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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 decrease in antioxidant defences (and possibly both), is associated with male infertility. A consequence of oxidative stress is the generation of 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 to 5 mM) of exogenous hydrogen peroxide, tert-buthyl 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 (LPC) were determined. Modified sperm proteins from cytosolic, Triton-soluble and – insoluble fractions were analysed by SDS-PAGE immunoblotting and immunocytochemistry with anti-glutathione and anti-nitro tyrosine antibodies. Levels of S-glutathionylation increased dose dependently after exposure to hydroperoxides ($p<0.05$), and were localised mainly in 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 in the Triton-insoluble fraction. ROS-treated spermatozoa showed impaired motility without affecting viability (hypoosmotic swelling test). These treated spermatozoa had tyrosine phosphorylation and AR levels similarly 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 of tyrosine nitration and S-glutathionylation and alters motility and the ability of spermatozoa to undergo capacitation.

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DECLARATION OF INTEREST
None of the authors have conflict of interest to disclosure.

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Keywords

reactive oxygen species; oxidative stress; redox protein modifications

INTRODUCTION

Human infertility is an important health and a social concern which affects 15% of couples in the reproductive age (WHO 2010; de Kretser 1997). 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 nonfunctional 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 (Anderson & Williamson 1988; Brennemann *et al.* 1997; Hasegawa *et al.* 1997; Smith *et al.* 2006). Interestingly, all the above mentioned conditions have the oxidative stress as an important component of their pathophysiological mechanisms (Agarwal *et al.* 2008; Turner 2001; Anderson & Williamson 1988; Smith *et al.* 2006; Brennemann *et al.* 1997; Hasegawa *et al.* 1997).

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 (Griveau & Le Lannou 1997; Sikka *et al.* 1995), 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 (Agarwal *et al.* 2006; Aitken 2006; Gagnon *et al.* 1991; de Lamirande & Gagnon 1995; 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 (Gong *et al.* 2012; Aitken & Curry 2011).

Paradoxically, the spermatozoon requires of low and controlled amounts of ROS to acquire fertilizing ability during capacitation (de Lamirande & O'Flaherty 2012). ROS trigger most of the recognized events associated with capacitation: activation of adenylyl cyclase, increase of intracellular calcium and phosphorylation events (protein kinases A, C, ERK and PI3K/Akt pathways) culminating with the late tyrosine phosphorylation (de Lamirande & O'Flaherty 2012; Leclerc *et al.* 1996; Visconti *et al.* 1995; O'Flaherty *et al.* 2006a). Noteworthy, failure to undergo tyrosine phosphorylation were observed in spermatozoa from infertile patients (Buffone *et al.* 2005). Hydrogen peroxide (H_2O_2) affected motility (de Lamirande & Gagnon C 1992) and sperm hemi-zona binding (Oehninger *et al.* 1995) at 0.5 or 0.2 mM, respectively. The incubation of human spermatozoa with sodium nitropruside (a 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 C 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 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-nitro-tyrosine 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 horseradish peroxidase) were provided by Cederlane Laboratories Ltd (Hornby, Canada). Nitrocellulose membranes (pore size, 0.22 um) were purchased from Osmonics Inc (Westborough, Massachusetts, USA) and the enhanced chemiluminescence kit Lumi-Light from Roche Molecular Biochemicals (Laval, QC Canada). Radiographic films (obtained brom 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 (Burlington, ON, Canada). 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 satvium agglutinin conjugated to fluorescein isothiocyanate (PSA-FITC) were purchased from Sigma- Aldrich Chemical Co (Milwaukee, WI, USA). Other chemicals used were of at least reagent grade.

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Subjects

Semen samples were obtained from healthy volunteers (n=21) after three 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 prior to participating. Following collection, samples were incubated at 37°C for 30 minutes 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).

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, white blood cells, from the sperm cells with the best motility and morphology (Kovalski *et al.* 1992) without increasing ROS levels (Zini *et al.* 1993; Plante *et al.* 1994; Iwasaki & Gagnon 1992). 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 medium (BWW, pH 8.0) (Biggers *et al.* 1971), and used for experimentation.

Induction of in vitro oxidative stress in spermatozoa

Spermatozoa were incubated during 30 min incubation at 37°C with increasing concentrations of either hydrogen peroxide (H_2O_2), tert-buthyl hydroperxide (tert-BHP; a synthetic organic hydroperoxides that can produce alkoxyl radical, $O_2^{\cdot-}$ and H_2O_2), or Da-NONOate (NO• donor) in BWW. 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, vanadate 0.1 mM, sodium floride 5 mM and glycerol phosphate 20 mM) supplemented or not with 100 mM diothiothreitol (DTT) was added to an 50-μl aliquot of the sperm suspension (100 $\times 10^6$/ml) of each sample. The absence of DTT in the sample buffer is indispensable to be able to see the S-glutathionilated 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.

Sperm motility and viability analysis

Spermatozoa were subjected to oxidative stress as previously described, washed and resuspened in 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 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 10 different fields.

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Both ROS-treated and control samples were centrifuged for 5 min at 600×g at 20°C. The supernatant was removed and replaced with a hypo-osmotic solution (HOS) at 37°C (WHO 2010). Samples were incubated for 30 minutes at 37°C. Following this, sperm samples were gently centrifuged for 5 minutes at 1,000×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 using 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 minutes at 600×g at 20°C. The supernatant was then discarded and replaced with fresh BWW containing either 10% fetal chord serum ultrafiltrate (FCSu), 3mg/ml bovine serum albumin (BSA) or 10 μM progesterone in order to induce capacitation and spermatozoa were then incubated for 3.5 hours 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.* 2006b). The remaining sample was centrifuged at 2,000×g for 5 min at 20°C. The resulting pellet was recuperated and resuspended in fresh BWW containing 2.5 μM lysophosphatidylcholine (LPC) and incubated for 30 minutes at 37°C in order to induce the acrosome reaction (de Lamirande *et al.* 1997). In these samples, the percentage of capacitated spermatozoa was determined by the fluorescein isothiocyanate-conjugated *Pissum sativum* agglutinin staining (FITC-PSA) (de Lamirande *et al.* 1997). Sperm samples were centrifuged for 5 minutes at 600×g at 20°C, and fixed with ethanol. An aliquot of $10{\times}10^6$ 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 coverslip. Slides were then observed under a Carl Zeiss (Oberkochen, Germany) Axiophot microscope (exciter filter BP450- 490) at 1,000 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 previously described (O'Flaherty & de Souza 2011). Briefly, cells were frozen at −80°C for 15 minutes, 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×g, and the supernatant was collected. The remaining pellet was resuspended (100×10^6/ml final concentration) in BWW containing 0.2% Triton-x100 (BWW-T) and incubated for 10 minutes on ice. This chilled sample was then centrifuged for 5 minutes at 12,000×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,

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Newtown, CT, USA) with net power output: 10 watts and 20 kHz. The cytosolic, Triton-soluble and – insoluble fractions were supplemented with sample buffer with (reducing conditions) or without (non-reducing conditions) 100 mM dithiothreitol (DTT) and boiled for 5 min at 96°C.

SDS-PAGE and Immunoblotting

Aliquots of supernatant containing sperm proteins from entire spermatozoa or subcelullar fractions (under non- or reducing conditions) were loaded in 12% polyacrylamide gels, electrophoresed and electrotransferred on to nitrocellulose membranes using transfer buffer (192 mM glycine and 25 mM Tris, pH 8.3) containing 20% methanol. The membranes were then blocked via a 30 minutes 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- or reducing conditions were then washed with TTBS and incubated overnight with anti-Nitro-tyrosine 1:10,000 or anti-glutathione 1:2,000 dilution prepared in 1% skim milk in TTBS, respectively. Following incubation, membranes were washed using 10 min incubations in fresh TTBS. This was followed by 1 hour incubation at room temperature with a horseradish peroxidase conjugated secondary antibody (1:5,000 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 for the equal loading for each sample.

Relative intensity of proteins bands were done as previously reported (O'Flaherty *et al.* 2005; Gong *et al.* 2012). Briefly, films with the same time of exposure were scanned using a Hewlett Packard scanjet G4010 (Hewlett Pakcard, Mississauga, ON, Canada) and the resulted 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 5mM H_2O_2 or 500μM 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 done before (O'Flaherty & de Souza 2011). Cells were rehydrated with PBS supplemented with Triton-X100 (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-nitro-tyrosine or anti-glutathione antibodies. Cells were then washed, and incubated for 1 hour at 20°C with their respective biotinylated anti-IgG antibody (dilution 3:1000). Following this, strepavidin conjugated to alexa fluor 555 (1:500) was applied to slides. Smears were mounted with prolong antifade, and sealed with a coverslip. Negative controls were prepared in the same

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way, except 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's test. Normal distribution was confirmed by using Anderson-Darling test. A difference was considered to be significant when the p value was equal to or less than 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, alkoxyl radical, $O_2^{\cdot-}$, 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 motility was observed in spermatozoa treated with H_2O_2 and a significant reduction in DA-NONOate-treated spermatozoa (Figure 1A and 1B). A significant decrease total motility was documented in spermatozoa treated with 0.5 and 1mM of H_2O_2 as compared to non-oxidized controls. In the case of sperm treated with DA-NONOate, a significant decrease was noted only after incubation with 1mM DA-NONOate (Figure 1A and 1B). 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 (Figure 1C).

Oxidative stress impairs sperm capacitation

Spermatozoa, treated with H_2O_2 or DA-NONOate prior to capacitation with FCSu01, had similar levels of tyrosine phosphorylation compared to non-capacitated controls (Figure 2). Hydrogen peroxide 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 acrosome reaction levels in spermatozoa previously treated with H_2O_2 (Figure 3A). However, spermatozoa treated with DA-NONOate showed no change in the percentage of acrosome reaction following incubation with 0.1 mM (Figure 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).

Reactive oxygen species 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 (Figure 4). DA-NONOate promoted a dose dependent increase of the tyrosine nitration in spermatozoa (Figure 4). Treatment with peroxides (H_2O_2 or tert-BHP) generated a dose dependent increase in S-glutathionylation in spermatozoa (Figure 5),

showing a minimum or no effect on inducing the tyrosine nitration modification (Figure 4). The levels of S-glutathionylation in spermatozoa treated with DA-NONOate (0.1 to 1 mM) were similar to those of the control and never exceeding those generated with 0.25 mM H_2O_2 (Figure 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 in human spermatozoa

Since exposure to high concentrations of different ROS induces an increase in the tyrosine nitration and S-glutathionylation (Figures 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 of tyrosine nitrated proteins in all of the cytosolic, Triton-soluble and -insoluble fractions following treatment with DA-NONOate (Figure 7A). The highest levels were found in the Triton-insoluble fraction. Immunocytochemistry experiments revealed a strong labeling within the tail region (Figure 8A). Moreover, permeabilized DA-NONOate-treated spermatozoa, displayed a complete labeling of the head and tail (Figure 8, lower panel).

The majority of S-glutathionylated proteins were found in the cytosolic and Triton-soluble fractions (Figure 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 (Figure 8B). After permeabilization, a strong labeling was visible within the acrosome, and throughout the tail region (Figure 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 acrosome reaction.

DISCUSSION

In this study we showed evidence that oxidative stress impacts differently on sperm function depending on the type of ROS involved and promotes redox dependent protein modifications that display differential localization in subcellular compartments of human spermatozoa. To our knowledge this is the first report to extensively studied 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 (O'Flaherty *et al.* 2006b; Leclerc *et al.* 1996). It was found that spermatozoa, treated with H_2O_2 prior to capacitation, displayed levels of tyrosine phosphorylation (Figure 2) and of LPC-iduced AR (Figure 3A) that were similar to non-capacitated controls. In the case of DA-NONOate, only

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concentrations higher or equal to 0.25 mM were able to prevent both tyrosine phosphorylation and capacitation (Figures 2 and 3B). These results suggests that oxidative stress negatively impact on the capability of spermatozoa to acquired fertilizing ability and thus, 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 is driven 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 of S-glutathionylation and of tyrosine nitration in spermatozoa (Figure 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 tyrosine nitrated-modified proteins were localized in the midpiece (weak labeling) and principal piece (strong labeling) of the sperm tail (Figure 8A). We can suggest that some of these modified proteins maybe present in the fiber sheath, as proteins present in these sperm structure can be found in the Triton-insoluble fraction where the tyrosine nitrated-modified are present at high levels (Figure 7). The decreased total and progressive motility and the evident labeling of the tail (Figures 1 and 8A) suggest that an increase of 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 dihydro lipoamide dehydrogenase (present in the pyruvate dehydrogenase that converts pyruvate into acetylCoA) may be targets since they can be altered by this modification (Shi *et al.* 2011; Gokulrangan *et al.* 2007; Lind *et al.* 2002)(Supplementary Table 2). The evidence that the size of bands obtained during western blot experiments for tyrosine nitrated-modified proteins (Figure 4 and 7A) that 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 in the head of permeabilized spermatozoa, high levels of tyrosine nitration after treatment with DA-NONOate (Figure 8). Tyrosine nitration activates metalloproteinase-9 (MMP-9) promoting astrocyte migration and 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 MMP-2 and MMP-9 found human spermatozoa (Buchman-Shaked *et al.* 2002) and 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 acrosome reaction (Ferrer *et al.* 2012). It is possible that a premature activation of MMPs by tyrosine nitration (Wang *et al.* 2011), decreases the capability of the spermatozoon to penetrate the zona pellucida.

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Most of the S-glutathionylated-modified proteins were found in the cytosolic and triton-soluble fractions (Figure 7B). Immunocytochemistry studies revealed a strong labeling throughout the midpiece and in the post acrosomal region (Figure 8B). Following permeabilization, a strong labeling was visible within the acrosome, and 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 acrosome reaction (Breitbart *et al.* 2005; Brener *et al.* 2003). 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 acrosome reaction to occur upon LPC treatment (Figure 3).

Since S-glutathionylation seemed to be present over the entirety of the sperm cell, similarly to the tyrosine nitration modification, we can also suggest that S-glutathionylation may impair sperm function by impeding the energy production. It was shown that enzymes of glycolysis and of the Krebs cycle can be S-glutahionylated 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 (Agarwal *et al.* 2006; de Lamirande E. & Gagnon 1994; Gagnon *et al.* 1991; Aitken & Baker 2006); however, little is known regarding the players and mechanisms affected by high levels of ROS and whether various sources of oxidative stress differentially impact on sperm function. Although high levels of ROS impair motility (Smith *et al.* 2006; Aitken *et al.* 1998), the ability to fuse to zona-free hamster oocytes (Aitken *et al.* 1998), promote increased DNA damage and inhibition of capacitation (present study), it is yet to be elucidated the molecular mechanisms associated with these functions that are impaired by high levels of ROS.

Besides the high sperm lipid peroxidation and DNA damage, it was 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 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 ~170kDa protein complexes detected in spermatozoa treated high H_2O_2 concentrations (0.25–5 mM) and also found in spermatozoa from infertile men (Gong *et al.* 2012; O'Flaherty & de Souza 2011). There is increasing evidence supporting the involvement of PRDXs in the regulation of redox signaling, especially H_2O_2-dependent signaling (Fourquet *et al.* 2008; Rhee *et al.* 2005) Since oxidative stress promotes S-glutathionylation of PRDXs (Lind *et al.* 2002; Noguera-Mazon *et al.* 2006) and because we found 130–170 kDa bands with high levels of S-glutathionylation, it is possible that S-glutathionylation of PRDX1 and PRDX6 promotes the impairment of sperm function observed in this study and previously (Gong *et al.* 2012; O'Flaherty & de Souza 2011). It is worth noting that the strong signal of tyrosine nitration

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and of S-glutathionylation on the sperm head (Figure 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 of levels of sperm DNA damage observed in infertile men (Gong *et al.* 2012). We recently found that males mice lacking PRDX6 show high levels of DNA fragmentation and oxidation (Ozkosem & O'Flaherty 2012); these data support 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 of S-glutathionylation of specific sperm proteins may be involved in the pathological mechanisms leading to impairment of sperm function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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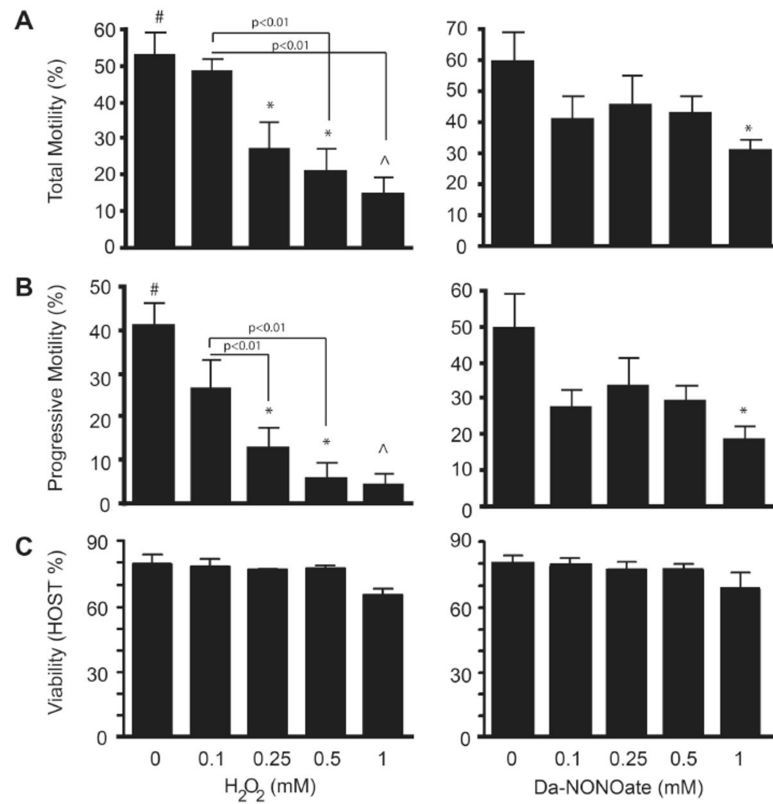

Figure 1. Total and progressive motility decreases dose dependently in sperm treated with H_2O_2 and DA-NONOate without affecting sperm viability

The motility of sperm cells was analyzed using the CASA sperm analysis system. Total motility (A), progressive motility (B) and sperm viability (C) were recorder from 6 different donors. # or ^means the highest and lowest values, respectively. * means lower than control (0 mM).

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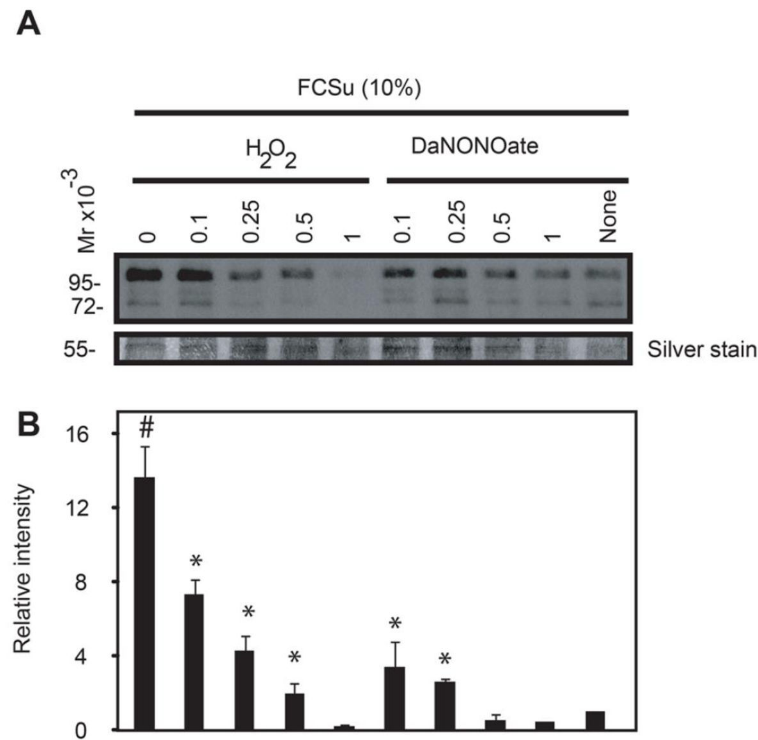

Figure 2. Spermatozoa treated with H_2O_2 or DA-NONOate showed lower levels of protein tyrosine phosphorylation after capacitation than non-oxidized controls

A) Treated spermatozoa were capacitated for 3.5h in BWW, pH 8.0, 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 polyacrylamyde gel under reducing conditions and immunoblotted with anti-phosphotyrosine antibody. Silver stain was used as 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 presented as the mean ± SEM. Results are representative of 3 others done with different healthy donors (n=4). [#]means the highest value, *means higher than non-capacitating control (None).

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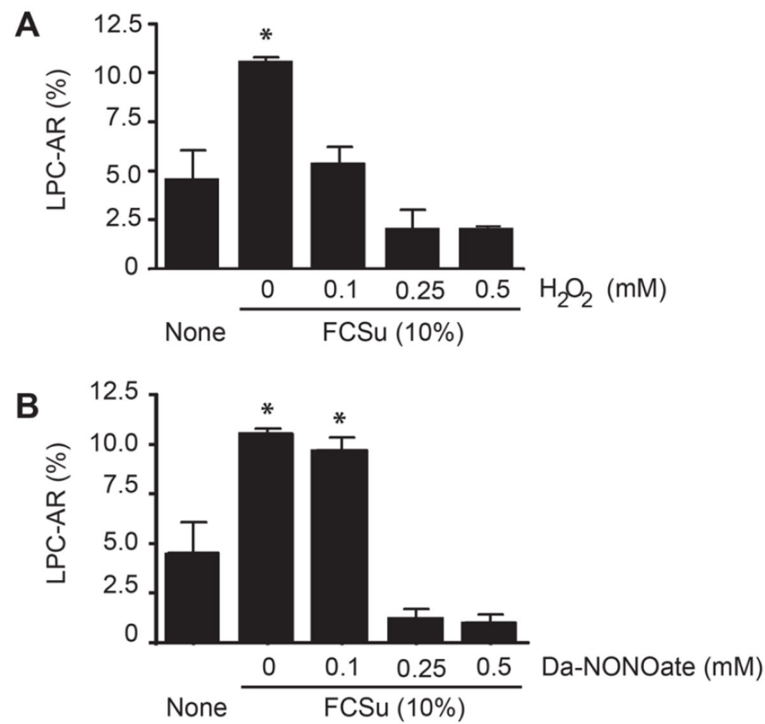

Figure 3. Oxidized spermatozoa displayed lower level of capacitation compared to untreated controls under capacitation conditions

Spermatozoa previously treated with H_2O_2 or DA-NONOate were capacitated with FCSu for 3.5h and then, with LPC for 30 minutes to induce the acrosome reaction. Sperm cells were stained with PSA-FITC to visualize the acrosome, and two hundred spermatozoa were observed under fluorescent microscope. Representative blot from 4 other experiments done with different donors (n=5). *Means higher than non-capacitating control (None).

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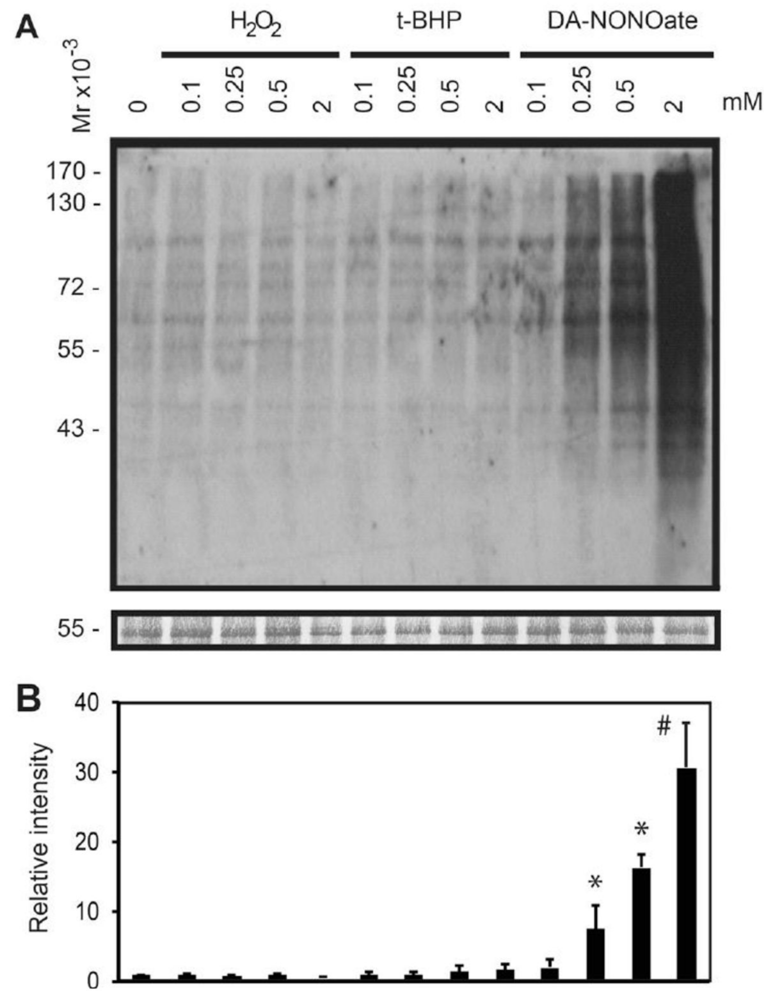

Figure 4. Dose dependent increase of tyrosine nitration in human spermatozoa following treatment with ROS

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 from sample incubated without ROS (0 mM) was used to normalize the values obtained with the other samples. Relative intensity of bands is presented as the mean ± SEM. Membranes were silver stained to confirm equal loading between lanes (band at 55kDa is shown in the bottom panel). Representative blot from 4 other experiments done with different donors (n=5). #means the highest value, *means higher than control (0 mM).

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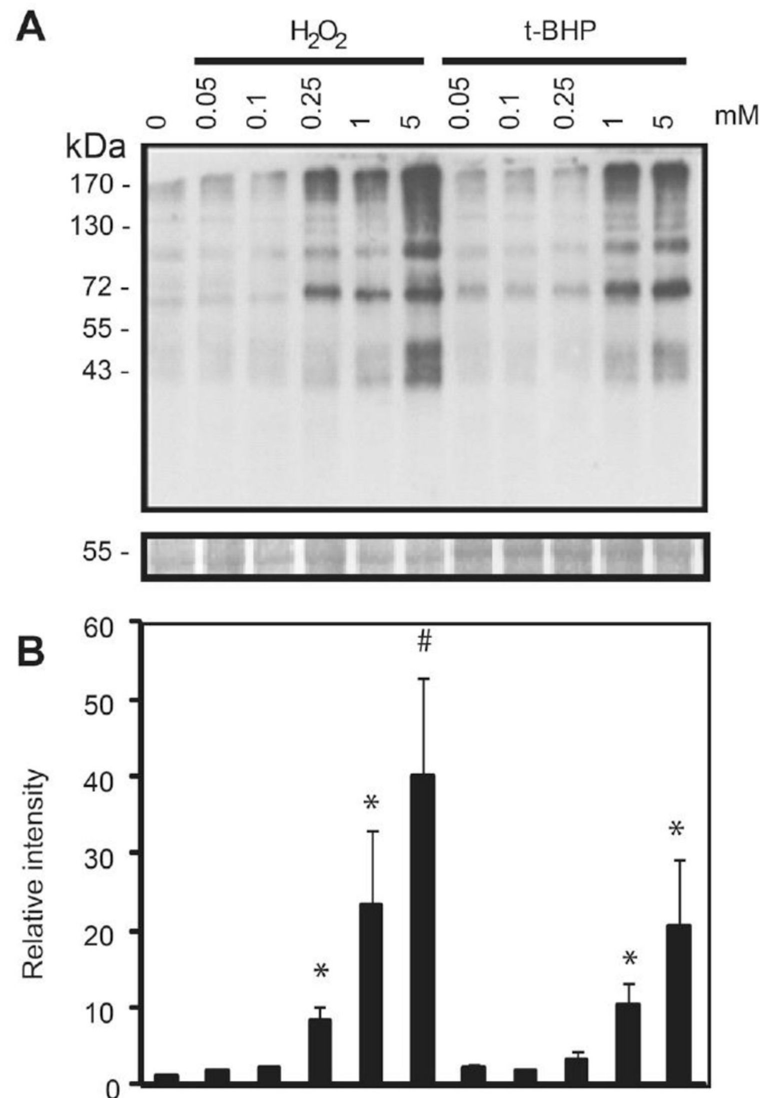

Figure 5. Dose dependent increase of S-glutathionylation modifications in human spermatozoa following treatment with ROS

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-gluthathionylated proteins. The density value of bands from sample incubated without ROS (0 mM) was used to normalize the values obtained with the other samples. Relative intensity of bands is presented as the mean ± SEM. $^{\#}$means the highest value, *means higher than control (0 mM).

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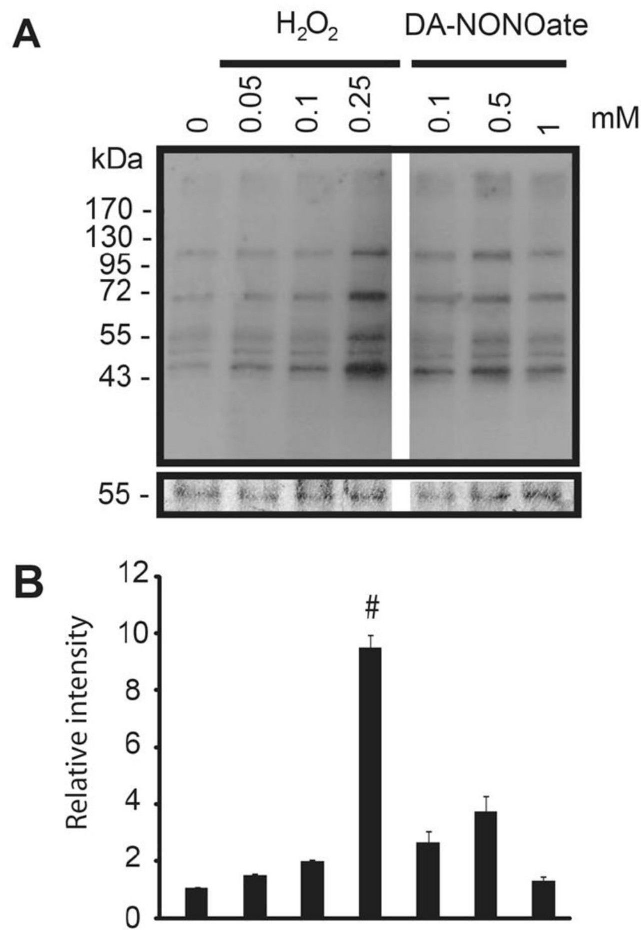

Figure 6. DA-NONOate does not increase the levels of S-glutathionylation in spermatozoa

A) Spermatozoa were treated with increasing concentrations of DA-NONOate or H_2O_2 and immunoblotted with anti-glutathione antibody. Membranes were silver stained to confirm equal loading between lanes (band at 55kDa is shown in the bottom panel). The density value of bands from sample incubated without ROS (0 mM) was used to normalize the values obtained with the other samples. Relative intensity of bands is presented as the mean ± SEM. All samples were loaded in the same gel. Representative blot from 2 other experiments done with different donors (n=3). [#]means the highest value.

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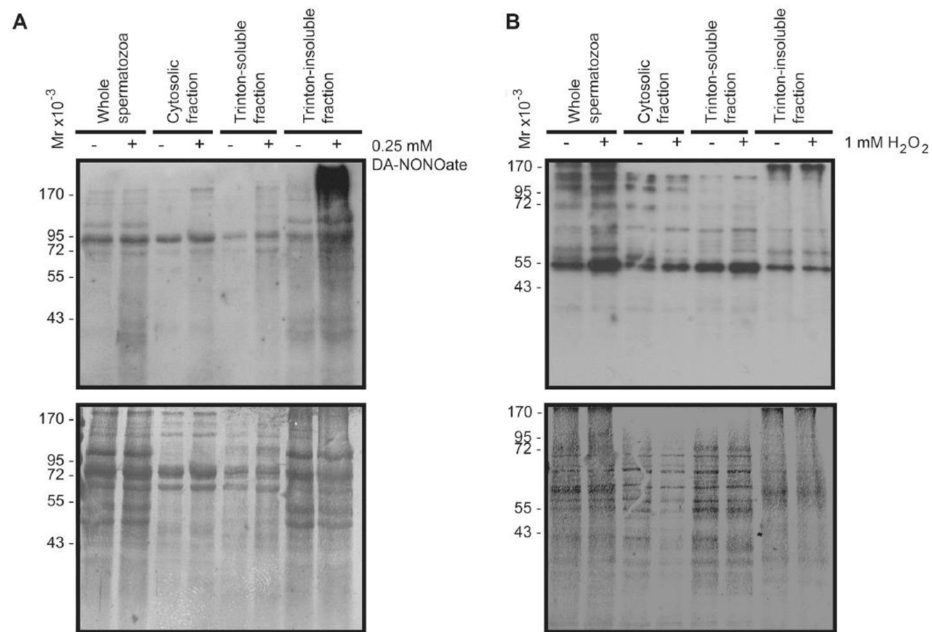

Figure 7. Tyrosine nitration and S-glutathionylation are differentially localized within the sperm cell, and most abundantly found in Triton-insoluble proteins of the tail and fibrous sheath

A) Sperm proteins treated with 0.25mM of DA-NONOate under reducing conditions, and immunoblotted with an anti-nitro tyrosine antibody (upper panel; 0.5×10^6 cells were loaded for the whole and cytosolic lanes, while 1 $x10^6$ cells were loaded for the triton-soluble and insoluble lanes). B) Sperm proteins treated with 1mM H_2O_2 under non-reducing conditions, and immunoblotted with anti-GSS-R antibody (upper panel; 0.5×10^6 cells were loaded for the whole and cytosolic lanes, while 1 $x10^6$ cells were loaded for the triton-soluble and insoluble lanes). Lower panel in A and B represent the respective membrane stained with colloidal gold as described in material methods. Representative blots from 3 other experiments done with different donors (n=4).

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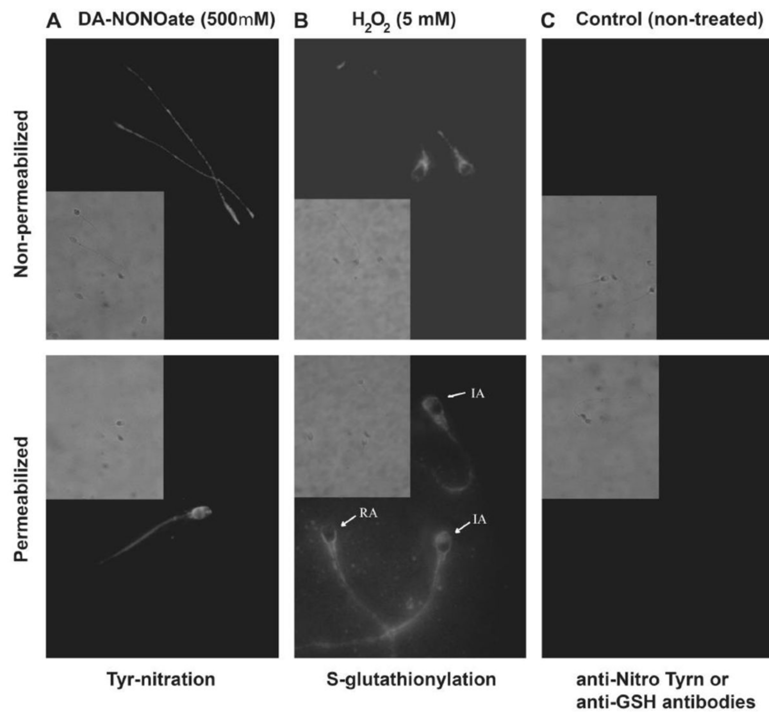

Figure 8. S-glutathionylated proteins were differentially localized within the sperm cell and preferentially found within the Triton-soluble and cytosolic fractions

Immunocytochemistry images were taken with fluorescence and phase contrast microscopy at 1000x magnification for sperm treated with 500μM DA-NONOate (A) or with 5mM 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).