; Agbani *et al.*, 2011).

In this study, the suitable generation of peroxynitrite with SIN-1 was monitored using DHR, which is selectively oxidized by peroxynitrite (Blanco Garcia *et al.*, 2009). Spectrophotometric measurements showed that the amount of SIN-1 added to the solution was directly correlated with the DHR oxidation and thus with the $ONOO^-$ generated amount, as previously reported (Blanco Garcia *et al.*, 2009). Then, the DHR oxidation was confirmed in the spermatozoa exposed to SIN-1 compared with the untreated sperm, demonstrating that with SIN-1 the effects of peroxynitrite could be analyzed on different cell variables.

We found that sperm viability was unchanged after treatment with concentrations up to 1.0 mmol/l of SIN-1 during an incubation time up to 4 h. These data are consistent with a previous study conducted on cryopreserved bovine spermatozoa, where incubation for 45 min at 37°C with up to 1.0 mmol/l of SIN-1 did not significantly reduce sperm viability (Rodriguez and Beconi, 2009). Considering that these SIN-1 concentrations did not alter sperm viability, we proceeded to evaluate motility.

Our results show a time- and dose-dependent decrease in progressive and total sperm motility and also in several sperm kinetic parameters after exposure to peroxynitrite. An association has been reported between sperm motility and the precursor of peroxynitrite, nitric oxide, high concentrations of which were associated with reduced sperm motility in *in vitro* studies (Tomlinson *et al.*, 1992; Herrero *et al.*, 1994; Rosselli *et al.*, 1995; Weinberg *et al.*, 1995; Rodriguez *et al.*, 2005) and in asthenozoospermic patients (Aksoy *et al.*, 2002; Balercia *et al.*, 2004). Thus, if NO can generate $ONOO^-$ *in vivo*, then this highly reactive radical could ultimately be responsible for impaired sperm function. Consistent with this, asthenozoospermic infertile men present significantly higher $ONOO^-$ concentrations than normospermic fertile donors, revealing a negative correlation between $ONOO^-$ concentration and sperm motility (Vignini *et al.*, 2006). Also, it has been affirmed that the high levels of peroxynitrite observed in patients together with high NOS activity are involved in the pathogenesis of idiopathic asthenozoospermia (Salvolini *et al.*, 2012).

In vitro studies have shown an association between high levels of peroxynitrite and impaired sperm motility. In a study using peroxynitrite, a decrease in human sperm motility was observed, which was associated with increased levels of lipid peroxidation (Oztezcan *et al.*, 1999). Later, in bovine spermatozoa, a significant decrease in the motility of cryopreserved bull spermatozoa was reported after incubation with 160 μmol/l SIN-1 (Rodriguez and Beconi, 2009). Although we also worked with SIN-1, higher SIN-1 concentrations were needed to significantly decrease sperm motility. This difference could be explained by species-specific characteristics and by the higher susceptibility of cryopreserved spermatozoa to structural and physiological alterations induced by free radicals (Medeiros *et al.*, 2002).

Considering the highly oxidizing nature of peroxynitrite and the multiple disorders caused by its exposure in somatic cells (Pacher *et al.*, 2007), we hypothesize that impairment of sperm motility caused by peroxynitrite may be produced at different levels, including lipid peroxidation (Radi *et al.*, 1991; Oztezcan *et al.*, 1999), damage to the structure or function of contractile proteins responsible for the movement, or alteration of ATP production by the sperm, either by the glycolytic (Souza and Radi, 1998; Buchczyk *et al.*, 2003; Koeck *et al.*, 2004) or mitochondrial pathways (Radi *et al.*, 2002). Possibly a combination of these or other factors may contribute to sperm motility impairment.

We also studied mitochondrial membrane potential and observed the decrease in sperm $\Delta\Psi m$ after incubation with SIN-1, demonstrating a negative effect of peroxynitrite on the mitochondrial function of human spermatozoa as well. The inhibition of cellular respiration has been previously reported in human spermatozoa induced by SIN-1 as a NO generator (Weinberg *et al.*, 1995). In somatic cells, it has been shown that mitochondrial enzymes are particularly vulnerable to damage by peroxynitrite. Peroxynitrite causes, among other things, inhibition of most components of the electron transport chain (ETC) because it causes cysteine oxidation, tyrosine nitration and damage to iron sulfur centers [reviewed in Pacher *et al.* (2007)]. Therefore, inhibition of ETC proteins that maintain a proper transmembrane proton gradient could be responsible for the decrease in $\Delta\Psi m$ observed in human spermatozoa. The decrease in $\Delta\Psi m$ could also be due to lipid peroxidation. It has been demonstrated in human spermatozoa that lipid peroxidation generates electrophilic aldehydes that disrupt enzymes of the ETC, thereby increasing mitochondrial ROS generation. This process induces a series of harmful changes in spermatozoa which include a loss of $\Delta\Psi m$ (Aitken *et al.*, 2012). Considering that peroxynitrite causes lipid peroxidation (Oztezcan *et al.*, 1999), we could hypothesize that the generation of electrophilic aldehydes and their damaging effects could also contribute to the decrease in $\Delta\Psi m$ observed in our study.

Likewise, a positive correlation has been reported between $\Delta\Psi m$ and routine semen analysis parameters such as motility (Kasai *et al.*, 2002; Espinoza *et al.*, 2009). Interestingly in our study the $\Delta\Psi m$ decreased after 1 h of exposure to peroxynitrite, whereas progressive motility significantly decreased after 3 h of incubation and with a higher concentration of peroxynitrite. This suggests that $\Delta\Psi m$ is a more sensitive variable than motility to detect a decrease in sperm quality, as has already been suggested (Marchetti *et al.*, 2002). Furthermore, the maintenance of sperm motility when the $\Delta\Psi m$ has already decreased may be consistent with glycolysis rather than oxidative phosphorylation as the primary energy source supporting sperm motility (Mukai and Okuno, 2004; Nascimento *et al.*, 2008).

In conclusion, peroxynitrite causes decreased motility and mitochondrial membrane potential of human spermatozoa, compromising vital functions of the male gamete without affecting viability. As already been mentioned, studies in somatic cells have revealed many alterations caused by peroxynitrite [for an extensive review, see Pacher *et al.* (2007)]. Thus, it is expected that in human sperm, peroxynitrite will affect other functional parameters such as capacitation, acrosome reaction and ATP synthesis. Signaling pathways and DNA integrity could also be affected. Peroxynitrite, at higher levels, might even induce apoptotic-like changes. In this way, earlier reports in spermatozoa have shown the negative effects of nitric oxide, a precursor of peroxynitrite, on cellular respiration (Weinberg *et al.*, 1995), DNA integrity (Amiri *et al.*, 2007) and sperm-zona binding (Wu *et al.*, 2004). A better understanding of the influence on sperm function of $ONOO^-$ and other RNS causing nitrosative stress is crucial to the future improvement of treating patients with impaired fertility. Anti-RNS drugs could be used to help patients affected by high levels of nitrosative stress, to improve semen quality in semen samples for fertilization therapies, and as cryoprotective agents in cryopreservation media. Alternatively, RNS could be used in male contraception.

Acknowledgements

The authors are very grateful to Dr Helen Lowry for providing language help.

Authors' roles

P.U.: study conception and design, data acquisition, analysis and interpretation, drafting of the article and final approval of the version to be published. R.B. and F.T.: data analysis and interpretation, revising of the article critically for important intellectual content and final approval of the version to be published. R.S.: study conception and design, revising it critically for important intellectual content and final approval of the version to be published. J.V.V.: study conception and design, data analysis and interpretation, drafting of the article, revising it critically for important intellectual content and final approval of the version to be published.

Funding

This work was supported by: Fondo Nacional de Ciencia y Tecnología (grant number 1130888 to R.S.); Dirección de Investigación, Universidad de La Frontera (grant number DI12-0102 to J.V.V); and a scholarship from Comisión Nacional de Ciencia y Tecnología (grant number 21110812 to P.U.).

Conflict of interest

The authors declare no conflict of interest.

References

Agbani E, Coats P, Wadsworth RM. Threshold of peroxynitrite cytotoxicity in bovine pulmonary artery endothelial and smooth muscle cells. *Toxicol In Vitro* 2011;**25**:1680–1686.

Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J Biol Chem* 2012;**287**:33048–33060.

Aksoy Y, Ozbey I, Aksoy H, Polat O, Akcay F. Seminal plasma nitric oxide concentration in oligo- and/or asthenozoospermic subjects with/without varicocele. *Arch Androl* 2002;**48**:181–185.

Amiri I, Sheikh N, Najafi R. Nitric oxide level in seminal plasma and its relation with sperm DNA damages. *Iran Biomed J* 2007;**11**:259–264.

Aziz N, Novotny J, Oborna I, Fingerova H, Brezinova J, Svobodova M. Comparison of chemiluminescence and flow cytometry in the estimation of reactive oxygen and nitrogen species in human semen. *Fertil Steril* 2010;**94**:2604–2608.

Balercia G, Moretti S, Vignini A, Magagnini M, Mantero F, Boscaro M, Ricciardo-Lamonica G, Mazzanti L. Role of nitric oxide concentrations on human sperm motility. *J Androl* 2004;**25**:245–249.

Belen Herrero M, Chatterjee S, Lefievre L, de Lamirande E, Gagnon C. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. *Free Radic Biol Med* 2000;**29**:522–536.

Blanco Garcia J, Aldinucci C, Maiorca SM, Palmi M, Valoti M, Buonocore G, Pessina GP. Physiopathological effects of the NO donor 3-morpholinosydnonimine on rat cortical synaptosomes. *Neurochem Res* 2009;**34**:931–941.

Buchczyk DP, Grune T, Sies H, Klotz LO. Modifications of glyceraldehyde-3-phosphate dehydrogenase induced by increasing concentrations of peroxynitrite: early recognition by 20S proteasome. *Biol Chem* 2003;**384**:237–241.

Crow JP. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. *Nitric Oxide* 1997;**1**:145–157.

Ehren I, Adolfsson J, Wiklund NP. Nitric oxide synthase activity in the human urogenital tract. *Urol Res* 1994;**22**:287–290.

Espinoza JA, Schulz MA, Sanchez R, Villegas JV. Integrity of mitochondrial membrane potential reflects human sperm quality. *Andrologia* 2009;**41**:51–54.

Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* 2011;**26**:1628–1640.

Herrero MB, Cebral E, Boquet M, Viggiano JM, Vitullo A, Gimeno MA. Effect of nitric oxide on mouse sperm hyperactivation. *Acta Physiol Pharmacol Ther Latinoam* 1994;**44**:65–69.

Herrero MB, Perez Martinez S, Viggiano JM, Polak JM, de Gimeno MF. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. *Reprod Fertil Dev* 1996;**8**:931–934.

Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S, Hoshi K. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. *Asian J Androl* 2002;**4**:97–103.

Khosravi F, Valojerdi MR, Amanlou M, Karimian L, Abolhassani F. Relationship of seminal reactive nitrogen and oxygen species and total antioxidant capacity with sperm DNA fragmentation in infertile couples with normal and abnormal sperm parameters. *Andrologia* 2012;**46**:17–23.

Koeck T, Levison B, Hazen SL, Crabb JW, Stuehr DJ, Aulak KS. Tyrosine nitration impairs mammalian aldolase A activity. *Mol Cell Proteomics* 2004;**3**:548–557.

Lewis SE, Donnelly ET, Sterling ES, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. *Mol Hum Reprod* 1996;**2**:873–878.

Makker K, Agarwal A, Sharma R. Oxidative stress and male infertility. *Indian J Med Res* 2009;**129**:357–367.

Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod* 2002;**17**:1257–1265.

Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? *Theriogenology* 2002;**57**:327–344.

Miraglia E, De Angelis F, Gazzano E, Hassanpour H, Bertagna A, Aldieri E, Revelli A, Ghigo D. Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway. *Reproduction* 2011;**141**:47–54.

Mitropoulos D, Deliconstantinos G, Zervas A, Villiotou V, Dimopoulos C, Stavrides J. Nitric oxide synthase and xanthine oxidase activities in the spermatic vein of patients with varicocele: a potential role for nitric oxide and peroxynitrite in sperm dysfunction. *J Urology* 1996;**156**:1952–1958.

Mukai C, Okuno M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biol Reprod* 2004;**71**:540–547.

Nascimento JM, Shi LZ, Tam J, Chandsawangbhuwana C, Durrant B, Botvinick EL, Berns MW. Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. *J Cell Physiol* 2008;**217**:745–751.

Nash KM, Rockenbauer A, Villamena FA. Reactive nitrogen species reactivities with nitrones: theoretical and experimental studies. *Chem Res Toxicol* 2012;**25**:1581–1597.

Nobunaga T, Tokugawa Y, Hashimoto K, Kubota Y, Sawai K, Kimura T, Shimoya K, Takemura M, Matsuzaki N, Azuma C *et al*. Elevated nitric oxide concentration in the seminal plasma of infertile males: nitric oxide inhibits sperm motility. *Am J Reprod Immunol* 1996;**36**:193–197.

O'Bryan MK, Zini A, Cheng CY, Schlegel PN. Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. *Fertil Steril* 1998;**70**:1143–1147.

Oztezcan S, Turkoglu UM, Kervancioglu E, Kocak T, Kocak-Toker N, Aykac-Toker G. In vitro effects of peroxynitrite on human spermatozoa. *Andrologia* 1999;**31**:195–198.

Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007;**87**:315–424.

Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 1991;**288**:481–487.

Radi R, Cassina A, Hodara R. Nitric oxide and peroxynitrite interactions with mitochondria. *Biol Chem* 2002;**383**:401–409.

Revelli A, Soldati G, Costamagna C, Pellerey O, Aldieri E, Massobrio M, Bosia A, Ghigo D. Follicular fluid proteins stimulate nitric oxide (NO) synthesis in human sperm: a possible role for NO in acrosomal reaction. *J Cell Physiol* 1999;**178**:85–92.

Rodriguez PC, Beconi MT. Peroxynitrite participates in mechanisms involved in capacitation of cryopreserved cattle. *Anim Reprod Sci* 2009;**110**:96–107.

Rodriguez PC, O'Flaherty CM, Beconi MT, Beorlegui NB. Nitric oxide-induced capacitation of cryopreserved bull spermatozoa and assessment of participating regulatory pathways. *Anim Reprod Sci* 2005;**85**:231–242.

Rosselli M, Dubey RK, Imthurn B, Macas E, Keller PJ. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. *Hum Reprod* 1995;**10**:1786–1790.

Salvolini E, Buldreghini E, Lucarini G, Vignini A, Di Primio R, Balercia G. Nitric oxide synthase and tyrosine nitration in idiopathic asthenozoospermia: an immunohistochemical study. *Fertil Steril* 2012;**97**:554–560.

Santiso R, Tamayo M, Gosalvez J, Johnston S, Marino A, Fernandez C, Losada C, Fernandez JL. DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide. *Mutat Res* 2012;**734**:41–49.

Sengoku K, Tamate K, Yoshida T, Takaoka Y, Miyamoto T, Ishikawa M. Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa. *Fertil Steril* 1998;**69**:522–527.

Souza JM, Radi R. Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. *Arch Biochem Biophys* 1998;**360**:187–194.

Szabo C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nat Rev Drug Discov* 2007;**6**:662–680.

Tomlinson MJ, East SJ, Barratt CL, Bolton AE, Cooke ID. Preliminary communication: possible role of reactive nitrogen intermediates in leucocyte-mediated sperm dysfunction. *Am J Reprod Immunol* 1992;**27**:89–92.

Uckert S, Stanarius A, Stief CG, Wolf G, Jonas U, Machtens S. Immunocytochemical distribution of nitric oxide synthase in the human seminal vesicle: a light and electron microscopical study. *Urol Res* 2003; **31**:262–266.

Vignini A, Nanetti L, Buldreghini E, Moroni C, Ricciardo-Lamonica G, Mantero F, Boscaro M, Mazzanti L, Balercia G. The production of peroxynitrite by human spermatozoa may affect sperm motility through the formation of protein nitrotyrosine. *Fertil Steril* 2006;**85**:947–953.

Vignini A, Buldreghini E, Nanetti L, Amoroso S, Boscaro M, Ricciardo-Lamonica G, Mazzanti L, Balercia G. Free thiols in human spermatozoa: are Na+/K+-ATPase, Ca2+-ATPase activities involved in sperm motility through peroxynitrite formation? *Reprod Biomed Online* 2009;**18**:132–140.

Weinberg JB, Doty E, Bonaventura J, Haney AF. Nitric oxide inhibition of human sperm motility. *Fertil Steril* 1995;**64**:408–413.

World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Geneva: WHO press, 2010.

Wu TP, Huang BM, Tsai HC, Lui MC, Liu MY. Effects of nitric oxide on human spermatozoa activity, fertilization and mouse embryonic development. *Arch Androl* 2004;**50**:173–179.

Zini A, De Lamirande E, Gagnon C. Low levels of nitric oxide promote human sperm capacitation in vitro. *J Androl* 1995;**16**:424–431.

Zini A, O'Bryan MK, Magid MS, Schlegel PN. Immunohistochemical localization of endothelial nitric oxide synthase in human testis, epididymis, and vas deferens suggests a possible role for nitric oxide in spermatogenesis, sperm maturation, and programmed cell death. *Biol Reprod* 1996;**55**:935–941.