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ORIGINAL ARTICLE

Sperm Biology

In vivo oxidative stress alters thiol redox status of peroxiredoxin 1 and 6 and impairs rat sperm quality

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Oxidative stress, the imbalance between the production of reactive oxygen species (ROS) and antioxidant activity is a major culprit of male infertility. Peroxiredoxins (PRDXs) are major antioxidant enzymes of mammalian spermatozoa and are thiol oxidized and inactivated by ROS in a dose-dependent manner. Their deficiency and/or inactivation have been associated with men infertility. The aim of this study was to elucidate the impact of oxidative stress, generated by the *in vivo* tert-butyl hydroperoxide (tert-BHP) treatment on rat epididymal spermatozoa during their maturation process. Adult Sprague-Dawley males were treated with 300 μmoles tert-BHP/kg or saline (control) per day intraperitoneal for 15 days. Lipid peroxidation (2-thibarbituric acid reactive substances assay), total amount and thiol oxidation of PRDXs along with the total amount of superoxide dismutase (SOD), motility and DNA oxidation (8-hydroxy-deoxyguanosine) were determined in epididymal spermatozoa. Total amount of PRDXs and catalase and thiol oxidation of PRDXs were determined in caput and cauda epididymis. While animals were not affected by treatment, their epididymal spermatozoa have decreased motility, increased levels of DNA oxidation and lipid peroxidation along with increased PRDXs (and not SOD) amounts. Moreover, sperm PRDXs were highly thiol oxidized. There was a differential regulation in the expression of PRDX1 and PRDX6 in the epididymis that suggests a segment-specific role for PRDXs. In conclusion, PRDXs are increased in epididymal spermatozoa in an attempt to fight against the oxidative stress generated by tert-BHP in the epididymis. These findings highlight the role of PRDXs in the protection of sperm function and DNA integrity during epididymal maturation.
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Keywords: antioxidant enzymes; DNA oxidation; epididymis; reactive oxygen species; spermatozoa; thiol oxidation

INTRODUCTION

Infertility is a medical condition that affects 16% of couples worldwide, and the cause in approximately half the cases can be found in men.[1] While the exact cause in around half of these cases is unknown, 30%–80% of infertile men have been found to have high levels of reactive oxygen species (ROS) in their semen.[2,3] ROS, such as hydrogen peroxide (H_2O_2), nitric oxide, hydroxyl radical, superoxide anion, and peroxynitrite ($ONOO^-$), which can arise in regular cellular metabolism, are controlled through the body's antioxidant defense systems.[4] A failure in these systems or an overload of ROS promote an oxidative stress that causes permanent damage to spermatozoa, causing low motility and mitochondrial malfunction leading to infertility or unviable spermatozoa.[5,6]

Peroxiredoxins (PRDXs) are 20–31 kDa enzymes that contain one or two cysteine (Cys) residues in their active site.[7,8] There are six isoforms divided into three groups: the 2-Cys PRDXs (PRDX1 to 4), the atypical 2-Cys PRDX (PRDX5) and the 1-Cys PRDX (PRDX6). These proteins are involved in the antioxidant protection and serve to regulate redox homeostasis.[7–10] The six members of the family are present in human spermatozoa and react with H_2O_2 and $ONOO^-$.[11,12] They are highly sensitive to oxidative stress becoming thiol oxidized and therefore inactivated by ROS in a dose-dependent manner.[11] The low amount and the high thiol oxidation levels of PRDX1 and PRDX6 have been associated with men infertility.[13] Recently, it has been demonstrated that $Prdx6^{-/-}$ males have spermatozoa with reduced motility and abnormal sperm chromatin (high oxidation and fragmentation of DNA and low DNA compaction) and these abnormalities are augmented as the male age.[14] The absence of PRDX4 promotes testicular atrophy with increased apoptosis during spermatogenesis and sperm DNA damage in mice.[15]

After the spermatozoa are produced in the testis through the process of spermatogenesis, they must transit through the epididymis to undergo epididymal maturation.[16,17] The duration of the sperm transit varies among species and in the rat occurs between 8 and 15 days in the rat promoting changes in motility and morphology in the spermatozoa so that it may become viable for fertilization.[16,18]

In this study, we studied the impact of an *in vivo* oxidative stress with tert-butyl hydroperoxide (tert-BHP) on epididymal spermatozoa during their maturation process.

MATERIALS AND METHODS

Materials

tert-butyl hydroperoxide (tert-BHP), SDS, phosphotungstic acid, buthylated hydroxytoluene, 2-thiobarbituric acid and malonaldehyde bis(dimethyl acetal), the Bicinchoninic protein determination assay and the anti-α-tubulin were purchased from Sigma-Aldrich Chemical

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Co. (St. Louis, MO, USA). The following were purchased from Abcam Inc., (Cambridge, MA, USA): rabbit polyclonal anti-PRDX1, monoclonal anti-PRDX4, monoclonal anti-PRDX6, the antigenic peptide used to raise the anti-PRDX1 antibody and 8-hydroxy-deoxyguanosine (8-OHdG). The anti-8-OHdG antibody was purchased from StressMarq Biosciences Inc., (Victoria, BC, Canada). Biotinylated horse anti-mouse antibody and Horse Serum were purchased from Vector Labs. Alexa-555 fluor streptavidin (1 mg ml^{-1} in H_2O) and ProLong Gold antifade with DAPI were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). Nitrocellulose (0.22 μm pore size; Osmonics Inc., MN, USA), donkey anti-rabbit IgG and goat anti-mouse IgG, both conjugated to horseradish peroxidase (Cedarlane Laboratories Ltd., Hornby, ON, Canada), an enhanced chemiluminescence kit (Lumi-Light; Roche Molecular Biochemicals, Laval, QC, Canada) and radiographic films (Fuji, Minamiashigara, Japan) were also used for immunodetection of blotted proteins. Other chemicals used were of at least reagent grade.

Animals and treatment

Adult male Sprague-Dawley rats (300–350 g) were treated with 300 μmoles tert-BHP/kg or saline (control) once a day intraperitoneally for 15 days. Treatment with tert-BHP showed to have no effects on the health of rats.[19] Twenty-four hours after the end of treatment, the rats were euthanized and reproductive organs and cauda epididymal spermatozoa were collected. After weighted, organs were kept at −80°C until further use. Cauda epididymes were placed in phosphate-buffered saline (PBS) (1 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} Na_2HPO_4, 137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, pH 7.4) and cut 1 time in the based with a surgical blade to allow spermatozoa to swim-out for 10 min at 37°C. Sperm motility was assessed by the same observer (CO) using the Olympus BH-2 microscope at 100 magnification with a thermal plate at 37°C. Sperm production was determined by counting spermatid heads in an aliquot from each testis homogenate using a hemocytometer. Briefly, a weighed portion of the decapsulated right testis was homogenized in 5 ml of 0.9% NaCl and 0.5% Triton X-100 with a glass homogenizer. All procedures were carried out in accordance with the regulations of the Canadian Council for Animal Care (CACC) and were approved by the Animal Care Committees of McGill University and the McGill University Health Centre.

2-thiobarbituric acid reactive substances

The level of Levels 2-thiobarbituric acid-reactive substances (TBARS) as a measurement of lipid peroxidation were determined in spermatozoa after tert-BHP treatment by spectrofluorometry using a microplate reader (Fluostar Optima; BMG Labtech, Durham, North Carolina) as done before.[11] The TBARS assay measures malondialdehyde (MDA) and other aldehydes that are predominantly generated from lipid hydroperoxides under acidic and high temperature (100°C) conditions. MDA, generated by the acid hydrolysis of malonaldehyde bis(dimethyl acetal), was used as standard,[20] and the values presented as nmol TBARS/10^6 spermatozoa.

Sperm DNA oxidation

Levels of 8-OHdG were determined by immunocytochemistry as previously done with modifications.[14] Briefly, sperm samples were centrifuged at 2000 *g* for 5 min to remove the PBS medium and resuspended in 20 mM phosphate buffer (pH 6.0) with 1 mM EDTA for 5 min. Samples were then centrifuged and resuspended in 50 mM Tris-HCl (pH 7.4), 1% SDS and 40 mmol l^{-1} dithiothreitol for 30 min. A final centrifugation to replace the mixture with PBS was performed. The sperm PBS solution was smeared on Superfrost Plus slides (Fischer Scientific, Ottawa, ON, Canada). Smears were fixated with 100% methanol at −20°C for 30 min. Slides were then incubated with 5% horse serum for 30 min, washed with PBS-T and incubated with anti-8-OHdG antibody (1:100) (SMC-155D, StressMarq Biosciences Inc., (Victoria, BC, Canada) diluted in 1% horse serum overnight at 4°C. After a wash with PBS, the samples were incubated with biotinylated horse anti-mouse antibody in 1% BSA and PBS-T for 1 h, washed and finally incubated with Alexa Fluor 555-streptavidin (1:500 in PBS) for 45 min at 20°C. ProLong Gold antifade with DAPI was added and smears sealed. Slides were analyzed with Zeiss Axiophot fluorescence microscopy (Carl Zeiss, Oberkochen, Germany). Positive control was generated by incubating spermatozoa with 2 mM H_2O_2 for 1 h at 20°C (**Figure 1d**). To test the specificity of the antibody, H_2O_2-treated spermatozoa were incubated with the primary antibody previously incubated with 8-hydroxy-guanosine (Abcam Inc., Toronto, ON, Canada) at a concentration 1000 times higher than the primary antibody for 1 h at 20°C.[21] At least 200 spermatozoa were counted as positive or negative for 8-OHdG labeling per sample.

Immunoblotting

Caput and cauda epididymis were homogenized in protease inhibitors containing RIPA buffer on ice with a glass potter. Samples were then sonicated for 20 s at 30% amplitude. Tissue homogenates were centrifuged at 21 000 *g* for 20 min at 4°C. Protein concentration was determined with the bicinchoninic protein determination assay as per manufacturer instructions. Caput and cauda epididymis and sperm samples were placed in electrophoresis sample buffer supplemented with 100 mM DTT, incubated at 95°C for 5 min, and then centrifuged at 21 000 *g* for 5 min. Sperm proteins present in the supernatant were then electrophoresed on 12% acrylamide gels and transferred to nitrocellulose membranes with associated transfer buffer (192 mmol l^{-1} glycine and 25 mmol l^{-1} Tris, pH 8.3). The membranes were incubated with a solution of skim milk (5%, *w/v*) in Tris (20 mM, pH 7.8)-buffered saline containing Tween 20 (0.1% (*v/v*); TTBS) for 30 min and incubated with anti-PRDX1 (1:1000), anti-PRDX4 (1:2000), anti-PRDX6 (1:10 000) or anti-superoxide dismutase 1 (SOD1; 1:2000) antibodies through overnight incubations. Secondary antibody blocking followed with anti-mouse or anti-rabbit IgG and the Lumi-Light chemiluminescence kit was used for detection. The equal loading was further confirmed by re-blotting the membrane with anti-tubulin antibody (1:10 000). Then, films with the same time of exposure were scanned using an Agfa SnapScan 1236 scanner (Agfa-Gevaert NV, Mortsel, Belgium) and the images were analyzed with the Un-Scan-It gel software version 5.1 (Silk Scientific Corporation, Orem, UT, USA) to obtain the intensity bands values. To compare the amounts of a given enzyme (PRDXs, SOD, CAT) band in control and treated samples, the band intensities were normalized to that of tubulin of each sample (ratio PRDX: tubulin band intensity). The thiol oxidation ratio of PRDXs was calculated by the intensity ratio of a given PRDX band under nonreducing (the thiol-oxidized PRDX) divided by that under reducing (representing total PRDX) conditions. The thiol oxidation ratio is an indication of the redox status of the cysteine residues present in the active site of PRDXs. In order to compared band intensities between control and treated groups, the value of enzyme: tubulin band intensity and of thiol oxidation ratio of PRDXs were normalized to 1 corresponding to the mean of relative intensity or thiol-oxidation ratio of control samples.

Statistical analysis

All graphical data are represented as the mean ± s.e.m.; statistical differences between group means were determined using *t*-test or Mann–Whitney test as appropriate using Sigma Systat 13 (Systat

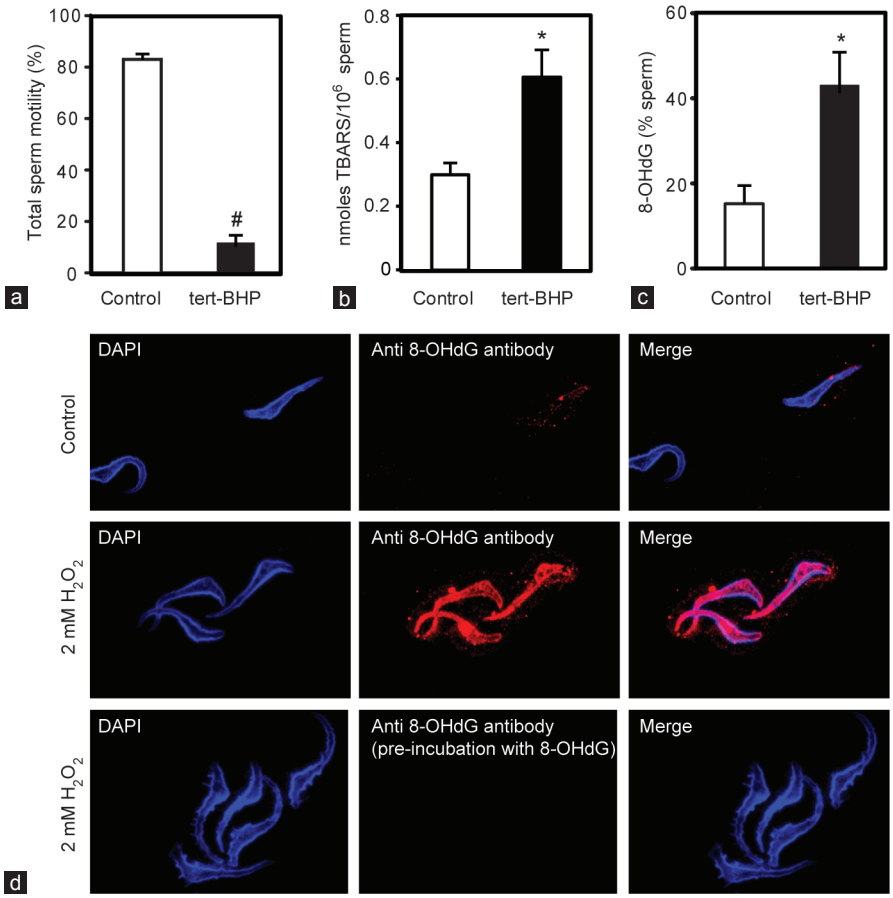

Figure 1: Reduced sperm motility and oxidative stress markers in tert-BHP-treated compared to controls rats. (**a**) Sperm motility, (**b**) lipid peroxidation observed in units of nmoles of TBARS and (**c**) sperm DNA oxidation expressed as percentage of cells showing strong 8-OHdG labeling. Results are expressed as mean ± s.e.m. #and *mean lower or higher than controls ($P < 0.05$), respectively (Mann–Whitney test, $n = 6$). (**d**) Rat spermatozoa nuclei showing 8-OHdG labeling. Spermatozoa were incubated without (control) or with 2 mmol l^{-1} hydrogen peroxide (H_2O_2) for 1 h at 20°C. Specificity of the antibody was demonstrated by incubating H_2O_2-treated samples with antibody previously incubated with 8-OHdG as described in material and methods.

software Inc., San Jose, CA, USA). Difference among samples was considered to be significant when the $P < 0.05$.

RESULTS

In vivo oxidative stress impairs epididymal sperm quality

The tert-BHP treatment during 2 weeks affected the spermatozoa during their transit through the epididymis. Twenty-four hours after treatment, epididymal spermatozoa showed a significant decrease in sperm motility in treated compared to controls rats (**Figure 1a**). Spermatozoa from treated rats showed increased levels of oxidative stress markers; indeed, high levels of TBARS, indicating lipid peroxidation (**Figure 1b**) and of 8-OHdG, marker of DNA oxidation were found in spermatozoa from treated compared to controls rats (**Figure 1c** and **1d**).

Organs collected at the same time point as spermatozoa did not show major changes; testis, epididymis, ventral prostate, seminal vesicles, and coagulating glands weights remain unchanged and no significant change in sperm production by the testis (**Table 1**).

PRDXs content and thiol oxidation increase in cauda epididymal spermatozoa due to oxidative stress

Rat spermatozoa contain PRDX1, PRDX4 and PRDX6 (**Figure 2**). The anti-PRDX1 antibody recognized PRDX1 (21 kDa) but also other bands (27, 54, 72 and 94 kDa). All these bands were not recognized by the primary antibody previously treated with the antigenic peptide, thus demonstrating the specificity of the antibody. The two isoforms of PRDX4 (27 and 31 kDa) present in the testis[22] and PRDX6 (26 kDa) were found in spermatozoa.

Spermatozoa from treated rats showed a significant increase only in the amount of PRDX1 (21 kDa) and PRDX6, and a trend of increase in the amount of PRDX1 (54 kDa) and PRDX4 compared to controls (**Figure 3**). Contrary to the increase in PRDXs amounts, the content of SOD1 (a marker of residual cytoplasm in spermatozoa) was unchanged in tert-BHP-treated animals compared to controls (**Figure 4**).

When sperm samples were electrophoresed under nonreducing conditions (in the absence of DTT) (**Figure 5**), a set of bands at 34–43 kDa and 54 kDa were recognized with the anti-PRDX1 antibody. Only 34–43 kDa PRDX1 bands showed increased levels of thiol oxidation in spermatozoa from treated rats compared to controls (**Figure 5a**). We also observed increased levels of thiol oxidation also in PRDX6 in spermatozoa from the treated groups compared to controls (**Figure 5b**). The thiol oxidation signal of PRDX4 was very weak due to low amount of this enzyme in rat spermatozoa, (data not shown). Moreover, we observed increased levels in the thiol oxidation ratio of PRDX1 and PRDX6 in spermatozoa from treated animals, indicating that the oxidative stress generated promoted oxidation and therefore inactivation of these enzymes (**Figure 5c**).

PRDXs are differentially expressed in caput and cauda epididymis

We analyzed the levels of PRDXs in caput and cauda epididymal tissues; an upregulation of PRDX 1 expression and downregulation of PRDX6 expression in both the caput and upregulation PRDX 6 expression in the cauda were observed in treated rats compared to controls (**Figure 6**). Noteworthy, the levels of thiol oxidized PRDX6 were higher in the cauda epididymis of treated rats compared to Controls. We did not observe changes in the expression of catalase in caput or cauda epididymis (**Figure 7**).

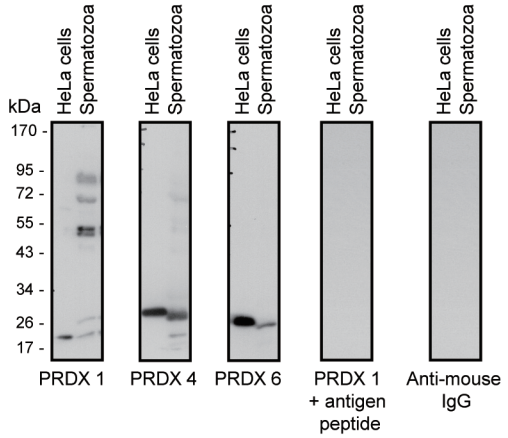

Figure 2: Expression of PRDXs in rat spermatozoa. Representative blots of rat sperm proteins under reducing conditions ($n = 4$); 0.1×10^6, 0.4×10^6 or 0.1×10^6 spermatozoa were loaded in each well. HeLa cells solubilized in electrophoresis sample buffer were used as positive control. To test the specificity, 0.4 μg ml^{-1} of anti-PRDX1 antibody were incubated with 2 μg ml^{-1} of its antigenic peptide in TBS-T supplemented with 3% BSA for 2 h at room temperature. The anti-mouse IgG antibody alone did not recognize any protein bands.

DISCUSSION

The present study illustrates the effects of *in vivo* oxidative stress on expression and thiol oxidation levels of PRDXs along with a decrease of sperm quality. We demonstrated for the first time that cauda epididymal spermatozoa displayed low motility and high levels of DNA oxidation along with increased amounts and of levels of thiol oxidation of sperm PRDXs in rats exposed to tert-BHP, suggesting an attempt by this organ to protect spermatozoa against high levels of ROS during their maturation.

Due to the 2 weeks duration of the tert-BHP treatment, the entire spermatozoa maturation through epididymal transit (8–15 days in the rat)[16,18] was affected and samples obtained 24 h thereafter display the effects of oxidative stress on fully formed but not yet mature spermatozoa. Indeed, cauda epididymis spermatozoa collected at that time point allow us to evaluate changes and possible damages occurring during epididymal maturation. The poor motility and high levels of lipid peroxidation and of DNA oxidation displayed by spermatozoa from treated rats compared to the unaffected controls along with no changes in epididymes weight suggest problems in the functionality of the epididymal epithelium rather than a systemic effect due to the treatment. Moreover, the inactivation of PRDXs by

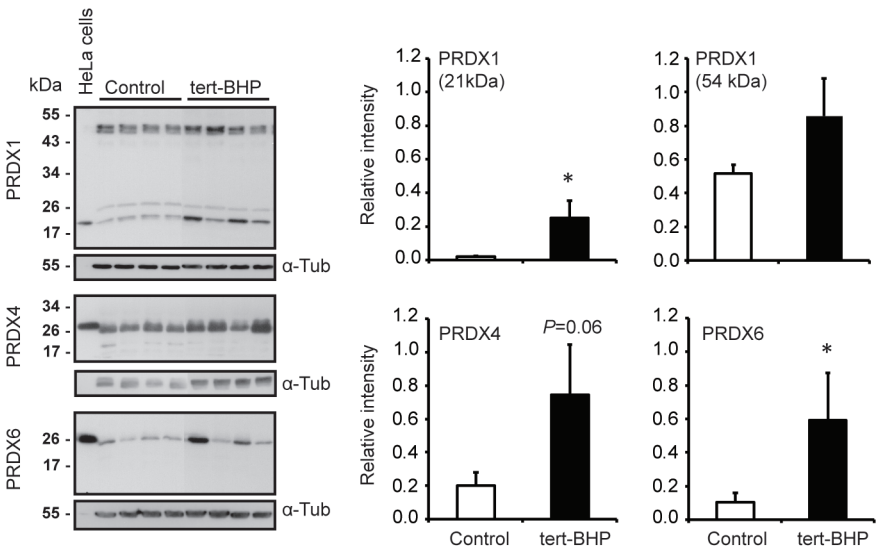

Figure 3: Increased total amount and relative intensity of PRDX1 and PRDX6 in spermatozoa from treated rats. On the left, representative immunoblots of sperm proteins under reducing conditions (sample buffer with 100 mmol l^{-1} DTT); 0.1×10^6, 0.4×10^6 or 0.1×10^6 spermatozoa were loaded in each well for PRDX1, PRDX4 and PRDX6, respectively. The loading control was done by re-blotting each membrane with an anti-α-tubulin (α-Tub) antibody. HeLa cells solubilized in electrophoresis sample buffer were used as positive control. On the right, relative intensities of PRDX1 (23 and 54 kDa), PRDX4 (27 kDa) and PRDX6 (26 kDa). PRDXs band Intensities were normalized to that of α-tubulin (α-Tub). Lanes correspond to the same gel. *$P < 0.05$ (*t*-test, $n = 6$).

Table 1: Body and reproductive organs weight and sperm production

	Body weight (g)	*Testis (g)*	*Epididymis (g)*	*Ventral prostate (g)*	*Seminal vesicles (g)*	*Coagulating glands (g)*	*Sperm production (×10^6 spermatids/g testis)*
Control	441.67±15.34	1.79±0.06	0.58±0.02	0.53±0.04	1.0±0.10	0.12±0.02	231.58±38.05
tert-BHP	432.4±12.57	1.77±0.05	0.56±0.02	0.47±0.07	0.75±0.1	0.10±0.01	223.84±14.48

Body and organ weights were not different between the groups (n=6, Mann–Whitney test, P>0.05). tert-BHP: tert-buthyl hydroperoxide

thiol oxidation indicates a failure of these antioxidant enzymes in the protection of membranes and DNA against oxidative stress. PRDX6 is critical to repair peroxidized phospholipids in cell membranes[23,24] and to avoid DNA oxidation in spermatozoa.[14] Murine embryonic fibroblasts derived from Prdx1 knockout mice had higher levels of DNA oxidation compared to wild type controls.[25] Sperm motility was reduced by tert-BHP treatment and this is in part due to increased lipid peroxidation of sperm membranes that has been associated with loss of motility[26–28] because of PRDX6 inactivation by ROS.[23]

The tert-BHP established an *in vivo* oxidative stress altering the epididymal environment and thus affecting direct or indirectly the maturation of sperm cells. The thiol oxidation of sperm PRDX1 and PRDX6 along with the increased TBARS and 8-OHdG levels and the thiol oxidation of PRDX6 in the cauda epididymis account for the ongoing oxidative stress in the epididymis. It is known that increased levels of ROS in spermatozoa have detrimental effects on motility and their ability to undergo capacitation and acrosome reaction, both processes needed to fertilize oocytes.[29,30] Indeed, redox-dependent modifications such as *S*-glutationylation of sperm proteins are promoted by oxidative stress and are associated with loss of motility and fertilizing ability.[27] The thiol oxidation of PRDXs promotes the impairment of the antioxidant system promoting higher levels of ROS within the spermatozoon and thus generating more damage.[31] Although other antioxidant enzymes play a protective role in mammalian spermatozoa such as glutathione peroxidases, glutathione S-transferases, thioredoxins, etc., the reduction in motility and the increased levels of oxidative stress markers (TBARS and 8-OHdG) clearly demonstrate that the system is override with the ultimate outcome of oxidative stress-dependent damage in these cells.

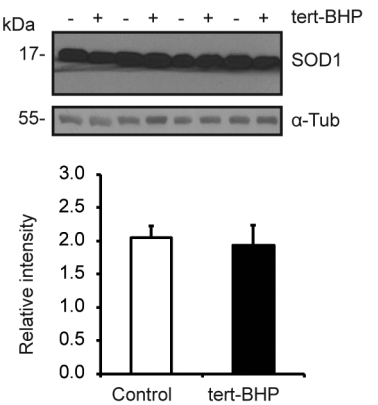

Figure 4: SOD1 is unchanged in spermatozoa from control or tert-BHP-treated rats. On the left, a representative immunoblotting of sperm proteins under reducing conditions; 0.2 × 10^6 spermatozoa were loaded in each well for SOD1. Membrane was re-blotted with anti-α-Tubulin (loading control). On the right, relative intensity of SOD1 normalized to that of α-tubulin (α-Tub); $n = 6$.

The observation that the quantity of the PRDX1 and PRDX6 (and the trend of increase of PRDX4) in spermatozoa from treated rats was higher than in those from controls males suggests that the oxidative stress triggered mechanisms in an attempt to react against the high levels of ROS in the epididymal lumen in order to protect spermatozoa when transit through the convoluted organ to complete their maturation. Without the ability to synthetize proteins on their own, the increased amount of PRDXs found in spermatozoa of treated rats can be explained by two phenomena: (1) the disruption of mechanisms of reabsorption of sperm cytoplasm by the epididymal epithelium and (2) an active supply of PRDXs form the epithelium to the maturing spermatozoa. Since SOD1 quantities did not change due to the tert-BHP treatment, we can conclude that the reabsorption of residual cytoplasm is little or not compromised and thus an active transfer of PRDXs from the epididymal epithelium to the maturing spermatozoa may occur. Although we did not probe a direct transfer of PRDXs to spermatozoa from treated rats, previous evidence strongly suggest this possibility; epididymosomes from bull and humans contain

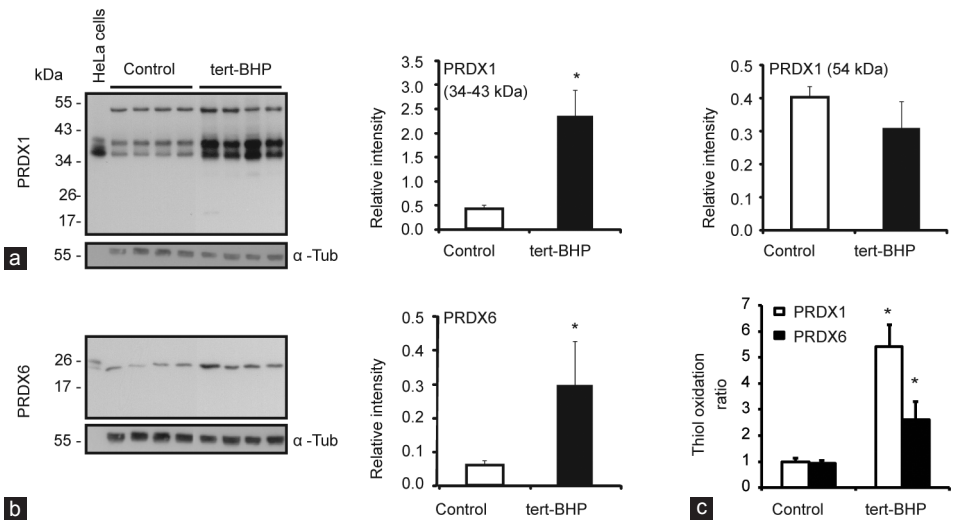

Figure 5: Thiol oxidation levels of PRDX1 and PRDX6 are increased in spermatozoa from treated rats. On the left, representative immunoblots of sperm proteins under nonreducing conditions (sample buffer without DTT) showing PRDX1 (**a**) and PRDX6 (**b**) labeling. 0.1, 0.4 or 0.1 × 10^6 spermatozoa were loaded in each well for PRDX1, PRDX4 and PRDX6, respectively. The loading control was done by re-blotting each membrane with an anti-α-tubulin (α-Tub) antibody. HeLa cells solubilized in electrophoresis sample buffer were used as positive control. On the right, relative intensities of thiol oxidized PRDX1 and PRDX6. PRDXs band intensities were normalized to that of α-tubulin (α-Tub). (**c**) Thiol oxidation ratio of PRDX1 and PRDX6. The thiol oxidation ratio was obtained by dividing the relative intensities of thiol-oxidized PRDXs (obtained from samples under nonreducing conditions) by those of reduced PRDX (total, obtained from samples under reducing conditions). Then, values were normalized to 1 corresponding to the mean thiol-oxidation ratio of that of controls. *$P < 0.05$ (*t*-test, $n = 6$).

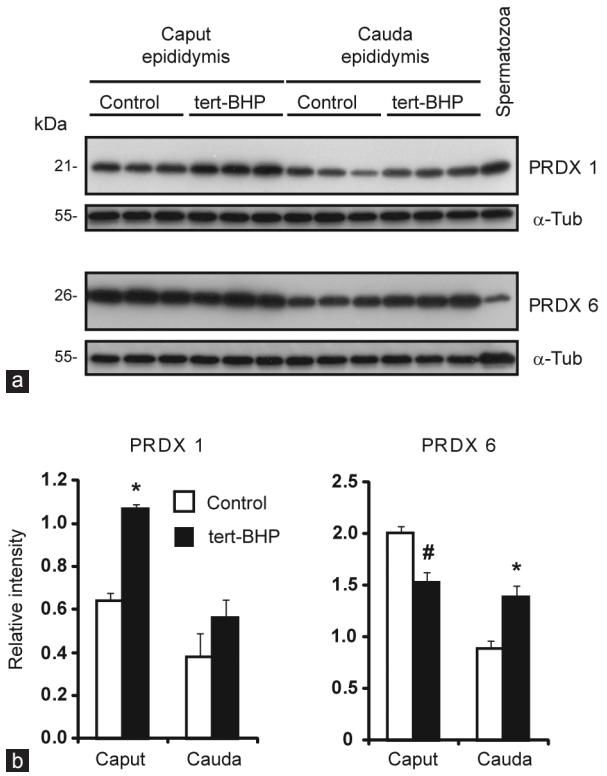

Figure 6: PRDX1 and PRDX6 are differentially expressed in rat epididymis after tert-BHP treatment. (**a**) Representative immunoblot of caput and cauda epididymis proteins (10 μg protein/lane). Loading controls were done re-blotting each membrane with anti-α-tubulin (α-Tub). 0.1×10^6 spermatozoa were loaded for PRDX1 and PRDX6 as positive controls. (**b**) Relative intensities of PRDX1 and PRDX6 normalized to that of α-Tubulin *and #mean higher or lower than controls ($P < 0.05$), respectively (*t*-test, $n = 6$).

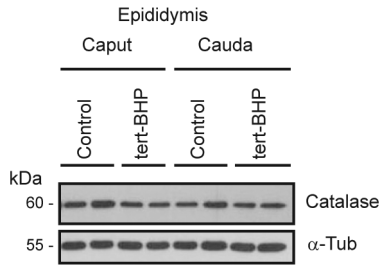

Figure 7: Catalase expression is not changed in epididymis due to tert-BHP treatment. Representative immunoblot of caput and cauda epididymis proteins (10 μg protein per lane). Loading controls were done re-blotting the membrane with anti-α-tubulin (α-Tub), $n = 3$.

PRDXs[32,33] and others already demonstrated that proteins can be transferred from epididymosomes to spermatozoa.[34–37]

It is also possible that other antioxidant enzymes may be transferred by epididymosomes, along with PRDXs; these organelles also carry SOD1, thioredoxins, glutathione peroxidase 5 and s-glutathione transferases.[32-35,37] However, this transfer seems to be well regulated since SOD1 amounts were not augmented in spermatozoa from treated rats. In any case, the high levels of thiol oxidation of PRDXs along with the high levels of lipid peroxidation and of sperm DNA oxidation clearly demonstrate that epididymal spermatozoa were damaged by the tert-BHP-dependent oxidative stress and, although attempts to increase the amount of PRDXs (and possible other antioxidant enzymes) in the sperm cells, the outcome result in a significant decrease of sperm quality. These results are in accordance on previous studies showing that high levels of thiol oxidized PRDXs are found in spermatozoa from infertile patients.[13] Moreover, the absence of PRDX6 is associated with impairment of mouse sperm motility and abnormal sperm chromatin quality and subfertility, and these abnormal outcomes worsen with age.[14] A possible explanation of the phenotypes observed in these three species is that when spermatozoa face a strong and continuous oxidative stress, the antioxidant system, particularly PRDXs, are overwhelmed and thus incapable of fight against the high levels of ROS produced.[31]

The epididymal epithelium reacted upon the oxidative stress generated by the tert-BHP treatment; certainly, increases of PRDX1 in the caput and of PRDX6 in the cauda were observed in treated rats. These segment specific increases of PRDXs highlight a differential role of PRDXs in the epididymis. PRDX6 was highly thiol oxidized in the cauda epididymis of treated rats, thus suggesting that although there was an attempt of the epididymis to respond to the oxidative stress by increasing the amount of the protein in this segment, the high levels of ROS oxidized the cysteine residue in the active site and therefore inactivated PRDX6. Considering that the amount of PRDX1 was similar in the cauda epididymis of treated compared to that of controls rats and that PRDX6 is thiol oxidized and therefore inactivated, we can conclude a major role of PRDX6 in the protection of both the epithelium and the spermatozoa in the cauda segment. We recently reported that mice lacking PRDX6 have abnormal spermatozoa showing low motility, sperm DNA fragmentation and oxidation and a low DNA compaction.[14] Moreover, PRDX6 was lower and highly oxidized in spermatozoa from infertile patients with varicocele or unknown cause of infertility.[13] Altogether, these findings stress the importance of PRDX6 in the assurance of sperm quality in mammals.

The effects of oxidative stress on the epididymis and spermatozoa observed in this study suggest a shut-down of the PRDX system during the time that spermatozoa enters into the epididymis until they reached the cauda epididymis awaiting for ejaculation. We recently suggested that the PRDX system is delicate and depends on the supporting activity of the thioredoxin-thioredoxin reductase system (for PRDX1-5) and glutathione-S-tranferases (for PRDX6), systems that rely on sufficient amount of NADPH and of reduced glutathione, to assure the recycling of PRDXs to fight against high levels of ROS.[38]

CONCLUSION

Tert-BHP dependent oxidative stress impact on the epididymis promoting a differential expression of PRDXs in different segments of the organ and the increase in the PRDXs content in spermatozoa. However, this response is insufficient since PRDXs become highly oxidized (displaying oxidation of the thiol groups in their active site) and unable to protect spermatozoa against the oxidative stress generated by the treatment. These findings are observed also in spermatozoa of infertile men. Altogether, these findings highlight a role of PRDXs in the antioxidant protection of sperm function.

AUTHOR CONTRIBUTIONS

YL carried out the determination of sperm DNA oxidation and determination of SOD expression in spermatozoa, participated in the analysis of the results and wrote the draft of the manuscript. CO conceived of the study, determined expression and thiol oxidation of PRDXs in rat spermatozoa, sperm motility, participated in the analysis of the results and wrote the final version of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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