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Oxidative stress impairs function and increases redox protein modifications in human spermatozoa

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Abstract

Oxidative stress, generated by excessive reactive oxygen species (ROS) or decreased antioxidant defenses (and possibly both), is associated with male infertility. Oxidative stress results in redox-dependent protein modifications, such as tyrosine nitration and *S*-glutathionylation. Normozoospermic sperm samples from healthy individuals were included in this study. Samples were incubated with increasing concentrations (0–5 mM) of exogenous hydrogen peroxide, tert-butyl hydroperoxide, or diethylamine NONOate (DA-NONOate, a nitric oxide (NO·) donor) added to the medium. Spermatozoa treated with or without ROS were incubated under capacitating conditions and then levels of tyrosine phosphorylation and percentage of acrosome reaction (AR) induced by lysophosphatidylcholine were determined. Modified sperm proteins from cytosolic, triton-soluble, and triton-insoluble fractions were analyzed by SDS–PAGE immunoblotting and immunocytochemistry with anti-glutathione and anti-nitrotyrosine antibodies. Levels of *S*-glutathionylation increased dose dependently after exposure to hydroperoxides ($P<0.05$) and were localized mainly to the cytosolic and triton-soluble fractions of the spermatozoa. Levels of tyrosine-nitrated proteins increased dose dependently after exposure to DA-NONOate ($P<0.05$) and were mainly localized to the triton-insoluble fraction. ROS-treated spermatozoa showed impaired motility without affecting viability (hypo-osmotic swelling test). These treated spermatozoa had tyrosine phosphorylation and AR levels similar to that of non-capacitated spermatozoa following incubation under capacitating conditions, suggesting an impairment of sperm capacitation by oxidative stress. In conclusion, oxidative stress promotes a dose-dependent increase in tyrosine nitration and *S*-glutathionylation and alters motility and the ability of spermatozoa to undergo capacitation.

Free Spanish abstract

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Reproduction (2015) **149** 113–123

Introduction

Human infertility is an important health and a social concern, which affects 15% of couples in the reproductive age (de Kretser 1997, WHO 2010). Of these cases of infertility, 50% can be attributed to the male factor (Abid *et al*. 2008). Male infertility is a multifactorial disorder, which is presented clinically as low or absent sperm counts, or the presence of mutated or non-functional sperm cells due to abnormal spermatogenesis (Tournaye & Cohlen 2012). This defective spermatogenesis can be linked to medical conditions such as varicocele, cryptorchidism, infections, nutritional deficiencies, or trauma. It can also be caused by exposure to environmental agents, chemotherapeutic agents, smoking, or even diseases (Brennemann *et al*. 1997, Hasegawa *et al*. 1997, Anderson & Williamson 1988, Smith *et al*. 2006). Interestingly, all the above-mentioned conditions have the oxidative stress as an important component of their pathophysiological mechanisms (Brennemann *et al*. 1997, Hasegawa *et al*. 1997, Anderson & Williamson 1988, Turner 2001, Smith *et al*. 2006, Agarwal *et al*. 2008).

Oxidative stress is the result of an excessive production of reactive oxygen species (ROS) and/or a decrease in the antioxidant defenses (Halliwell 2006, Halliwell & Gutteridge 2007) and targets all cell components decreasing sperm motility and mitochondrial activity (Sikka *et al*. 1995, Griveau & Le Lannou 1997), promoting peroxidation of membrane lipids (Storey 1997), and DNA fragmentation and oxidation (Aitken *et al*. 1998, Barroso *et al*. 2000). The ROS-mediated damage to sperm is a significant contributing factor in 30–80% of infertile men (Gagnon *et al*. 1991, de Lamirande & Gagnon 1995,

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-14-0240
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Agarwal *et al*. 2006, Aitken 2006, Tremellen 2008). Low levels of antioxidant enzymes in both seminal plasma and spermatozoa are associated with impairment of sperm function, DNA integrity, and men infertility (Aitken & Curry 2011, Gong *et al*. 2012).

Paradoxically, the spermatozoon requires low and controlled amounts of ROS to acquire fertilizing ability during capacitation (de Lamirande & O'Flaherty 2012). ROS triggers most of the recognized events associated with capacitation: activation of adenylyl cyclase, increase in intracellular calcium, and phosphorylation events (protein kinases A, C, ERK, and PI3K/Akt pathways) culminating with the late tyrosine phosphorylation (Visconti *et al*. 1995, Leclerc *et al*. 1996, de O'Flaherty *et al*. 2006*a*, Lamirande & O'Flaherty 2012). Noteworthy, failure to undergo tyrosine phosphorylation was observed in spermatozoa from infertile patients (Buffone *et al*. 2005). Hydrogen peroxide (H_2O_2) affected motility (de Lamirande & Gagnon 1992) and sperm hemizona binding (Oehninger *et al*. 1995) at a concentration of 0.5 or 0.2 mM respectively. The incubation of human spermatozoa with sodium nitroprusside (a nitric oxide (NO·) donor) promoted similar results but at higher concentrations (1 mM) (Wu *et al*. 2004).

S-glutathionylation of proteins is a post-translational modification that occurs under normal conditions as well as under conditions of oxidative stress. This modification occurs by the addition of glutathione (GSH) to cysteine residues of certain target proteins; the disulfide linkage between the glutathione and the protein is reversible affecting the functionality of enzymes, receptors, and structural proteins, thus altering normal cell biology (Halliwell & Gutteridge 2007).

Nitrotyrosine is formed by the reaction of peroxynitrite or donors of NO· with tyrosine residues (Halliwell & Gutteridge 2007). It can be produced by the sperm cell by the reaction of superoxide anion ($O_2^{\cdot -}$) and NO· (Herrero *et al*. 2001). The nitrotyrosine protein modification can result in alteration of protein function or structure and thus may affect sperm motility (Vignini *et al*. 2006), but is required at low amounts in the spermatozoon in order to undergo capacitation (Herrero *et al*. 2001).

Although it is known that high levels of ROS are detrimental for sperm motility and zona-binding ability (de Lamirande & Gagnon 1992, Oehninger *et al*. 1995), there are no studies in the literature specifically elucidating the effects of ROS on the ability of human spermatozoa to undergo capacitation. Moreover, little is known regarding the promotion of redox-dependent protein modifications in human spermatozoa; thus, the objectives of this work were to determine the effect of different ROS on the production of tyrosine nitration and *S*-glutathionylation and their subcellular localization and whether an increase in modified proteins is associated with an impairment of the function of human spermatozoa.

Materials and methods

Materials

Percoll was obtained from GE Healthcare (Baie-d'Urfe, QC, Canada). Mouse monoclonal anti-glutathione antibody (clone G8) was purchased from Virogen (Watertown, MA, USA). Mouse monoclonal anti-phosphotyrosine (clone 4G10) and mouse monoclonal anti-nitrotyrosine antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA) and from Abcam (Toronto, ON, Canada) respectively. Donkey anti-rabbit immunoglobulin IgG and goat anti-mouse IgG antibodies (both conjugated with HRP) were provided by Cederlane Laboratories Ltd (Hornby, ON, Canada). Nitrocellulose membranes (pore size, 0.22 µm) were purchased from Osmonics, Inc. (Westborough, MA, USA) and the ECL Kit Lumi-Light from Roche Molecular Biochemicals. Radiographic films (obtained from Fuji; Minami-Ashigara, Japan) were used for immunodetection of blotted proteins. For immunocytochemistry studies, both biotinylated goat anti-rabbit IgG (H+L) and biotinylated horse anti-mouse IgG (H+L) were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA) and Alexa Fluor 555 conjugate of streptavidin and Prolong Antifade were purchased from Life Technologies, Inc. Diethylamine NONOate (DA-NONOate) was obtained from Calbiochem (San Diego, CA, USA); Bis(dimethyl acetal), 1,4-diazabicyclo-[2.2.2] octane (DABCO), and *Pisum sativum* agglutinin conjugated to FITC (PSA–FITC) were purchased from Sigma-Aldrich Chemical Co. Other chemicals used were of at least reagent grade.

Subjects

Semen samples were obtained from healthy volunteers ($n=21$) after 3 days of sexual abstinence. This study was approved by the Ethics Board of the Royal Victoria Hospital–McGill University Health Centre, and all participants signed an informed consent form before participating. Following collection, samples were incubated at 37 °C for 30 min to induce liquefaction. The liquefied semen was then analyzed by computer-assisted semen analysis system (CASA; Sperm vision HR Software v1.01, Penetrating Innovations, Ingersoll, ON, Canada) and the quality of the sample was determined according to the parameters set out by the World Health Organization guidelines (WHO 2010) (Supplementary Table 1, see section on supplementary data given at the end of this article).

Sperm sample preparation

Liquefied semen samples were centrifuged for 30 min at 2300× ***g*** at 20 °C over a four-layer Percoll gradient (95-65-40-20%, made with isotonic HEPES-balanced saline (HBS)). This step was used to separate the abnormal sperm cells, seminal plasma, and white blood cells from the sperm cells with the best motility and morphology (Kovalski *et al*. 1992) without increasing ROS levels (Iwasaki & Gagnon 1992, Zini *et al*. 1993, Plante *et al*. 1994). Highly motile spermatozoa recovered from the 95% layer and the 65–95% interface were diluted to 50×10^6 cell/ml in Biggers, Whitten, and Whittingham (BWW, pH 8.0) medium (Biggers *et al*. 1971) and used for experimentation.

Induction of in vitro *oxidative stress in spermatozoa*

Spermatozoa were incubated for 30 min at 37 °C with increasing concentrations of either H_2O_2, or tert-butyl hydroperoxide (tert-BHP; a synthetic organic hydroperoxide that can produce alkoxy radical, $O_2^{\cdot -}$,and H_2O_2), or Da-NONOate (NO· donor) in the BWW medium. Concentrations were selected to mimic both mild (0.1–0.25 mM) and strong (0.5–5 mM) oxidative stress. After treatment, electrophoresis sample buffer (Tris–HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.0025% bromophenol, 0.1 mM vanadate, 5 mM sodium fluoride, and 20 mM glycerol phosphate) supplemented with or without 100 mM dithiothreitol (DTT) was added to a 50-µl aliquot of the sperm suspension (100×10^6/ml) of each sample. The absence of DTT in the sample buffer is indispensable to be able to see the *S*-glutathionylated proteins; as a reducing agent, DTT will cleave the glutathione from the protein and thus eliminating the signal. The rest of the aliquot was used to determine motility, viability, and ability to undergo capacitation and acrosome reaction (AR).

Sperm motility and viability analysis

Spermatozoa were subjected to oxidative stress as described previously, washed, and resuspended in the fresh BWW medium and an aliquot of 10×10^6 cells/ml was smeared onto collodion-coated slides. Sperm motility was analyzed using the CASA system (Sperm Vision HR software v1.01, Penetrating Innovations, Ingersoll, ON, Canada) according to the WHO guidelines (WHO 2010) (Supplementary Table 1). This was achieved by averaging the motility parameters for rapid progressive, slow progressive, non-progressive, and immotile sperm, obtained from ten different fields.

Both ROS-treated and control samples were centrifuged for 5 min at $600\times$ ***g*** at 20 °C. The supernatant was removed and replaced with a hypo-osmotic solution at 37 °C (WHO 2010). Samples were incubated for 30 min at 37 °C. Following this, sperm samples were gently centrifuged for 5 min at $1000\times$ ***g***. The supernatant was removed, and the pellet was resuspended in ethanol; the fixed cells were smeared onto superfrost plus slides, and viability was assessed by bright field microscopy to observe the presence or absence of tail curl (WHO 2010). Only sperm with a visible tail curl were considered viable and at least 200 cells were counted for each treatment.

Induction of sperm capacitation

ROS-treated spermatozoa were centrifuged for 5 min at $600\times$ ***g*** at 20 °C. The supernatant was then discarded and replaced with the fresh BWW medium containing either 10% fetal chord serum ultrafiltrate (FCSu), 3 mg/ml BSA, or 10 µM progesterone in order to induce capacitation and spermatozoa were then incubated for 3.5 h at 37 °C (O'Flaherty *et al.* 2004). Then, an aliquot was taken from each sample, supplemented with reducing sample buffer containing DTT as explained above, and used for immunoblotting in order to determine capacitation-associated tyrosine phosphorylation using an anti-phosphotyrosine antibody (1:10 000 dilution) (O'Flaherty *et al.* 2006*b*). The remaining sample was centrifuged at $2000\times$ ***g*** for 5 min at 20 °C. The resulting pellet was recuperated and resuspended in the fresh BWW medium containing 2.5 µM lysophosphatidylcholine (LPC) and incubated for 30 min at 37 °C in order to induce the AR (de Lamirande *et al.* 1997). In these samples, the percentage of capacitated spermatozoa was determined by the FITC-conjugated *Pisum sativum* agglutinin staining (FITC–PSA) (de Lamirande *et al.* 1997). Sperm samples were centrifuged for 5 min at $600\times$ ***g*** at 20 °C, and fixed with ethanol. An aliquot of 10×10^6 cells was then smeared onto superfrost plus slides (Fischer Scientific, Montreal, QC, Canada) and air dried. Following this, slides were incubated for 5 min with PSA–FITC. Slides were then washed with water. A 1,4-diazabicyclo [2.2.2]octane (DABCO) solution was then applied to each slide, and they were sealed with a cover slip. Slides were then observed under a Carl Zeiss (Oberkochen, Germany) Axiophot microscope (exciter filter BP450-490) at 1000 magnifications. Two hundred cells per duplicate were counted for presence or absence of an intact acrosome.

Cellular fractionation and localization of tyrosine nitration and S*-glutathionylation in spermatozoa*

ROS-treated spermatozoa were fractionated into cytosolic, triton-soluble, and triton-insoluble fractions as described previously (O'Flaherty & de Souza 2011). Briefly, cells were frozen at −80 °C for 15 min and then thawed at 37 °C in order to disrupt sperm membranes and allow the release of the cytosolic content. Sperm suspensions (50×10^6 cells/ml) were then centrifuged for 5 min at $12\,000\times$ ***g***, and the supernatant was collected. The remaining pellet was resuspended (100×10^6/ml final concentration) in the BWW medium containing 0.2% triton × −100 (BWW-T) and incubated for 10 min on ice. This chilled sample was then centrifuged for 5 min at $12\,000\times$***g*** at 5 °C, and the supernatant, containing the triton-soluble fraction, was collected. The remaining pellet (triton-insoluble fraction) was resuspended (100×10^6/ml final concentration) in BWW-T, and sonicated (three cycles of 5 min at 30% output) with a Sonic Vibracell (Sonics and Materials, Inc., Newtown, CT, USA) with net power output: 10 W and 20 kHz. The cytosolic, triton-soluble and -insoluble fractions were supplemented with a sample buffer with (reducing conditions) or without (non-reducing conditions) 100 mM DTT and boiled for 5 min at 96 °C.

SDS–PAGE and immunoblotting

Aliquots of supernatant containing sperm proteins from entire spermatozoa or subcellular fractions (under non-reducing or reducing conditions) were loaded on 12% polyacrylamide gels, electrophoresed, and electrotransferred onto nitrocellulose membranes using a transfer buffer (192 mM glycine and 25 mM Tris, pH 8.3) containing 20% methanol. The membranes were then blocked via a 30-min incubation in 5% skim milk dissolved in 2 mM Tris (pH 7.8)-buffered saline and 0.1% Tween 20 (TTBS). Membranes containing proteins under non-reducing or reducing conditions were then washed with TTBS and incubated overnight with anti-nitrotyrosine diluted

1:10 000 or anti-glutathione diluted 1:2000 in 1% skim milk in TTBS respectively.

Following incubation, membranes were washed by 10-min incubations in fresh TTBS. This was followed by 1-h incubation at room temperature with a HRP-conjugated secondary antibody (1:5000 dilution). Positive immunoreactive bands were detected using chemiluminescence (Lumi-light; Roche Molecular Biochemicals). After detection, membranes were washed with distilled water and silver stained (Jacobson & Karsnas 1990) to assure equal loading for each sample.

The relative intensity of protein bands was determined as described previously (O'Flaherty *et al*. 2005, Gong *et al*. 2012). Briefly, films with the same duration of exposure were scanned using a Hewlett Packard scanjet G4010 (Hewlett Packard) and the resulting images were analyzed using the Un-Scan-It gel Software version 5.1 (Silk Scientific Corporation, Orem, Utah). Each band's intensity was obtained and normalized to the respective intensity of the 55 kDa band present in the membrane after staining with colloidal silver as explained above. Then, the total value of all the normalized intensity bands were obtained and again normalized with that of the control sample. Therefore, the intensity of each sample is a proportion of the intensity of the respective control for each experiment. This last normalization allowed us to determine the relative increases or decreases in intensities obtained under various experimental conditions.

Immunocytochemistry

Sperm suspensions were treated with either 5 mM H_2O_2 or 500 μM DA-NONOate and incubated for 30 min at 37 °C to induce oxidation. Aliquots containing 10×10^6 cells were then smeared onto superfrost plus slides (Fisher Scientific, Montreal, QC, Canada), and allowed to dry at room temperature. Dried cells were permeabilized with methanol as described previously (O'Flaherty & de Souza 2011). Cells were rehydrated with PBS supplemented with triton × −100 (PBS-T) and blocked with 5% goat serum in PBS-T for 30 min at 20 °C. Slides were washed in PBS-T and incubated overnight at 4 °C with anti-nitrotyrosine or anti-glutathione antibodies. Cells were then washed and incubated for 1 h at 20 °C with their respective biotinylated anti-IgG antibody (dilution 3:1000). Following this, streptavidin conjugated to Alexa Fluor 555 (1:500) was applied to slides. Smears were mounted with prolong antifade and sealed with a cover slip. Negative controls were prepared in the same way, except that samples were incubated solely with the respective biotinylated anti-IgG antibody.

Statistical analysis

Percentages of capacitation, motility, and viability were transformed as arcsin square root of the proportion value and analyzed using ANOVA and the Bonferroni test. Normal distribution was confirmed using Anderson–Darling test. A difference was considered to be significant when the *P* value was ≤ 0.05. Systat 13 for Windows (Systat Software, Inc.) was used for all statistical analyses.

Results

Impact of oxidative stress on sperm motility and viability

We generated an *in vitro* mild to strong oxidative stress with exogenous sources of ROS, generating H_2O_2, alkoxy radical, $O_2^{\bullet-}$, NO•, and peroxynitrite to mimic what is happening to spermatozoa of infertile men affected by high levels of ROS. A dose-dependent decrease ($P<0.05$) in both total and progressive motilities was observed in spermatozoa treated with H_2O_2 and a significant reduction in DA-NONOate-treated spermatozoa (Fig. 1A and B). A significant decrease in total motility was documented in spermatozoa treated with 0.5 and 1 mM of H_2O_2 when compared with non-oxidized controls. In the case of sperm treated with DA-NONOate, a significant decrease was noted only after incubation with 1 mM DA-NONOate (Fig. 1A and B). Noteworthy, H_2O_2 showed a stronger negative effect on sperm motility at higher doses than DA-NONOate.

Sperm viability was then assessed using a hypo-osmotic swelling test in order to determine whether the loss of motility is due to cell death. Our results confirmed that sperm viability was not affected following oxidation with both H_2O_2 and DA-NONOate (Fig. 1C).

Oxidative stress impairs sperm capacitation

Spermatozoa, treated with H_2O_2 or DA-NONOate before capacitation with FCSu01, had similar levels of tyrosine phosphorylation compared with non-capacitated controls (Fig. 2). H_2O_2 produced a greater decrease in tyrosine phosphorylation than DA-NONOate, particularly at concentrations of 1 mM. There was a dose-dependent decrease in LPC-induced AR levels in spermatozoa previously treated with H_2O_2 (Fig. 3A). However, spermatozoa treated with DA-NONOate showed no change in the percentage of AR following incubation with 0.1 mM (Fig. 3B), while this percentage drastically dropped in spermatozoa treated with 0.25 or 0.5 mM. Similar results were obtained with BSA or progesterone as capacitation inducers (data not shown).

ROS promote tyrosine nitration and S-glutathionylation of sperm proteins

Percoll-washed spermatozoa were exposed to increasing concentrations of DA-NONOate, H_2O_2, or tert-BHP (Fig. 4). DA-NONOate promoted a dose-dependent increase in the tyrosine nitration in spermatozoa (Fig. 4). Treatment with peroxides (H_2O_2 or tert-BHP) generated a dose dependent increase in *S*-glutathionylation in spermatozoa (Fig. 5), showing a minimum or no effect on inducing the tyrosine nitration modification (Fig. 4). The levels of *S*-glutathionylation in spermatozoa treated with DA-NONOate (0.1–1 mM) were similar to

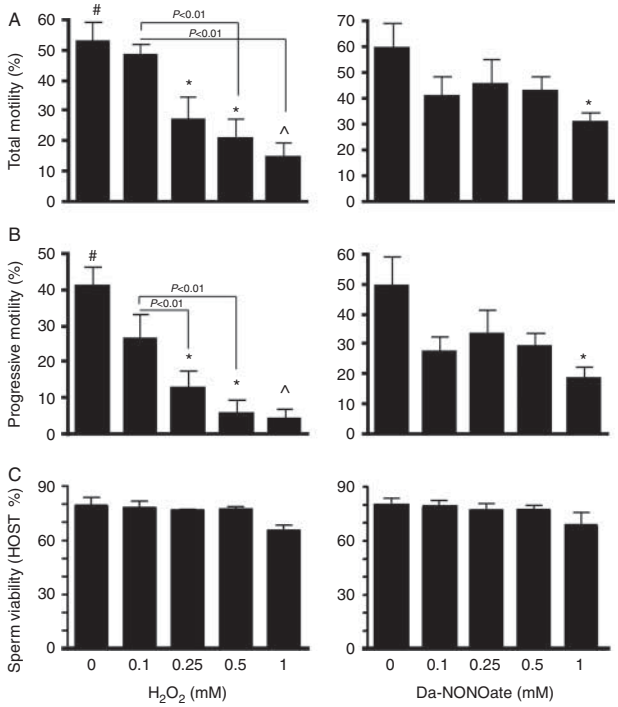

Figure 1 Total and progressive motilities decrease dose dependently in sperm treated with H_2O_2 and DA-NONOate without affecting sperm viability. The motility of sperm cells was analyzed using the CASA sperm analysis system. Total motility (A), progressive motility (B), and sperm viability (C) were recorded from six different donors. # or ^Means the highest and lowest values respectively. *Lower than control (0 mM).

those of the control and never exceeding those generated with 0.25 mM H_2O_2 (Fig. 6), suggesting that NO• is not a major inducer of *S*-glutathionylation in human spermatozoa.

Tyrosine nitration- and S-glutathionylation-modified proteins are differentially localized to human spermatozoa

As exposure to high concentrations of different ROS induces an increase in the tyrosine nitration and *S*-glutathionylation (Figs 4 and 5), the next step was to determine the localization of these modified proteins within the compartments of the sperm cell. Following fractionation, we found an increase in tyrosine-nitrated proteins in all of the cytosolic, triton-soluble, and triton-insoluble fractions following treatment with DA-NONOate (Fig. 7A). The highest levels were found in the triton-insoluble fraction. Immunocytochemistry experiments revealed a strong labeling within the tail region (Fig. 8A). Moreover, permeabilized DA-NONOate-treated spermatozoa displayed a complete labeling of the head and tail (Fig. 8, lower panel).

The majority of *S*-glutathionylated proteins were found in the cytosolic and triton-soluble fractions (Fig. 7B). Noteworthy, a strong signal was observed in proteins of high molecular mass (~170 kDa). The H_2O_2 treatment promoted the highest levels of *S*-glutathionylation in sperm proteins found in the triton-soluble fraction and with a less extent in the cytosolic fraction. The *S*-glutathionylated sperm proteins found at 170 kDa were present in the triton-insoluble fraction. Non-permeabilized spermatozoa displayed a strong labeling throughout the midpiece and in the post-acrosomal region (Fig. 8B). After permeabilization, a strong labeling was visible within the acrosome and throughout the tail region (Fig. 8B, lower panel). It was also noted that treatment with high concentrations of H_2O_2 caused the acrosome labeling to disappear in some cells, which may be indicative of a spontaneous AR.

Discussion

In this study, we showed evidence that oxidative stress affects sperm function differently depending on the type of ROS involved and promotes redox-dependent protein modifications that display differential localization to subcellular compartments of human spermatozoa. To our knowledge, this is the first report to extensively

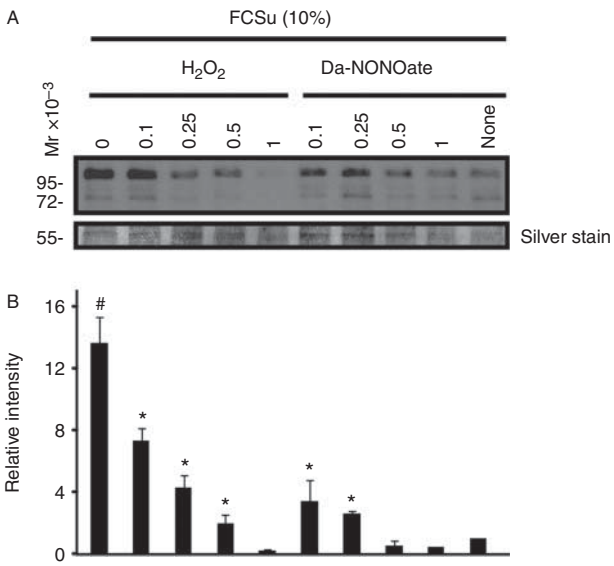

Figure 2 Spermatozoa treated with H_2O_2 or DA-NONOate showed lower levels of protein tyrosine phosphorylation after capacitation than non-oxidized controls. (A) Treated spermatozoa were capacitated for 3.5 h in the BWW medium, pH 8.0, at 37 °C supplemented without (none) or with the capacitation inducer FCSu (10%). Sperm proteins from 0.1×10^6 spermatozoa were loaded in each well, electrophoresed in SDS–polyacrylamide gel under reducing conditions and immunoblotted with an anti-phosphotyrosine antibody. Silver stain was used as a loading control (band at 55 kDa is shown in the bottom panel). (B) Relative intensity of protein tyrosine phosphorylation. The density value of bands from sample incubated under non-capacitating conditions (none) was used to normalize the values obtained with the other samples. Relative intensity of bands is expressed as mean ± S.E.M. Results are representative of three other experiments performed with different healthy donors ($n=4$). #Highest value and *higher than non-capacitating control (none).

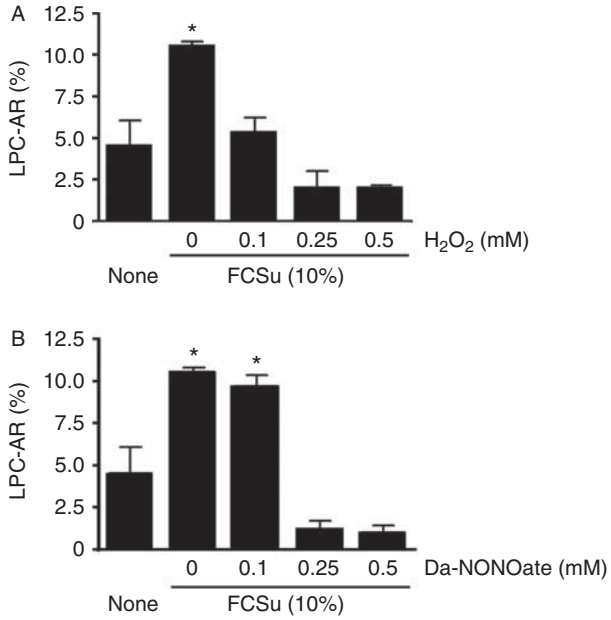

Figure 3 Oxidized spermatozoa displayed a lower level of capacitation compared with untreated controls under capacitation conditions. Spermatozoa previously treated with H_2O_2 or DA-NONOate were capacitated with FCSu for 3.5 h and then with LPC for 30 min to induce the acrosome reaction. Sperm cells were stained with PSA–FITC to visualize the acrosome, and 200 spermatozoa were observed under a fluorescent microscope. Representative blot was obtained from four other experiments performed with different donors ($n=5$). *Higher than non-capacitating control (none).

study tyrosine nitration and *S*-glutathionylation protein modifications in light of impairment of sperm motility and capacitation.

Based on the motility and viability analysis, we confirmed that the impairment of sperm motility is not due to cell death; thus, there is a direct effect of ROS on motility machinery. We examined the effect of oxidative stress on capacitation by determining the percentage of capacitated spermatozoa and the levels of tyrosine phosphorylation (Leclerc *et al.* 1996, O'Flaherty *et al.* 2006*b*). It was found that spermatozoa, treated with H_2O_2 before capacitation, displayed levels of tyrosine phosphorylation (Fig. 2) and LPC-induced AR (Fig. 3A) that were similar to non-capacitated controls. In the case of DA-NONOate, only concentrations higher or equal to 0.25 mM were able to prevent both tyrosine phosphorylation and capacitation (Figs 2 and 3B). These results suggest that oxidative stress negatively affect the capability of spermatozoa to acquire fertilizing ability, thereby explaining why men with high levels of ROS in semen are infertile. The inhibition of motility and capacitation was less severe with DA-NONOate than with H_2O_2; therefore, it is important to determine which type of ROS drives the oxidative stress at the time to establish an antioxidant therapy. These results emphasize the importance of seeking ROS-targeted therapy to treat male infertility.

Peroxides (H_2O_2 or tert-BHP) and DA-NONOate (NO· donor) generated a dose-dependent increase in *S*-glutathionylation and tyrosine nitration in spermatozoa (Fig. 4), suggesting that human spermatozoa actively produce significant levels of redox-dependent protein modifications when they are challenged with an oxidative stress. These results suggest that the oxidative stress impairs motility without affecting sperm viability when high levels of tyrosine nitration and *S*-glutathionylation are present in human spermatozoa.

The majority of nitrated tyrosine-modified proteins were localized to the midpiece (weak labeling) and principal piece (strong labeling) of the sperm tail (Fig. 8A). We can suggest that some of these modified proteins may be present in the fiber sheath, as proteins present in these sperm structure can be found in the triton-insoluble fraction where the tyrosine-nitrated-

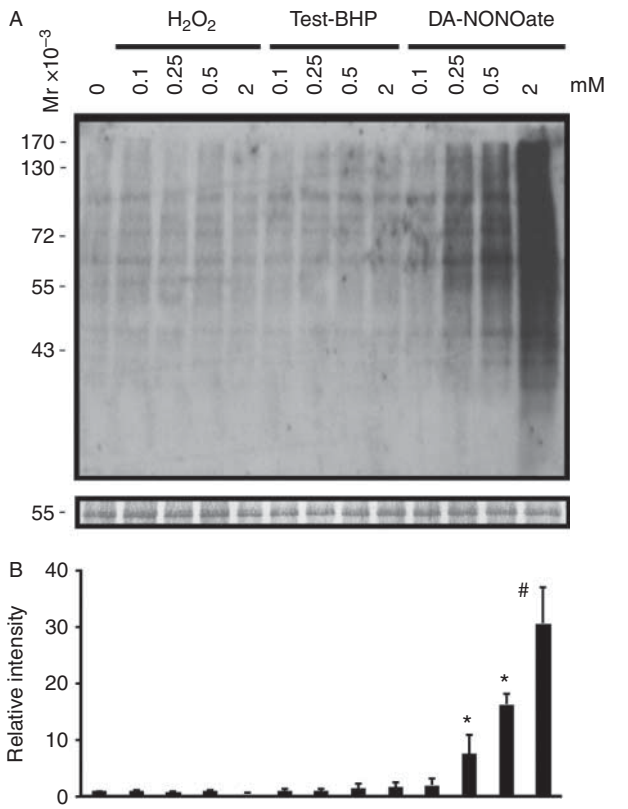

Figure 4 Dose-dependent increase in tyrosine nitration in human spermatozoa following treatment with ROS. (A) Spermatozoa were treated with increasing concentrations of H_2O_2, tert-BHP, or DA-NONOate and immunoblotted with an anti-nitrotyrosine antibody. (B) Relative intensity of tyrosine-nitrated proteins. The density value of bands obtained from a sample incubated without ROS (0 mM) was used to normalize the values obtained with the other samples. The relative intensity of bands is expressed as mean ± S.E.M. Membranes were silver stained to confirm equal loading between lanes (band at 55 kDa is shown in the bottom panel). Representative blot was obtained from four other experiments performed with different donors ($n=5$). #Highest value and *higher than control (0 mM).

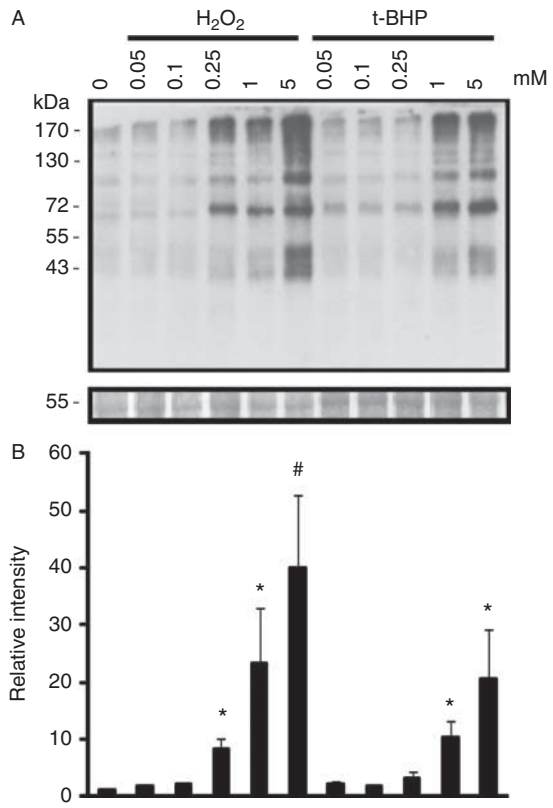

Figure 5 Dose-dependent increase in *S*-glutathionylation modifications in human spermatozoa following treatment with ROS. (A) Spermatozoa treated with increasing concentrations of H_2O_2 or t-BHP and immunoblotted with an anti-glutathione antibody. (B) Relative intensity of *S*-glutathionylated proteins. The density value of bands obtained from a sample incubated without ROS (0 mM) was used to normalize the values obtained with the other samples. The relative intensity of bands is expressed as mean ±S.E.M. #Highest value and *higher than control (0 mM).

modified proteins are present at high levels (Fig. 7). The decreased total and progressive motilities and the evident labeling of the tail (Figs 1 and 8A) suggest that an increase in tyrosine-nitrated-modified proteins may disrupt the function of proteins that are important for the motility machinery. While future experiments would be required to determine the identity of the modified proteins, our results suggest that glycolytic enzymes such as glyceraldehyde 3-P dehydrogenase, enolase, enzymes involved in the Krebs cycle such as aconitase, α-ketoglutarate dehydrogenase, malate dehydrogenase, and dihydrolipoamide dehydrogenase (present in the pyruvate dehydrogenase that converts pyruvate into acetyl CoA) may be the targets as they can be altered by this modification (Lind *et al*. 2002, Gokulrangan *et al*. 2007, Shi *et al*. 2011; Supplementary Table 2, see section on supplementary data given at the end of this article). The evidence that sizes of bands obtained during western blot experiments for tyrosine-nitrated-modified proteins (Fig. 4 and 7A) are similar to the molecular mass of the mentioned enzymes accounts for the possibility that these energy production-related enzymes may be affected by tyrosine nitration in spermatozoa affected by oxidative stress.

We found high levels of tyrosine nitration after treatment with DA-NONOate in the head of permeabilized spermatozoa (Fig. 8). Tyrosine nitration activates metalloproteinase 9 (MMP9), promoting astrocyte migration and the subsequent inflammation of the brain under oxidative stress (Wang *et al*. 2011). The localization of tyrosine nitration in the sperm head is similar to that of metalloproteinases MMP2 and MMP9 found in human spermatozoa (Buchman-Shaked *et al*. 2002), which are suggested as important proteins for sperm zona penetration (Ferrer *et al*. 2012). These proteins form the extracellular coat on the inner acrosomal membrane that is exposed after AR (Ferrer *et al*. 2012). It is possible that premature activation of MMPs by tyrosine nitration (Wang *et al*. 2011) decreases the capability of the spermatozoon to penetrate the zona pellucida.

Most of the *S*-glutathionylated-modified proteins were found in the cytosolic and triton-soluble fractions (Fig. 7B). Immunocytochemistry studies revealed a strong labeling throughout the midpiece and in the post-acrosomal

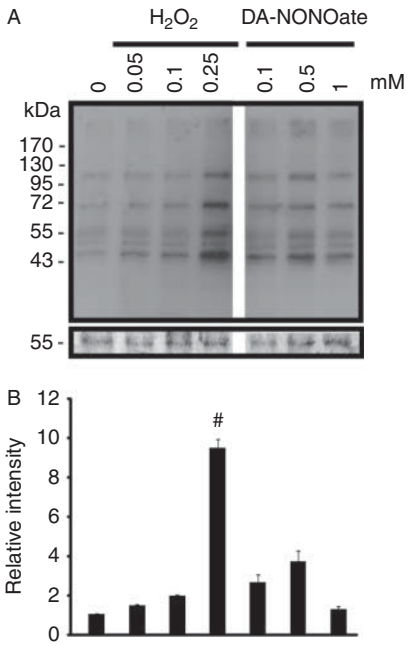

Figure 6 DA-NONOate does not increase the levels of *S*-glutathionylation in spermatozoa. (A) Spermatozoa were treated with increasing concentrations of DA-NONOate or H_2O_2 and immunoblotted with an anti-glutathione antibody. Membranes were silver stained to confirm equal loading between lanes (band at 55 kDa is shown in the bottom panel). The density value of bands obtained from a sample incubated without ROS (0 mM) was used to normalize the values obtained with the other samples. The relative intensity of bands is expressed as mean±S.E.M. All samples were loaded on the same gel. Representative blot was obtained from two other experiments performed with different donors ($n=3$). #Highest value.

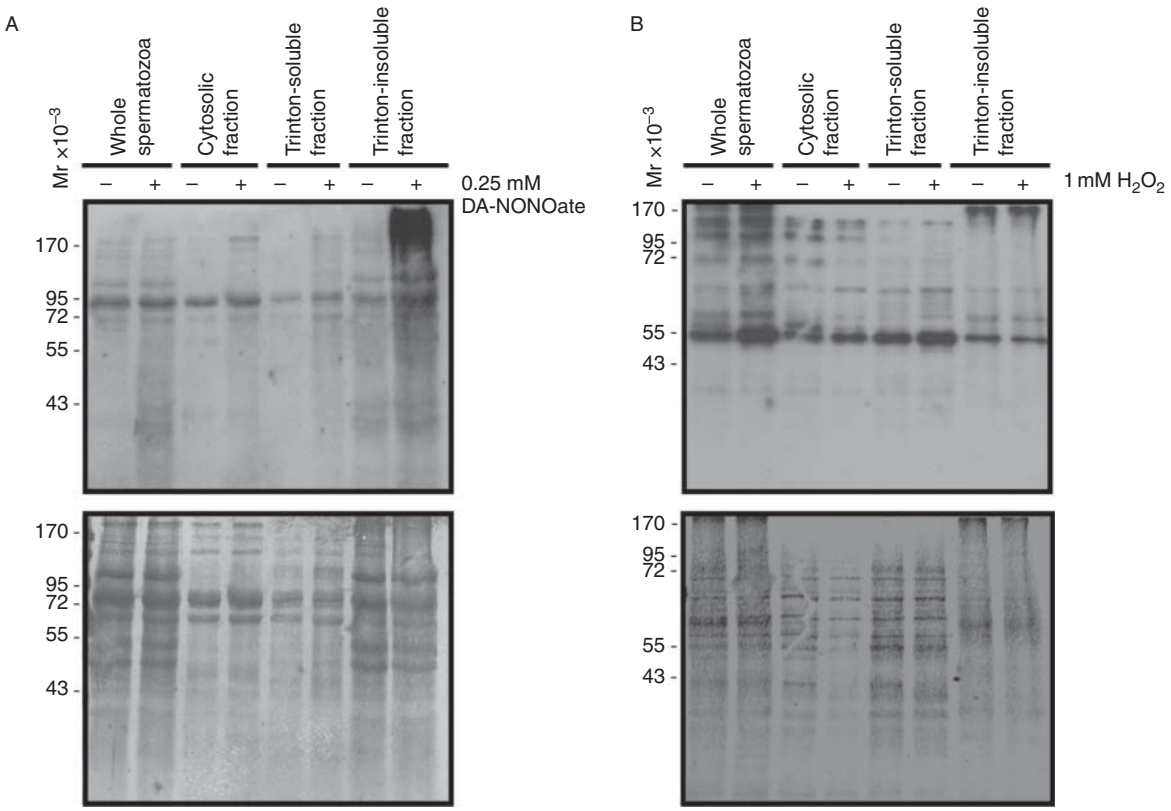

Figure 7 Tyrosine nitration and *S*-glutathionylation are differentially localized within the sperm cell, and most abundantly found in triton-insoluble proteins of the tail and fibrous sheath. (A) Sperm proteins treated with 0.25 mM of DA-NONOate under reducing conditions and immunoblotted with an anti-nitrotyrosine antibody (upper panel; 0.5×10^6 cells were loaded for the whole and cytosolic lanes, while 1×10^6 cells were loaded for the triton-soluble and -insoluble lanes). (B) Sperm proteins treated with 1 mM H_2O_2 under non-reducing conditions and immunoblotted with an anti-GSS-R antibody (upper panel; 0.5×10^6 cells were loaded for the whole and cytosolic lanes, while 1×10^6 cells were loaded for the triton-soluble and -insoluble lanes). Lower panels in A and B represent the respective membrane stained with colloidal gold as described in material methods. Representative blots were obtained from three other experiments performed with different donors ($n=4$).

region (Fig. 8B). Following permeabilization, a strong labeling was visible within the acrosome and in the tail region. Within the head, components of the cytoskeleton play a role during the activation of spermatozoon; during capacitation, there is polymerization of the actin filaments that is necessary for the spermatozoon to undergo the AR (Brener *et al*. 2003, Breitbart *et al*. 2005). Actin can be *S*-glutathionylated (Dalle-Donne *et al*. 2003) and its modified form has been associated with Friedreich's ataxia (Pastore *et al*. 2003). The strong labeling in the acrosome may also be indicative of *S*-glutathionylation of actin. This redox-dependent modification of actin promotes the impossibility of actin polymerization and thus preventing the AR to occur upon LPC treatment (Fig. 3).

As *S*-glutathionylation seemed to occur over the entire sperm cell, similar to the tyrosine nitration modification, we can also suggest that *S*-glutathionylation may impair sperm function by impeding the energy production. It has been shown that enzymes of glycolysis and of the Krebs cycle can be *S*-glutathionylated under oxidative stress (Supplementary Table 2; Fratelli *et al*. 2004). Noteworthy, tubulin, the major component of the sperm flagellum, can be modified by tyrosine nitration and *S*-glutathionylation (Landino *et al*. 2004); thus, our results suggest that sperm motility is affected also at the level of flagellar structure.

Oxidative stress affects human sperm function (Gagnon *et al*. 1991, de Lamirande & Gagnon 1994, Agarwal *et al*. 2006, Aitken & Baker 2006); however, little is known regarding the factors involved and mechanisms affected by high levels of ROS and whether various sources of oxidative stress differentially affect the sperm function. Although high levels of ROS impair motility (Aitken *et al*. 1998, Smith *et al*. 2006) and the ability to fuse with zona-free hamster oocytes (Aitken *et al*. 1998), and promote increased DNA damage and inhibition of capacitation (this study), the molecular mechanisms associated with these functions that are impaired by high levels of ROS are yet to be elucidated.

Besides the high sperm lipid peroxidation and DNA damage, it has been recently suggested that the antioxidant enzymes, peroxiredoxins (PRDXs), are highly oxidized and thus inactive in infertile patients (Gong *et al*. 2012). PRDXs are highly abundant and differentially distributed in all sub-compartments of the

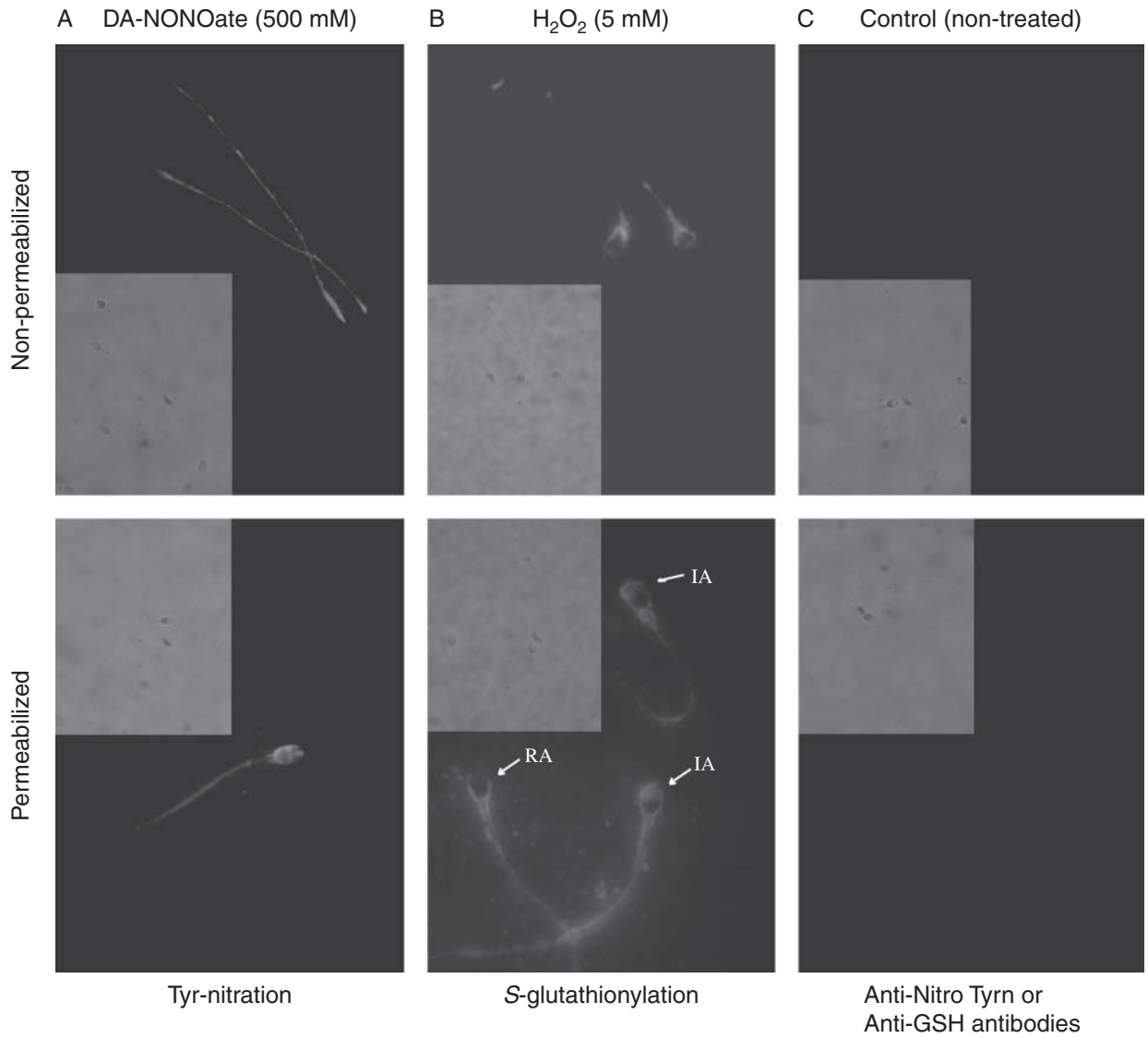

Figure 8 *S*-glutathionylated proteins were differentially localized within the sperm cell and preferentially found within the triton-soluble and cytosolic fractions. Immunocytochemistry images were taken by fluorescence and phase-contrast microscopy at 1000× magnification for sperm treated with 500 μM DA-NONOate (A) or with 5 mM of H_2O_2 (B). Treated and untreated sperm were photographed at the same time of exposure. Absence of unspecific labeling by the secondary antibody was also confirmed (data not shown) ($n=4$).

human spermatozoon and are considered the first line of defense against oxidative stress in human spermatozoa (O'Flaherty & de Souza 2011). Our laboratory reported that PRDX1 and PRDX6 form ~170 kDa protein complexes detected in spermatozoa treated with high concentrations of H_2O_2 (0.25–5 mM) and also in spermatozoa from infertile men (O'Flaherty & de Souza 2011, Gong *et al*. 2012). There is increasing evidence supporting the involvement of PRDXs in the regulation of redox signaling, especially H_2O_2-dependent signaling (Rhee *et al*. 2005, Fourquet *et al*. 2008). As oxidative stress promotes *S*-glutathionylation of PRDXs (Lind *et al*. 2002, Noguera-Mazon *et al*. 2006) and we found 130–170 kDa bands with high levels of *S*-glutathionylation, it is possible that *S*-glutathionylation of PRDX1 and PRDX6 worsens the impairment of sperm function observed in this and previous studies (O'Flaherty & de Souza 2011, Gong *et al*. 2012). It is noteworthy that the strong signal of tyrosine nitration and *S*-glutathionylation on the sperm head (Fig. 8) corresponds with the localization of PRDXs (O'Flaherty & de Souza 2011, O'Flaherty 2014). It is then plausible that the redox-dependent modifications of PRDXs are causing the increase in levels of sperm DNA damage observed in infertile men (Gong *et al*. 2012). We have recently found that male mice lacking *Prdx6* show high levels of DNA fragmentation and oxidation (Ozkosem & O'Flaherty 2012); these data provide evidence in support of the role of PRDXs and particularly PRDX6 in the protection of paternal genome against oxidative stress.

In conclusion, tyrosine nitration and *S*-glutathionylation increase dose dependently in spermatozoa after treatment with ROS, and they are differentially localized within spermatozoa. The oxidative stress prevents spermatozoa from undergoing capacitation, impairs sperm motility, and increases tyrosine nitration and *S*-glutathionylation. Excessive levels of tyrosine nitration and *S*-glutathionylation of specific sperm proteins may be involved in the pathological mechanisms leading to impairment of sperm function.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0240.

Declaration of interest

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

Funding

This study was supported by the Canadian Institutes of Health Research (MOP 133661), the Fonds de Recherché en Santé Quebec (FRSQS #22151), and a Chercheur Boursier Junior 1 Salary Award from FRSQS (20482) to C O'Flaherty and a studentship from the Research Institute-MUHC (#4696) to T Morielli.

Acknowledgements

The authors thank donors who participated in this study.

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Received 5 May 2014
First decision 10 June 2014
Revised manuscript received 10 October 2014
Accepted 28 October 2014