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Source: Biology of Reproduction, 94(3)

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.115.137646

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BIOLOGY OF REPRODUCTION (2016) **94**(3):68, 1–10
Published online before print 20 January 2016.
DOI 10.1095/biolreprod.115.137646

Absence of Peroxiredoxin 6 Amplifies the Effect of Oxidant Stress on Mobility and SCSA/CMA3 Defined Chromatin Quality and Impairs Fertilizing Ability of Mouse Spermatozoa[1]

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ABSTRACT

Oxidative stress, the imbalance between reactive oxygen species production and antioxidant defenses, is associated with male infertility. Peroxiredoxins (PRDXs) are antioxidant enzymes with a wide distribution in spermatozoa. PRDX6 is highly abundant and located in all subcellular compartments of the spermatozoon. Infertile men have lower levels of sperm PRDX6 associated with low sperm motility and high DNA damage. In order to better understand the role of PRDX6 in male reproduction, the aim of this study was to elucidate the impact of the lack of PRDX6 on male mouse fertility. Spermatozoa lacking PRDX6 showed significantly increased levels of cellular oxidative damage evidenced by high levels of lipid peroxidation, 8-hydroxy-deoxyguanosine (DNA oxidation), and protein oxidation (S-glutathionylation and carbonylation), lower sperm chromatin quality (high DNA fragmentation and low DNA compaction, due to low levels of protamination and a high percentage of free thiols), along with decreased sperm motility and impairment of capacitation as compared with wild-type (WT) spermatozoa. These manifestations of damage are exacerbated by tert-butyl hydroperoxide treatment in vivo. While WT males partially recovered the quality of their spermatozoa (in terms of motility and sperm DNA integrity), *Prdx6*$^{-/-}$ males showed higher levels of sperm damage (lower motility and chromatin integrity) 6 mo after the end of treatment. In conclusion, *Prdx6*$^{-/-}$ males are more vulnerable to oxidative stress than WT males, resulting in impairment of sperm quality and ability to fertilize the oocyte, compatible with the subfertility phenotype observed in these knockout mice.

chromomycin A3, fertility, gamete biology, male infertility, oxidative stress, reactive oxygen species, sperm capacitation, sperm chromatin structure assay, sperm DNA fragmentation, sperm function

INTRODUCTION

Excessive levels of reactive oxygen species (ROS) in spermatozoa are associated with infertility [1–4]. Oxidative stress is the result of an excessive production of ROS and/or a decrease in the antioxidant defense system [5, 6], and may cause serious cell injury and even cell death [6, 7]. Oxidative stress in spermatozoa targets all cell components, decreasing sperm motility and mitochondrial activity [8, 9].

The frequency of infertility in the human population has been increasing over the past few decades. However, treatment efficacy is poor, because the underlying causes are unknown in 40%–50% of cases [10]. Oxidative stress is a common feature of factors, such as environmental pollutants, chemicals, drugs, smoke, toxins, radiation, and diseases related to infertility [11–22]. In such conditions, vital cell components (proteins, lipids, and DNA) are oxidized, compromising cell function and survival [6, 7]. ROS-mediated damage to sperm is a significant contributing factor in 30%–80% of infertile men [1–4, 23]. In these cases, the antioxidant system present in semen [24, 25] is not sufficient to protect sperm from ROS-dependent damage, such as peroxidation of membrane lipids [26], oxidation of proteins [27], DNA fragmentation and oxidation of bases [28, 29], low mitochondrial membrane potential [30, 31], and inactivation of enzymes associated with motility [32, 33].

Peroxiredoxins (PRDXs) are ancestral thiol-dependent, selenium- and heme-free peroxidases that are highly expressed in almost all living species [34, 35]. They have either one (PRDX6) or two (PRDX1-5) cysteine (Cys) residues that participate in the redox cycle and are essential for their activity [36]. In contrast to glutathione peroxidases (GPXs), PRDXs do not require metals ions for their activity [37–39]. They can reduce both organic and inorganic hydroperoxides [40] and peroxynitrite [41, 42] by coupling with either the thioredoxin/thioredoxin reductase system (PRDX1-5) [43, 44] or the glutathione/glutathione reductase system mediated by glutathione S-transferase (PRDX6) [45–47]. They have a wide subcellular distribution (cytosol, nucleus, mitochondria, endoplasmic reticulum, and plasma membrane) facilitating regulation of ROS action [48–51]. In human spermatozoa, PRDXs

[1]Supported by the Canadian Institutes of Health Research grant MOP 133661 and the Fonds de Recherché en Santé Québec grant 22151 to C.O. B.O. was funded by a studentship from the Reseau Quebecois en Reproduction, Fonds de recherche du Québec - Nature et Technologies. Support for the generation of *Prdx6*$^{-/-}$ mice was provided by the National Heart, Lung, and Blood Institute of the National Institutes of Health.
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Received: 14 December 2015.
First decision: 8 January 2016.
Accepted: 15 January 2016.
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eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

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are highly reactive with H_2O_2 and other ROS (i.e., tert-butyl hydroperoxide [tert-BHP], peroxynitrite), and are differentially located in specific subcellular sperm compartments [52–54]. We recently reported that infertile men have lower quantities of PRDXs in seminal plasma and spermatozoa than healthy donors [55]. The total quantity of PRDX1 and PRDX6, but not PRDX4 and PRDX5, is lower in spermatozoa from varicocele patients (prior to surgery) and in men with idiopathic infertility than healthy donors [55]. It is noteworthy that thiol-oxidized PRDX1, PRDX5, and PRDX6 levels were higher in spermatozoa from men with idiopathic infertility than those from donors [55]. Due to the lower amount of total PRDX1 and PRDX6 and the high thiol oxidation of these PRDXs, very little (less than 20%) of their antioxidant activity remains, and this is associated with impaired sperm function and poor DNA integrity [55]. Recently, we demonstrated that the absence of PRDX6 leads to abnormal mouse sperm quality that translates into a low number of pups, and that this abnormal reproductive phenotype is exacerbated as the male mice age [56].

In view of the great abundance of PRDX6 in normal semen, its implications for the protection against oxidative stress of human spermatozoa [53, 55], and its necessity to assure normal spermatozoa, as seen in our *Prdx6*$^{-/-}$ mouse model [56], the objective of this study was to evaluate whether the absence of PRDX6 has implications for the quality and fertilizing ability of spermatozoa of male mice lacking PRDX6 exposed to an in vivo oxidative stress.

MATERIALS AND METHODS

Materials

Acridine orange, chromomycin A3 (CMA3), tert-BHP, and 2,4-dinitrophenyl hydrazine (DNPH) were purchased from Sigma-Aldrich Co. (Milwaukee, WI). The anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody was purchased from StressMarq Biosciences Inc (Victoria, BC, Canada). Donkey anti-rabbit IgG and goat anti-mouse IgG antibodies (both conjugated with horseradish peroxidase) were provided by Cedarlane Laboratories Ltd. (Hornby, ON, Canada). Other chemicals used were of at least reagent grade.

Animals

The *Prdx6*$^{-/-}$ mouse model was generated by Dr. Ye Shih Ho (Wayne State University) in collaboration with the laboratory of Dr. Aron Fisher at the University of Pennsylvania [57]; the mice were subsequently backcrossed to the C57Bl/6 background with >99.9% genetic identity, as determined by microsatellite analysis performed by the Jackson Laboratory [58]. A colony of these mice was established at The Research Institute, McGill University Health Centre. We also breed C57Bl/6J wild-type (WT) mice, and males from this other colony of the same age were used as controls. Mice lacking PRDX6 developed normally. No differences were noted in the body weights between the knockout and WT animals at the same age (8 wk). Mice were maintained on a 14L:10D cycle and provided with food and water ad libitum. All procedures were carried out in accordance with the regulations of the Canadian Council for Animal Care and were approved by the Animal Care Committees of McGill University and the McGill University Health Centre.

Tert-BHP-Induced In Vivo Oxidative Stress

The 8-wk-old *Prdx6*$^{-/-}$ (n = 6) and WT (n = 6) males received an intraperitoneal injection of 60 μmol/100 g body weight/day tert-BHP or saline (controls) for 1 wk [59]. Treated WT and *Prdx6*$^{-/-}$ mice and their controls were observed twice each day during the treatment period. Mice were euthanized 24 h or 6 mo after the last injection by cervical dislocation under anesthesia. Body weights were recorded on the days of treatment and euthanasia. Immediately after euthanasia, testes, epididymis, seminal vesicle, and prostate organ weights were recorded.

Sperm Preparation and Determination of Sperm Motility

Sperm suspensions were obtained from the caudal region of the epididymis by poking with a 27-G needle five times and allowing them to disperse for 10 min at 37°C in 500 μl of PBS (pH 7.4).

Progressive and total motility were determined using computer-assisted sperm analysis system (Sperm Vision HR software v1.01; Penetrating Innovations, Ingersoll, ON, Canada) [56]. The percentage of cauda epididymal spermatozoa with cytoplasmic droplet was determined by microscopy at 400× magnification [56].

Redox-Mediated Protein Modifications

Sperm samples were supplemented with a sample buffer with (reducing conditions) or without (nonreducing conditions) 100 mM dithiothreitol and boiled for 5 min at 96°C. Sperm proteins were loaded on 12% polyacrylamide resolving gel, electrophoresed, and electrotransferred using a buffer containing 192 mM glycine, 25 mM Tris, and 20% methanol (pH 8.3) onto nitrocellulose membranes. The S-glutathionylation levels were determined by immunoblotting under nonreducing conditions with an antioxidized glutathione (1:2000) antibody. Protein oxidation (formation of carbonyl groups) levels were determined by immunoblotting after the derivatization of sperm proteins with DNPH and using anti-DNP-KLH (1:2500) antibody. Staining of membranes with colloidal silver was used to determine equal loading in each well. Relative intensity semiquantitative analysis was performed using UnScanIt software v5.1 (Silk Scientific Corp., Orem, UT); each band's intensity was obtained and normalized to the respective intensity of the 250-kDa band present in the silver-stained membrane. The total value of all the normalized intensity bands was then obtained and again normalized with that of the control sample from nontreated WT males. 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 increase in intensities obtained in each sample.

Sperm DNA Oxidation

The oxidation of DNA has been used as a marker of oxidative stress in spermatozoa from different species, including the mouse [60–63]. The levels of 8-OHdG were determined by immunocytochemistry, as done previously [56]. Briefly, the sperm suspension was smeared onto superfrost plus slides (Fisher Scientific, Montreal, PQ, Canada), and allowed to air dry at room temperature. Dried cells were permeabilized with methanol as described previously [53]. Cells were rehydrated with PBS supplemented with Triton X-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-8-OHdG antibody (1:100). Cells were then washed and then incubated for 1 h at 20°C with biotinylated secondary antibody horse anti-mouse IgG (1:2000). Following this, streptavidin conjugated to Alexa Fluor 555 (1:500) was applied to slides. Smears were mounted with ProLong Antifade containing 1.0 ug/ml 4′,6-diamidin-2′-phenylindole dihydrochloride (Molecular Probes, Eugene, OR), and coverslipped. The positive controls were produced by incubating spermatozoa with 5 mM H_2O_2 and 1 mM $FeSO_4 \bullet 7H_2O$ (1 mM) in Biggers, Whitten, and Whittingham (BWW) medium composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, and 20 mM Hepes for 30 min at 37°C [56]. Negative controls were obtained by incubating smeared samples without the primary antibody. Fluorescent signals were examined under an epifluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany). All images were captured with a digital camera (Retiga 1300; QImaging, Burnaby, BC, Canada) and processed with Northern Eclipse digital imaging software, version 6.0 (Empix Imaging, Mississauga, ON, Canada). As demonstrated previously in rat spermatozoa [63], no distinct signals were seen when the primary antibody was omitted (negative control). A total of 200 cells per duplicate was counted per sample and results are presented as percent of cells with positive signal.

Lipid Peroxidation

Levels of lipid peroxidation were determined in spermatozoa after tert-BHP treatment by 2-thiobarbituric acid-reactive substances (TBARS) assay by spectrofluorometry using a microplate reader (Fluostar Optima; BMG Labtech, Durham, NC) [64]. Malondialdehyde, generated by the acid hydrolysis of malonaldehyde bis (dimethyl acetal), was used as standard (nmol TBARS/10^6 spermatozoa) [65].

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Evaluation of Sperm Chromatin Quality

Acridine orange/sperm chromatin structure assay. Altered chromatin structure measured by the susceptibility of sperm DNA to acid-induced denaturation at sites of existing DNA strand breaks [66] was assessed with the sperm chromatin structure assay (SCSA) [56, 67–69]. A minimum of 10 000 of AO-labeled spermatozoa were analyzed for each sample using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Inc., Auburn, CA) equipped with a 585/625-nm filter and a laser tuned to 488-nm line excitation. Raw data were processed using WinList Software (Verity Software, Topsham, ME). The extent of DNA denaturation was expressed as the percentage of DNA fragmentation (DFI) corresponding to the percentage of spermatozoa outside the main population, and sperm DNA compaction was assessed by calculating the high DNA stainability (HDS).

CMA3 labeling. The level of sperm chromatin packaging was determined on the basis of the accessibility of the fluorochrome, CMA3, to bind to protamine-free sites in the sperm DNA [70]. Thus, CMA3 labeling is an indirect indicator of the level of protamination in the sperm nucleus. As previously described [56], aliquots of epididymal spermatozoa (5×10^6 ml^{-1}) were incubated with 0.25 mg/ml CMA3 in McIlvaine buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 10 mM $MgCl_2$; pH 7.0) and incubated for 20 min at 25°C in the dark. A total of 10 000 cells was analyzed per sample per duplicate using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Inc.).

Reproductive outcomes. For the aim of investigating the effect of lacking PRDX6 on male reproductive performance in males challenged with oxidative stress, 24 h after the treatment with tert-BHP or saline for 1 wk, *Prdx6*$^{-/-}$-treated, WT-treated, and control males were paired with nontreated 8-wk-old WT females (1:1 ratio) and monitored for litter numbers and litter size for 6 mo.

Determination of Sperm Capacitation

Cauda epididymal sperm were collected from 2-mo-old *Prdx6*$^{-/-}$ males and their age-matched WT littermates. Briefly, cauda epididymides from each animal were poked with 27-G needle five times and the sperm were allowed to disperse for 10 min at 37°C in BWW medium without bovine serum albumin (BSA) or $NaHCO_3$. Then, sperm were centrifuged at 600 × *g* for 10 min at room temperature and the pellet resuspended at a concentration of 1–2 × 10^6 sperm/ml in BWW medium (pH 7.4) with or without 5 mg/ml BSA and 20 mM $NaHCO_3$ during a 60-min incubation at 37°C. Then, controls (spermatozoa in BWW alone) and capacitated spermatozoa were centrifuged and resuspended in BWW and incubated with 10 μM progestrerone for 30 min to determine the percentage of acrosome reaction. To test whether PRDX6 PLA_2 activity is involved in sperm capacitation, noncapacitating and capacitating spermatozoa (as described above) were incubated in the presence or absence of 10 μM 1-hexadecyl-3-trifluorethylglycero-sn-2-phospho-methanol (MJ33), a specific inhibitor of PRDX6 PLA_2 activity [71]. After allowing 60 min of incubation for capacitation to take place, spermatozoa were treated with progesterone to induce the acrosome reaction, as described earlier. In order, to determine the percentage of spermatozoa that undergo the acrosome reaction, spermatozoa were fixed in ice-cold 100% methanol for 5 min, and then placed on a glass slide, air dried, and the acrosomal status determined by FITC-conjugated *Pisum sativum* agglutinin (FITC-PSA) staining. Sperm were incubated for 20 min with FITC-PSA [72]. A 1,4-diazabicyclo [2.2.2]octane solution was then applied to each slide, and they were sealed with cover slips. The acrosomal status of viable spermatozoa was assessed by epifluorescence microscopy (Zeiss Axiophot) at 1000× magnification. A total of 200 cells per duplicate was counted for the presence or absence of an intact acrosome. The capacitation levels were expressed as the percentage of spermatozoa that undergo acrosome reaction stimulated by progesterone.

Determination of Membrane Fluidity During Capacitation

We assessed the changes in membrane fluidity monitored by flow cytometry using the fluorescent amphiphilic probe, Merocyanine 540 (M540) [73, 74] during sperm capacitation. Briefly, spermatozoa incubated with or without MJ33 under noncapacitating and capacitating conditions for 60 min at 37°C were centrifuged and resuspended in PBS and incubated in the dark with 3 μM M540 for 30 min at 37°C. Hoechst 33258, an indicator of dead cells, was used in conjunction with M540 and was added to the cells at a concentration of 10 μg/ml immediately prior to flow cytometry analysis (Ex/Em of M540: ~560/590 nm). Hoechst 33258-positive cells were measured with ultraviolet laser (Ex/Em of Hoescht 33258: ~352/461) and excluded from M540 analysis. A minimum of 10 000 events was analyzed for each sample using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Inc.).

Statistical Analysis

All graphical data are represented as the mean ± SEM; statistical differences between group means were determined using an ANOVA (one- or two-way ANOVA) followed by Bonferroni post hoc test or Friedman two-way analysis, as appropriate to the data distribution. All *P* values less than or equal to 0.05 were considered statistically significant. Multiple linear regression analyses were performed to establish relationships between sperm DNA damage (susceptibility to fragmentation or oxidation) and the level of DNA compaction (CMA3 labeling). The median test was used to determine differences in litter size among groups. Statistical analyses were performed with Sigma Systat 13 (Systat Software Inc., San Jose, CA) and Statistix for Windows V.1 (Analytical Software, Tallahassee, FL).

RESULTS

Impact of tert-BHP Treatment on Sperm Motility

Prdx6$^{-/-}$ spermatozoa had lower motility than WT controls before treatment, indicating an impairment of the motility machinery in *Prdx6*$^{-/-}$ males (Fig. 1, A and B). The in vivo oxidative stress generated by tert-BHP treatment lowered the motility significantly in both *Prdx6*$^{-/-}$ spermatozoa and in WT spermatozoa collected 24 h after the end of treatment. The detrimental effect due to the treatment was exacerbated in *Prdx6*$^{-/-}$ males. Interestingly, a recovery of motility was observed in WT males at 6 mo after the end of treatment. This recovery was not observed in treated *Prdx6*$^{-/-}$ males in which the sperm motility values were unchanged at 6 mo compared to the value at 24 h after the end of tert-BHP treatment.

Prdx6$^{-/-}$ Spermatozoa Are Sensitive to In Vivo Oxidative Stress

We determined the levels of sperm lipid peroxidation, protein oxidation (S-glutathionylation and carbonylation), and DNA oxidation (8-OHdG levels) as markers of oxidative stress (Figs. 2 and 3). *Prdx6*$^{-/-}$ spermatozoa from nontreated males had higher levels of lipid peroxidation (Fig. 2A) compared to their respective WT controls. Interestingly, spermatozoa from WT-treated animals evidenced similar high levels of lipid peroxidation than the *Prdx6*$^{-/-}$ controls. The tert-BHP treatment promoted the highest levels of lipid peroxidation observed in *Prdx6*$^{-/-}$ spermatozoa.

The levels of S-glutathionylation and of protein carbonylation were higher in spermatozoa from *Prdx6*$^{-/-}$ mice compared to WT controls (Fig. 2, B–E). The tert-BHP treatment of WT males increased the levels of these protein modifications in their spermatozoa to similar levels as those found in nontreated *Prdx6*$^{-/-}$ mice. We found an increasing trend in the level of protein modifications in tert-BHP-treated *Prdx6*$^{-/-}$ males.

Nontreated *Prdx6*$^{-/-}$ males had a significantly higher percentage of spermatozoa with DNA oxidation compared to WT controls (Fig. 3), and similar to those of WT-treated males at 24 h after the end of treatment. It was also observed that spermatozoa from treated WT males showed lower levels of DNA oxidation at 6 mo compared to 24 h after the end of treatment. Interestingly, DNA oxidation was even higher in Prx6$^{-/-}$ spermatozoa after tert-BHP treatment compared to the respective WT control ($P < 0.05$), regardless of the time that these spermatozoa were collected.

Sperm Chromatin Quality Is Impaired in Prdx6$^{-/-}$ Spermatozoa

Sperm DNA fragmentation. Sperm DNA fragmentation (evaluated by DFI) was higher in spermatozoa from nontreated *Prdx6*$^{-/-}$ males compared with their respective WT controls

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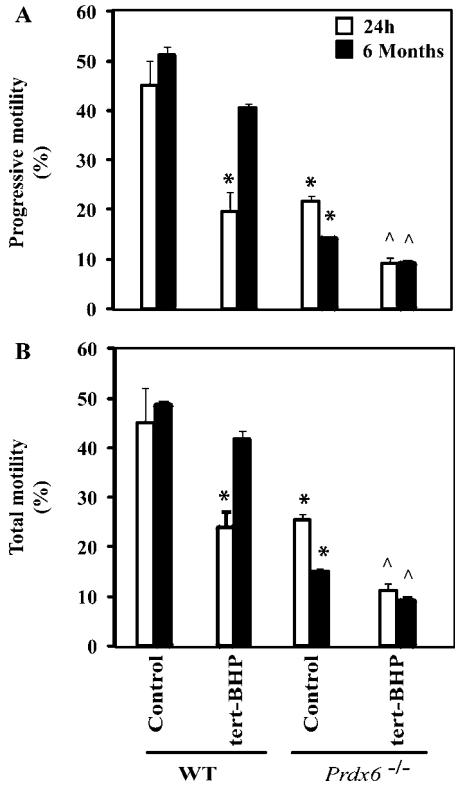

FIG. 1. Progressive (**A**) and total (**B**) sperm motility in *Prdx6*$^{-/-}$ and WT males treated or not with tert-BHP. Spermatozoa were collected at 24 h and 6 mo after the end of treatment. *Lower than WT controls; ^lower than all other values (n = 6; $P < 0.05$).

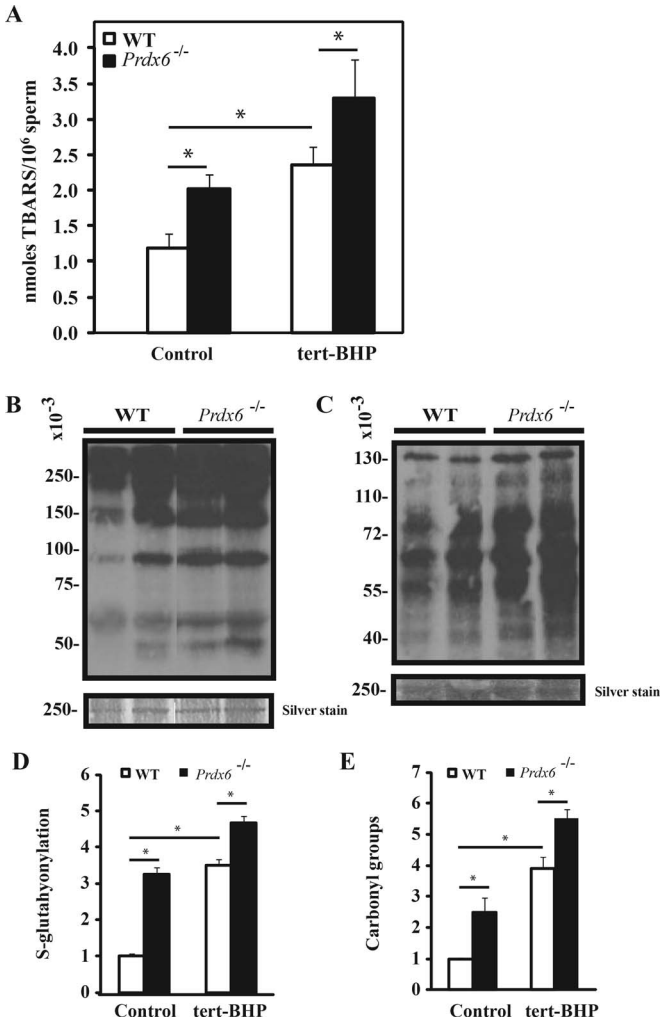

FIG. 2. Levels of lipid peroxidation (**A**), S-glutathionylation (**B**), and carbonylation (**C**) in sperm proteins of WT and *Prdx6*$^{-/-}$ males at 6 mo after the end of tert-BHP treatment. Lanes presented in **B** and **C** are from the same blot. **D** and **E**) The relative intensities of samples from controls and tert-BHP-treated WT and *Prdx6*$^{-/-}$ males. Values were first normalized to the 250-kDa band from the colloidal silver-stained membrane and then all the bands were normalized again to the respective normalized intensity of WT control. *Significantly different (n = 6; $P < 0.05$).

(Fig. 4A). At 24 h after the end of tert-BHP treatment, both groups showed increased levels of DFI; however, *Prdx6*$^{-/-}$ spermatozoa showed the highest levels of DNA fragmentation ($P < 0.05$). Spermatozoa recovered from WT males at 6 mo had lower levels than those from WT obtained at 24 h after the end of treatment. This recovery of sperm DNA integrity was not observed in *Prdx6*$^{-/-}$-treated males that showed even higher levels of DFI after 6 mo compared to 24 h. Interestingly, nontreated *Prdx6*$^{-/-}$ males had spermatozoa with higher levels of DFI at 6 mo compared to WT mice at 24 h after the end of treatment.

Sperm DNA compaction. Compaction of the DNA in spermatozoa is influenced by the amount of protamines (level of protamination) present in the sperm chromatin, and by their ability to form disulfide bridges among themselves to make an even more compacted structure. The level of protamination was assessed by CMA3 labeling assay (Fig. 4B). We found increased levels of CMA3 labeling in spermatozoa from treated WT animals compared to their respective control. Interestingly, the values from WT males were similar to those observed in spermatozoa from *Prdx6*$^{-/-}$ control animals (before treatment). Moreover, the levels of CMA3 labeling increased in *Prdx6*$^{-/-}$ spermatozoa from nontreated males at 6 mo from the beginning of the study. When *Prdx6*$^{-/-}$ males were treated with tert-BHP, we observed a greater increase in CMA3 labeling in their spermatozoa at both collection times after the treatment. It is worth noting that the highest levels of CMA3 labeling were observed in *Prdx6*$^{-/-}$ spermatozoa collected at 6 mo after the treatment.

The levels of sperm DNA compaction were also determined by the HDS parameter of SCSA (Fig. 4C). The HDS fraction contains sperm with an increased histone/protamine ratio and the presence of unprocessed protamines [22] and greater round morphology [69]. The HDS values were higher in treated WT compared to nontreated WT spermatozoa ($P < 0.05$). The HDS in spermatozoa from WT-treated males at 24 h after treatment were similar to those of nontreated *Prdx6*$^{-/-}$ males recovered after 6 mo of the end of treatment. The treatment triggered an increase in HDS values observed in *Prdx6*$^{-/-}$ spermatozoa at both endpoints compared to the respective *Prdx6*$^{-/-}$ controls.

Multiple regression analyses indicated that the levels of 8-OHdG ($R^2 = 0.79$, $P = 0.001$) and of DFI ($R^2 = 0.31$, $P = 0.02$) depend on the level of protamination (CMA3 labeling) and on the level of DNA compaction measured by HDS ($R^2 = 0.49$, $P = 0.001$ and $R^2 = 0.36$, $P = 0.01$, respectively).

Prdx6$^{-/-}$ *males are subfertile*. *Prdx6*$^{-/-}$ males showed lower fertility than the WT controls during the 6-mo mating period (Fig. 5). *Prdx6*$^{-/-}$ males produced fewer litters compared to WT mice (Fig. 5, A and B). The tert-BHP treatment affected the litter number and size in WT mice, resulting in numbers similar to nontreated *Prdx6*$^{-/-}$ males. The treated *Prdx6*$^{-/-}$ males showed lower litter size compared to

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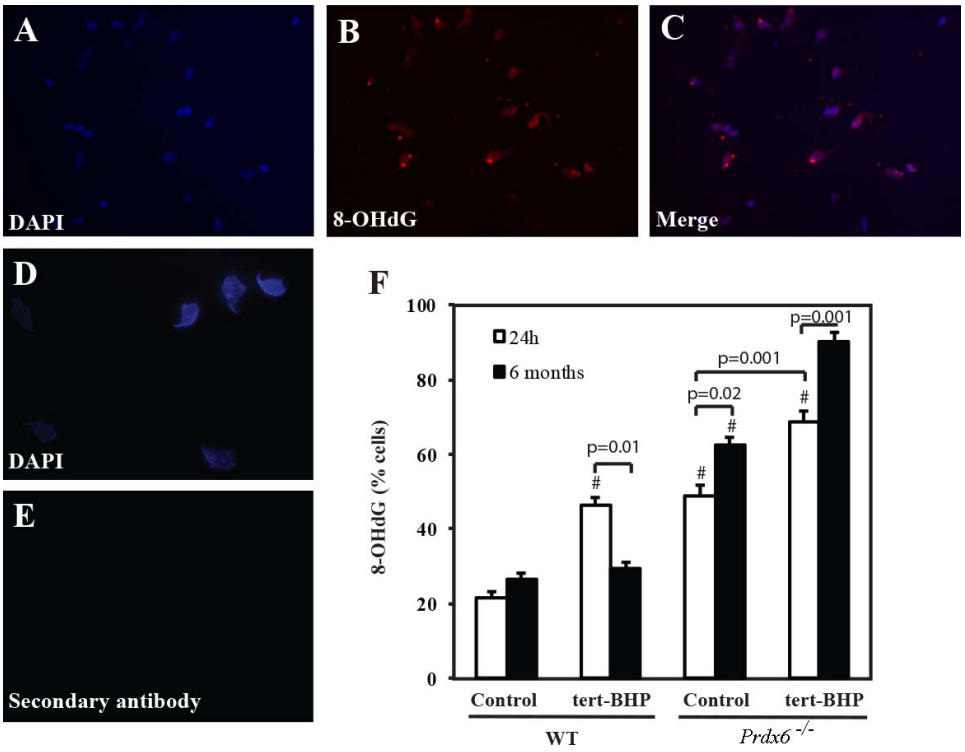

FIG. 3. The 8-OHdG levels in mouse spermatozoa. **A**–**C**) Cauda epididymis were treated with 5 mM H_2O_2 and 1 mM $FeSO_4$•$7H_2O$ during 30 min at 37°C as positive controls. Sperm were smeared and permeabilized with methanol and incubated with the anti-8-OHdG antibody overnight. Samples were then incubated with biotin-labeled anti-mouse antibody followed by streptavidin conjugated to Alexa Fluor 555 for 1 h. **D** and **E**) In order to show specificity of the primary antibody, sperm samples were incubated with the secondary antibody alone. Pictures were taken at the same exposure at 1000× magnification (**A**–**C**) and 400× magnification (**D** and **E**), respectively. **F**) Percentage of spermatozoa with 8-OHdG labeling. Cauda epididymis spermatozoa were collected from control and tert-BHP-treated WT and *Prdx6*$^{-/-}$ males (n = 6 for each group) and processed for detection of DNA oxidation, as explained in *Materials and Methods*. #Significantly higher than the WT control group ($P < 0.05$).

the respective controls (Fig. 5A). A similar decreasing trend was seen after 6 mo of matings. Only 33% or 42% of mating involving treated *Prdx6*$^{-/-}$ males produced pups at 1 or 6 mo after the treatment, respectively. A great variability was observed in the reproductive outcomes in this group compared to control: the majority of matings (58%) produced 0 pups, while 14% of matings produced large litters (10–11 pups). The mechanism for the unexpectedly large litters is unknown.

We found positive correlations among sperm chromatin quality parameters (Table 1): DFI positively correlated with DNA oxidation (8-OHdG) and with DNA compaction (level of protamination [CMA3 labeling] and HDS). DNA oxidation positively correlated with levels of protamination and HDS. Moreover, these sperm chromatin quality parameters negatively correlated with reproductive outcomes (total of litters and pups per male and cumulative n of litters; Table 1).

Poisson regression analyses revealed that the total n of pups/male depends on the sperm DNA compaction (determined by CMA3 labeling and HDS; likelihood ratio = 61.3, $P < 0.0001$) and on sperm DNA damage (determined by DFI and 8-OHdG; likelihood ratio = 54.6, $P < 0.0001$). Similarly, cumulative n of litters/male depends on the sperm DNA compaction (likelihood ratio = 50.25, $P < 0.0001$) and on sperm DNA damage (likelihood ratio = 36.4, $P < 0.0001$).

Capacitation Is Impaired in Prdx6$^{-/-}$ *Spermatozoa*

The capacitation levels were significantly lower in *Prdx6*$^{-/-}$ compared to WT spermatozoa (Fig. 6A). The presence of MJ33, an inhibitor of PLA_2 activity of PRDX6, in the incubation medium inhibited capacitation in WT at similar levels to those observed in *Prdx6*$^{-/-}$ spermatozoa (Fig. 6A), providing evidence for an important role of PRDX6 PLA_2 activity in sperm capacitation.

The increase in membrane fluidity is an event associated with capacitation and is necessary to prepare the sperm to undergo the acrosome reaction [74, 75]. We found an impairment of remodeling of sperm plasma membrane in *Prdx6*$^{-/-}$ males compared to WT controls (Fig. 6B), as evaluated by the incorporation of M450. Moreover, the presence of MJ33 in the capacitating medium promoted a significant decrease in WT spermatozoa compared to WT controls.

DISCUSSION

The results presented in this study highlight, for the first time, the important role of PRDX6 in maintaining sperm quality and fertilizing ability, protecting sperm vital components, including proteins and the paternal genome against cellular oxidative damage, to assure normal reproductive performance. *Prdx6*$^{-/-}$ males have lower sperm chromatin quality and motility, which translates into a lower number of pups/male and of litters/male compared to WT controls. Furthermore, *Prdx6*$^{-/-}$ males are susceptible to in vivo oxidative stress generated by tert-BHP treatment, showing higher levels of DNA oxidation and poorer sperm quality and capacitation than the nontreated *Prdx6*$^{-/-}$ controls.

The most significant decline in sperm motility and chromatin quality was observed in treated *Prdx6*$^{-/-}$ males compared to the other groups (Figs. 1, 3, and 4). These changes in sperm quality could account for the observation

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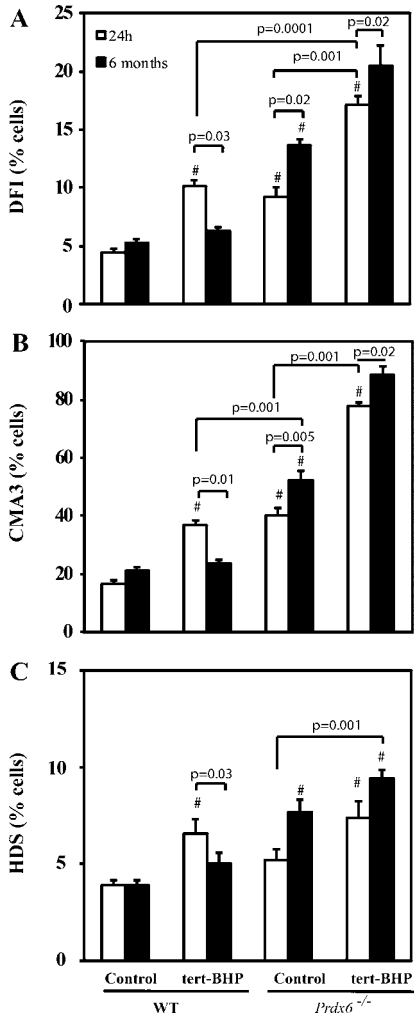

FIG. 4. Sperm chromatin quality in *Prdx6*$^{-/-}$ mice. Sperm DNA damage: (**A**) susceptibility to acid-induced fragmentation, (**B**) level of protamination, and (**C**) level of DNA compaction measured by HDS. #Higher than WT controls (n = 6; $P < 0.05$).

that *Prdx6*$^{-/-}$ males generate a lower number of litters and of pups compared to WT controls (Fig. 5, A and B), similar to results that we recently observed in young and old *Prdx6*$^{-/-}$ males [56]. We recently demonstrated the need for normal levels of reduced PRDX6 in human spermatozoa in order to maintain fertility [55]; infertile men have reduced sperm motility and high levels of sperm DNA fragmentation associated with low levels of PRDX6. Altogether, these results support the participation of PRDX6 in the prevention of oxidative stress and chromatin remodeling to assure normal fertility. As mentioned above, these changes in sperm quality translated into the observation that *Prdx6*$^{-/-}$ males generate a lower number of litters and of pups compared to WT controls (Fig. 5). Indeed, we found negative associations among sperm chromatin parameters and the reproductive outcomes (Table 1). The dependency of the number of pups produced with the level of sperm DNA damage (fragmentation and oxidation) and the level of sperm chromatin compaction (CMA3 labeling and HDS) supports this conclusion. These results are in accordance with those of our previous study that demonstrated the need for normal levels of reduced PRDX6 in human spermatozoa in order to maintain fertility [55]; infertile men have reduced sperm motility and high levels of sperm DNA fragmentation associated with low levels of PRDX6. Thus, our results with the *Prdx6*$^{-/-}$ mouse model, in light of the findings in humans, support the participation of PRDX6 in the prevention of oxidative stress to assure normal fertility. It is of interest that GSH depletion potentiates methanesulfonate-induced susceptibility of rat sperm DNA denaturation in situ [76]. This observation highlights the necessity for a functional GSH/GST system in the testis and spermatozoa to reactivate PRDX6 in order to fight against oxidative stress [45–47].

PRDX6, similar to GPX4, removes phospholipid hydroperoxides, thus quenching the chain reaction of lipid peroxidation or repairing peroxidized cell membranes [77]. Increased levels of lipid peroxidation in spermatozoa have been associated with reduction of motility and male infertility [64, 78]. The increased lipid peroxidation and reduced motility observed in *Prdx6*$^{-/-}$ spermatozoa (Figs. 1 and 2A) suggest an important role of PRDX6 in the protection of the spermatozoon against oxidative stress. The participation of GPX4 in antioxidant protection is unlikely, because this enzyme is insoluble and tightly bound in the mitochondrial sheath of spermatozoa [79]. An interesting observation is that sperm motility had partially recovered at 6 mo after the end of the oxidant treatment in WT-treated animals. However, this improvement was not observed in *Prdx6*$^{-/-}$ males, indicating the necessity of active PRDX6 to assure normal sperm motility (Fig. 1, A and B). This result may reflect the recent demonstration that PRDX6 activity is required for repair of cell membrane lipid peroxidation [80].

Recently, we described redox-protein modifications that are associated with a loss of motility by human spermatozoa [27]. The increased levels of S-glutathionylation and protein oxidation in spermatozoa (Fig. 2, B and C), along with the reduced percentage of motility (Fig. 1) observed in *Prdx6*$^{-/-}$ males, suggest that key proteins associated with the motility machinery may be affected by these oxidative stress-dependent protein modifications. It is known that tubulin, the major protein present in the sperm flagellum, is susceptible to S-glutathionylation and carbonylation due to oxidative stress [81, 82]. We recently found that tubulin is highly oxidized (data not shown) when human spermatozoa are incubated under the same oxidative stress conditions, as previously reported [27]. Moreover, immunocytochemistry studies revealed the presence of S-glutathionylation along the human sperm flagellum [27], thus supporting the possible S-glutathionylation of tubulin due to oxidative stress. Other possible protein targets for redox-dependent modifications are enzymes involved in glycolysis and the Krebs cycle [27, 82], needed for the supply of energy to the spermatozoon. Since we recognize several bands of oxidative stress-modified proteins, and that high levels of ROS affect sperm function, such as capacitation and binding to the zona pellucida [27, 83], it is possible that other key proteins involved in these processes may be affected in *Prdx6*$^{-/-}$ spermatozoa. From the studies presented here, it is possible to conclude that PRDX6 plays a significant role in the antioxidant protection of the motility machinery in mouse spermatozoa.

It was previously shown that *Prdx6*$^{-/-}$ males had decreased number of litters and litter size, and reduced total number of pups compared to WT males, and that these reproductive phenotypes worsen as the males age [56]. In the present study, we confirmed that *Prdx6*$^{-/-}$ males had decreased numbers of litters, litter size, and reduced total number of pups compared to WT males (Fig. 5, A and B). Furthermore, the percentage of males that produced pups was significantly lower in *Prdx6*$^{-/-}$ than in WT males (Fig. 5C). The tert-BHP treatment of WT males promoted a reduction in the number of pups produced, similar to observations in nontreated *Prdx6*$^{-/-}$ males. Moreover, there was a trend toward a greater decrease in the number of pups produced by treated *Prdx6*$^{-/-}$ males. Although the

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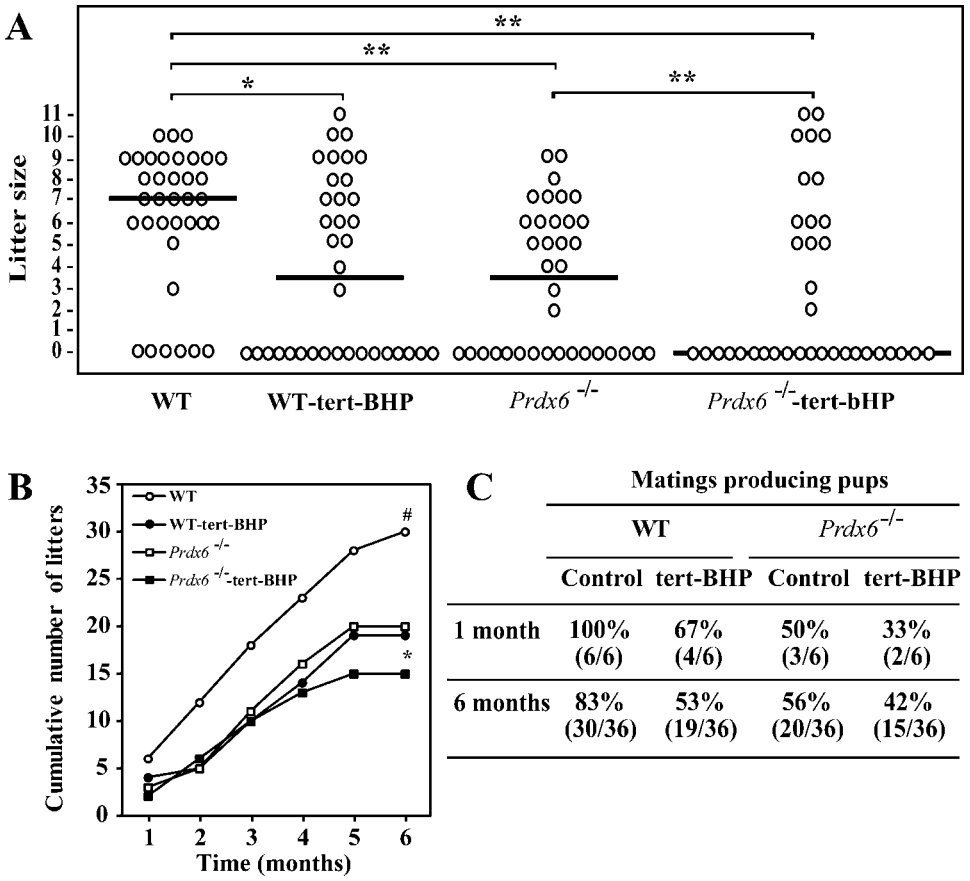

FIG. 5. Reproductive outcomes of *Prdx6*$^{-/-}$ and WT males during 6 mo of matings. **A**) Litter size. **B**) Cumulative number of litters. **C**) Percentage of matings producing pups. Control and tert-BHP-treated WT or *Prdx6*$^{-/-}$ males were mating with nontreated 8-wk-old WT females (1:1 ratio) for 6 mo. Gross horizontal black bars in **A** indicate the median for each group. *$P = 0.005$; **$P = 0.0001$; # and * indicate higher and lower than all other values, respectively ($n = 6$; $P < 0.05$).

TABLE 1. Correlations between sperm chromatin assays and reproductive outcomes.*

Parameter	HDS	8-hydroxy-2′-deoxyguanosine (% cells)	CMA3 (mean fluorescence)	Total n litters/male	Cumulative n litters	Total n pups/male
DFI	0.59 (0.01)	0.53 (0.02)	0.55 (0.02)	−0.55 (0.03)	−0.57 (0.02)	−0.50 (0.04)
HDS		—	0.42 (0.08)	−0.88 (0.0001)	−0.59 (0.02)	−0.58 (0.01)
8-OHdG			0.96 (0.0001)	−0.94 (0.0001)	−0.92 (0.0001)	−0.80 (0.0001)
CMA3				−0.88 (0.0001)	−0.88 (0.0001)	−0.76 (0.0001)

* Pearson correlation or Spearman rank order correlation coefficients. *P* value of significance is presented in parentheses ($P < 0.05$ was considered significant).

number of pups in successful litters was not significantly decreased by the treatment, ~60% of *Prdx6*$^{-/-}$ males were markedly impaired (based on the number of litters), while the remainder appeared normal—at least in terms of litter size. Although these values were quite variable, the oxidant treatment promoted a tendency to reduce the fertility of most *Prdx6*$^{-/-}$ males compared to nontreated *Prdx6*$^{-/-}$ mice. Since the spermatozoa are highly compartmentalized cells, the stronger negative effects of the tert-BHP-induced oxidative stress on sperm motility and oxidation of sperm DNA in *Prdx6*$^{-/-}$ mice compared to WT controls could account for the antioxidant protection of PRDX6 locally in the sperm head and tail.

In contrast to what was observed in WT males, the negative effects driven by tert-BHP in *Prdx6*$^{-/-}$ males remain even 6 mo after the treatment, indicating the production of long-term effects on the testis and highlighting the necessity of PRDX6 for normal spermatogenesis. It is known that tert-BHP treatment will affect fertility of male mice without altering testis weight and number of spermatozoa produced, but altering the development of spermatozoa during spermiogenesis and generating significant head abnormalities [59]. Similar to the effects of tert-BHP, the damage observed in *Prdx6*$^{-/-}$ males without treatment did not involve a reduction in the number of spermatozoa produced, but of the quality, since *Prdx6*$^{-/-}$ spermatozoa at 6 mo after the oxidant treatment showed lower motility and poorer chromatin integrity than WT controls.

Sperm chromatin integrity is essential to assure normal pregnancy and health of offspring [84, 85]. *Prdx6*$^{-/-}$ spermatozoa carry significant DNA fragmentation and oxidation (Figs. 3 and 4A), along with low levels of DNA compaction due to poor protamination (Fig. 4B). Interestingly, all of this damage is exacerbated in spermatozoa from *Prdx6*$^{-/-}$ males treated with tert-BHP. The regression analyses revealed that the sperm DNA integrity (measured by the levels of DNA fragmentation and oxidation) depends on the level of DNA

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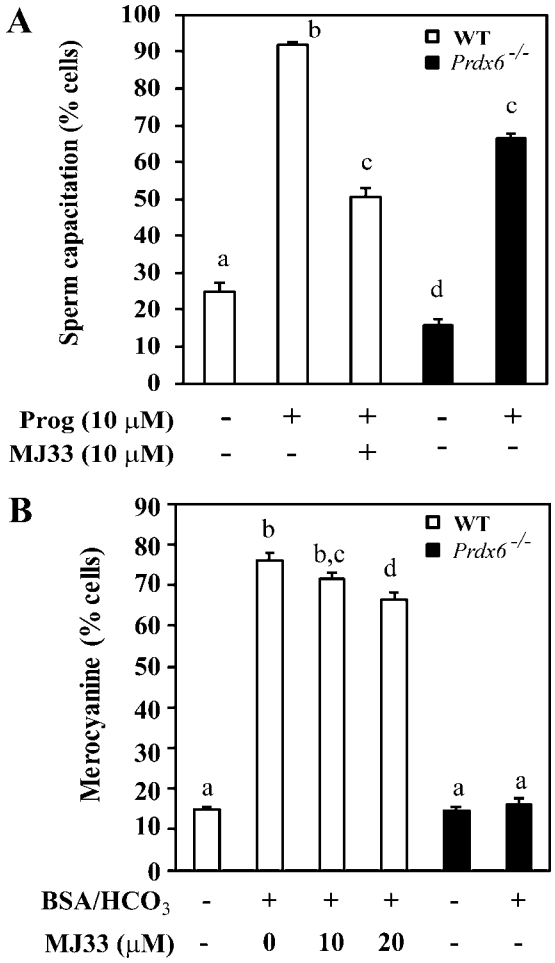

FIG. 6. Sperm capacitation and membrane fluidity are impaired in *Prdx6*$^{-/-}$ mice. **A**) Capacitated WT and *Prdx6*$^{-/-}$ spermatozoa were treated with 10 μM progesterone to induce the acrosome reaction. **B**) WT spermatozoa, in the absence or presence of MJ33 and *Prdx6*$^{-/-}$ spermatozoa were incubated under capacitating conditions (BSA/HCO_3^-) to determine the incorporation of the fluorescent probe M540, denoting increase of membrane fluidity. *Significantly lower than WT controls ($P < 0.05$). Different letters denote significant differences (n = 6; $P < 0.05$).

compaction measured by CMA3 labeling and HDS. These alterations of sperm chromatin are obvious after several cycles of spermatogenesis, evidencing a need for PRDX6 in the process of chromatin modeling during this process.

PRDX6 has wide localization in the subcellular compartments of human spermatozoa, including the head [52, 53]. This localization and the poorer sperm DNA compaction observed in this knockout mouse model suggest that PRDX6 is important in maintaining the appropriate thiol oxidation status needed to secure normal DNA compaction. This action was already suggested for the nuclear isoform of GPX4 (nGPX4), since spermatozoa from nGPX4 knockout males also have higher levels of free thiols in the head [86]. Since these knockouts are fertile [86], we can conclude that PRDX6 is important for maintaining the level of sperm DNA compaction to guarantee normal fertility. PRDX6 contains a lysine-rich C terminus; thus, it is reasonable to believe that PRDX6 binds to DNA and/or protamines to stabilize the sperm chromatin in a similar way to nGPX4 [86]. This possibility is supported by the interaction recognized between GPX4 and PRDX6 in hepatocytes [87].

The DNA damage observed in the *Prdx6*$^{-/-}$ spermatozoa can also be attributed to a lack of local protection by PRDX6 against oxidative stress in the knockout males. The *Plasmodium falciparum* nuclear PRDX (PfnPrx), a 1-Cys PRDX, is a genome-wide chromatin-associated nuclear PRDX that binds to DNA by its lysine-rich C terminus, and thus protects the parasite genome against oxidative stress [88]. This interaction with DNA may also occur for PRDX6 in spermatozoa, as it contains a lysine-rich C terminus.

During spermatogenesis, the sperm chromatin is tightly compacted by the exchange of histones by small basic protamines and by the formation of disulfide groups among these protamines occurring during epididymal maturation [89, 90]. The low protamination (evidenced by high levels of CMA3 labeling) observed in *Prdx6*$^{-/-}$ spermatozoa indicate an alteration of chromatin remodeling in these animals compared to WT controls. Similarly to nGPX4, it is possible then to ascribe a possible role of PRDX6 during spermatogenesis and epididymal maturation in the formation of disulfide bridges among protamines to assure the appropriate level of DNA compaction required by the spermatozoon. This is supported by the fact that *Prdx6*$^{-/-}$ males are subfertile, in contrast to nGPX4$^{-/-}$ males that show normal fertility, therefore highlighting the need for an active PRDX6 to assure normal sperm chromatin development. The finding of reducing protamination levels could be due to an alteration in the production of protamines or in the translocation mechanism to remove histones during spermiogenesis; the negative correlations between levels of ROS and of 8-OHdG with protamine quantities observed in spermatozoa from smokers compared to nonsmokers suggest that protamine production is susceptible to oxidative stress [91]. It is possible, then, that the lack of PRDX6 generates an oxidative stress in the testis that will alter protamine production and/or replacement of histones that will lead to a lesser DNA compaction, oxidation, and fragmentation.

Capacitation is a requirement for the spermatozoon to be able to fertilize the oocyte. *Prdx6*$^{-/-}$ spermatozoa failed to undergo in vitro capacitation, since they cannot undergo acrosome reaction when treated with progesterone (Fig. 6A) and they have lower levels of tyrosine phosphorylation compared to WT controls (data not shown). The failure to undergo capacitation and the acrosome reaction may reside in the possible oxidation of key proteins involved in these processes. We demonstrated that oxidative stress impairs sperm capacitation in humans, even when ROS levels are low enough not to affect sperm motility, but impair the ability to undergo the acrosome reaction [27]. Moreover, spermatozoa lacking PRDX6 failed to increase membrane fluidity when incubated under capacitating conditions (Fig. 6B). This failure impacts directly on the ability to undergo the acrosome reaction, as was demonstrated in this study (Fig. 6A). Human spermatozoa incubated with MJ33, inhibitor of PRDX6 PLA_2 activity, are unable to undergo capacitation [92]. Here, this inhibitor prevented both capacitation and the increase in membrane fluidity measured by M540 (Fig. 6), indicating that this activity is necessary for both remodeling of the plasma membrane and capacitation. Altogether, these studies in our animal model and humans suggest an important role of PRDX6 in the redox regulation of mammalian sperm capacitation.

In conclusion, we report, for the first time, a major role for PRDX6 in reducing oxidative stress to maintain sperm function and chromatin integrity in order to assure normal reproductive outcomes in mice. These findings, along with the low levels of PRDX6 in spermatozoa from infertile men [55], provide

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confirmatory evidence for an important role of PRDX6 in male reproduction.

ACKNOWLEDGMENT

We thank Dr. M. O'Flaherty (University of Liverpool) for his assistance on some of the statistical analyses.

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