Peroxiredoxin 6 Peroxidase and Ca^{2+}-Independent Phospholipase A_2 Activities Are Essential For Male Mouse Fertility

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Table of Contents

List of Figures……5

List of Abbreviations……7

Abstract……11

Résumé……13

Acknowledgements…… 15

1 Introduction……16

1.1 From spermatogenesis to fertilization: the spermatozoon's journey……16

1.1.1 Spermatogenesis…… 16

1.1.2 Structural components of spermatozoa…….. 16

1.1.3 Sperm epididymal maturation……18

1.1.4 Sperm ejaculation……...19

1.1.5 Sperm capacitation, acrosome reaction…….. 20

1.2 Male fertility…… 21

1.2.1 Oxidative stress in male fertility…… 21

1.2.2 Negative effects of ROS within spermatozoa…… 22

1.2.3 Positive effects of ROS within spermatozoa……. 24

1.3 Antioxidant system of human spermatozoa……. 26

1.3.1 ROS scavengers within spermatozoa…… 26

1.3.2 Vulnerability of spermatozoa to oxidative stress……... 26

1.3.3 Characterization of Peroxiredoxins (PRDXs) ……... 27

1.3.4 PRDX6: primary protector of spermatozoa against oxidative stress……. 31

2 Research Rationale…….34

2.1 Involvement of PRDX6 in sperm motility ……...34

2.2 Involvement of PRDX6 in sperm capacitation …….34

2.3 Involvement of PRDX6 in mice's reproductive outcomes …….. 35

2.4 Involvement of PRDX6 in sperm epididymal maturation ……35

2.5 Involvement of PRDX6 in prevention of sperm DNA oxidation ……..36

3 Hypothesis and Objective 37

4 Materials and Methods 38

4.1 Materials 38

4.2 Animals 38

4.3 Thermoresistance test to assess sperm motility 39

4.4 Assessment of sperm capacitation 39

4.5 Assessment of sperm epididymal maturation 40

4.6 Quantification of reproductive outcomes 40

4.7 SDS-PAGE and immunoblotting to determine 4HNE and nitrotyrosine levels in spermatozoa 40

4.8 Immunocytochemistry to determine 4HNE and nitrotyrosine levels in spermatozoa 41

4.9 Immunocytochemistry to determine DNA oxidation levels in spermatozoa 42

4.10 Statistical analysis 42

5 Results 44

5.1 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ and oxidative stress on male mice fertility 44

5.2 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ on sperm epididymal maturation 46

5.3 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ on lipid peroxidation levels in spermatozoa 47

5.4 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ on tyrosine nitration levels in spermatozoa 51

5.5 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ on sperm DNA oxidation 56

5.6 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ on sperm motility 57

5.7 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ on sperm capacitation 58

6 Discussion ……59
7 Conclusion ……...68
8 Future Directions ……69
9 References……70
10 Appendix……..92

List of Figures

Figure 1: Structural morphology of human spermatozoa……18

Figure 2: Schematic representation of the mechanisms……. 25
involved in sperm capacitation

Figure 3: Schematic representation of the reactivation……...28
system of 2-Cys PRDXs

Figure 4: Schematic representation of the reactivation……...29
system of PRDX6

Figure 5: Schematic representation of PRDX6 structure……31

Figure 6: Schematic depicting the involvement of PRDX6 in……33
regulating parameters of human spermatozoa associated
with male fertility

Table 1: PRDX6 peroxidase or $iPLA_2$ absence leads to impaired……...44
mating of mice

Figure 7: PRDX6 peroxidase or $iPLA_2$ absence leads to decreased……45
litter sizes

Figure 8: Removal of sperm cytoplasm is impaired in C47S……..46
and D140A mutant mice

Figure 9: PRDX6 peroxidase or $iPLA_2$ absence leads to……47
increased formation of 4HNE adducts

Figure 10A: Representative pictures depicting the intensity of 4HNE……49
labeling in non-permeabilized spermatozoa from each group

Figure 10B: PRDX6 peroxidase or $iPLA_2$ absence leads to……50
increased lipid peroxidation

Figure 11: 4HNE labeling is observed throughout the entire structure……...51
of permeabilized spermatozoa

Figure 12: PRDX6 peroxidase or $iPLA_2$ absence leads to increased……...52
tyrosine nitration of sperm proteins

Figure 13A: Representative pictures depicting the intensity of nitrotyrosine…….....54
labeling in non-permeabilized spermatozoa from each group

Figure 13B: PRDX6 peroxidase or iPLA$_2$ absence leads to increased……..55
nitrotyrosine levels of non-permeabilized spermatozoa in
response to oxidative stress

Figure 14: PRDX6 peroxidase or iPLA$_2$ absence leads to increased……...56
vulnerability of mice spermatozoa to DNA oxidation

Figure 15: PRDX6 peroxidase or iPLA$_2$ absence leads to decreased……..57
sperm motility

Figure 16: PRDX6 peroxidase or iPLA$_2$ absence leads to impaired……58
sperm capacitation.

Table 2: PRDX6 peroxidase and iPLA$_2$ are involved in the regulation……...67
of parameters that are associated with male fertility

List of Abbreviations

AKAP4	A-Kinase Anchoring Protein 4
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis Of Variance
Arg-X-X-(Ser/Thr)	Arginine-X-X-(Serine/Threonine)
ATP	Adenosine Triphosphate
ATP1A4	Na^+/K^+ Transporting ATPase Subunit Alpha-4
BSA	Bovine Serum Albumin
BWW	Biggers, Whitter and Whittingham
C47	Cysteine 47
C47S mice	PRDX6 Peroxidase-Deficient Knock-In Strain
C57Bl6/J mice	Wild-type Mice
Ca^{2+}	Calcium Ion
$CaCl_2$	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
CAP	Capacitated
CASA	Computer-Assisted Sperm Analysis
CDR	Cytoplasmic Droplet Retention
cKIT	Tyrosine-Protein Kinase KIT
CoA	Coenzyme A
Cu/Zn SOD	Copper/Zinc Superoxide Dismutase
Cys	Cysteine
D140	Aspartate 140
D140A mice	$iPLA_2$-Deficient Knock-In Strain
Da-NONOate	Diethylamine NONOate
DAPI	4′,6-Diamidino-2-Phenylindole
ddH_20	Double Distilled Water
DICER1	Endoribonuclease DICER
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol

ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular Signal-Regulated Kinase
ERp57	Protein Disulfide-Isomerase A3
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPX	Glutathione Peroxidase
GPX1	Glutathione Peroxidase 1 (same for GPX2/3/4/5/6/7/8/9)
G6PDH	Glucose-6-Phosphate Dehydrogenase
GSH	Glutathione
GSTpi	Glutathione S-Transferase Pi
H26	Histidine 26
H_2O_2	Hydrogen Peroxide
HBS	HEPES-Buffered Saline
HCl	Hydrochloric Acid
HCO_3^-	Bicarbonate
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
$iPLA_2$	Ca^{2+}-Independent Phospholipase A_2
IZUMO1	Izumo Sperm-Egg Fusion 1
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
LDH-C4	Lactate Dehydrogenase-C4
LPCAT	Lysophosphatidylcholine Acyltransferase
ME	Malic Enzyme
MEK	Mitogen Activated Kinase
mGPX4	Mitochondrial Glutathione Peroxidase 4
$MgSO_4$	Magnesium Sulfate
MJ33	1-Hexadecyl-3-(Trifluoroethyl)-*sn*-Glycero-2-Phosphomethanol Lithium
MnSOD	Manganese Superoxide Dismutase
MMP9	Matrix Metallopeptidase 9
Na_2HPO_4	Disodium Phosphate

NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NADP-ICDH	Nicotinamide Adenine Dinucleotide Phosphate-Dependent Isocitrate Dehydrogenase
$NAHCO_3$	Sodium Bicarbonate
NC	Non-Capacitated
nGPX4	Nuclear Glutathione Peroxidase 4
$NO^{\bullet}$	Nitric Oxide
NOS	Nitric Oxide Synthase
$O_2^{\bullet-}$	Superoxide Anion
$ONOO^-$	Peroxynitrite
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate Buffered Saline + 0.1% Triton
pH	Potential Hydrogen
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PP	Protein Phosphatases
PRDX	Peroxiredoxin
PRDX1	Peroxiredoxin 1 (same for PRDX2/3/4/5/6)
Prdx6$^{-/-}$ mice	*PRDX6*-Knockout Mice
PSA	Prostate Specific Antigen
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatases
PUFA	Polyunsaturated Fatty Acid
RAF	Rapidly Accelerated Fibrosarcoma
RFI	Relative Fluorescence Intensity
ROS	Reactive Oxygen Species
ROOH	Lipid Hydroperoxide
S32	Serine 32

sAC	Soluble Adenylyl Cyclase
SEM	Standard Error Of The Mean
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SNPs	Single Nucleotide Polymorphisms
SOD	Superoxide Dismutase
SOD1	Superoxide Dismutase 1 (same for SOD2/3)
SRX	Sulfiredoxin
tBHP	Tert-butyl Hydroperoxide
TRX/TRD	Thioredoxin/Thioredoxin Reductase
TTBS 1X	2 mM Tris (pH 7.8)-buffered saline and 0.1% Tween 20
VDAC2	Voltage-Dependent Anion-Selective Channel Protein 2
Zn^{2+}	Zinc Ion
1-Cys	1 Cysteine Residue Within Active Site
2-Cys	2 Cysteine Residues Within Active Site
4HNE	4-Hydroxynonenal
8-OHdG	8-Hydroxy-2'-Deoxyguanosine
%AR	Percentages of Acrosome Reaction

Abstract

Currently, one in six couples suffers from infertility, and half of the cases are caused by male infertility. Oxidative stress due to high reactive oxygen species (ROS) levels and/or low levels of antioxidant enzymes promotes infertility by inducing lipid peroxidation, DNA damage, impaired sperm motility and capacitation, and disrupted mitochondrial activity. 4-hydroxynonenal (4HNE), a lipid peroxidation product, increases levels of superoxide anion and nitric oxide responsible for tyrosine nitration of proteins, thus impairing sperm motility and capacitation. Peroxiredoxin 6 (PRDX6) protects spermatozoa from oxidative stress through its peroxidase and Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) activities that scavenge ROS and repair oxidized membranes, respectively. Spermatozoa from infertile patients have low PRDX6 levels that are associated with impaired motility, high lipid peroxidation levels and DNA damage. These abnormal parameters were also observed in spermatozoa of *Prdx6*$^{-/-}$ mice, resulting in smaller litter sizes compared to wild-type mice, and were worsened when males were treated with tert-butyl hydroperoxide (tBHP), an *in vivo* oxidative stress inducer. We hypothesize that PRDX6 peroxidase or $iPLA_2$ absence leads to infertility observed in *Prdx6*$^{-/-}$ male mice. Spermatozoa from two-month-old male mice of the C57Bl6/J (wild-type), *Prdx6*$^{-/-}$, C47S and D140A (peroxidase- and $iPLA_2$-deficient) mouse strains were incubated in PBS to assess motility. Percentages of acrosome-reaction of non- and capacitated spermatozoa (BWW medium + BSA/bicarbonate) were quantified using Giemsa staining. The number of unsuccessful matings, litter sizes and percentages of spermatozoa with cytoplasmic droplet retention (CDR) were recorded for mice treated with saline (control) and with 60 μM tBHP/100 g body weight (treated) daily for 9 days. Whole sperm 4HNE and nitrotyrosine levels were quantified using Western blot. 4HNE and nitrotyrosine levels of non-permeabilized spermatozoa and sperm DNA oxidation levels were quantified using immunocytochemistry. Mutant spermatozoa had impaired capacitation and motility. Percentages of spermatozoa with CDR, whole sperm 4HNE and nitrotyrosine levels, number of unsuccessful matings and litter sizes of control and treated mutant mice were higher compared to that of their wild-type counterparts. All mutant groups, except for control D140A mice, had higher nitrotyrosine levels within their non-permeabilized spermatozoa and percentages of spermatozoa with positive DNA oxidation labelling compared to control wild-type mice. Control mutants had higher 4HNE levels within their non-permeabilized

spermatozoa compared to control wild-type mice. In conclusion, the peroxidase and $iPLA_2$ activities of PRDX6 are essential to ensure male mouse fertility.

During the preparation of this thesis, I contributed to the experimental design, performed all experiments and data analysis.

Résumé

Actuellement, un couple sur six souffre d'infertilité et la moitié des cas sont dus à l'infertilité masculine. Le stress oxydatif dû à des niveaux élevés d'espèces réactives de l'oxygène (ROS) et/ou à de faibles niveaux d'enzymes antioxydantes favorise l'infertilité en induisant une peroxydation lipidique, des dommages à l'ADN, une altération de la motilité et de la capacitation des spermatozoïdes et une perturbation de l'activité mitochondriale. Le 4-hydroxynonénal (4HNE), un produit de peroxydation lipidique, augmente les niveaux d'anion superoxyde et d'oxyde nitrique responsables de la nitration de la tyrosine des protéines, altérant ainsi la motilité et la capacitation des spermatozoïdes. La Peroxiredoxine 6 (PRDX6) protège les spermatozoïdes du stress oxydatif grâce à ses activités de peroxydase et de phospholipase A_2 indépendante du Ca^{2+} ($iPLA_2$) qui enlèvent les ROS et réparent les membranes oxydées respectivement. Les spermatozoïdes de patients infertiles ont de faibles niveaux de PRDX6 qui sont associés à une motilité altérée, des niveaux élevés de peroxydation lipidique et des dommages à l'ADN. Ces paramètres anormaux ont également été observés dans les spermatozoïdes de souris *Prdx6*$^{-/-}$, entraînant des tailles de portée plus faibles par rapport aux souris de type sauvage, et ont été aggravés lorsque les mâles ont été traités avec de l'hydroperoxyde de tert-butyle (tBHP), un inducteur de stress oxydatif *en vivo*. Nous émettons l'hypothèse que l'absence de PRDX6 peroxydase ou d'$iPLA_2$ conduit à l'infertilité observée chez les souris *Prdx6*$^{-/-}$. Des spermatozoïdes provenant de souris (âgées de deux mois) C57Bl6/J (type sauvage), *Prdx6*$^{-/-}$, C47S et D140A (peroxydase et $iPLA_2$-déficientes) ont été incubés dans du PBS pour évaluer la motilité. Les pourcentages de spermatozoïdes non capacités et capacités (traités avec milieu BWW + BSA/bicarbonate) ayant subi la réaction acrosomique ont été quantifiés par coloration au Giemsa. Le nombre d'accouplements infructueux, la taille des portées et les pourcentages de spermatozoïdes avec rétention de gouttelettes cytoplasmiques (CDR) ont été enregistrés pour les souris traitées avec une solution saline (contrôles) et avec 60 μM de tBHP/100 g de poids corporel (traitées) quotidiennement pendant 9 jours. Les niveaux de 4HNE et de nitrotyrosine dans le spermatozoïde entier ont été quantifiés par Western blot. Les niveaux de 4HNE et de nitrotyrosine de spermatozoïdes non perméabilisés, ainsi que les niveaux d'oxydation de l'ADN des spermatozoïdes ont été quantifiés par immunocytochimie. Les spermatozoïdes mutants avaient une capacitation et une motilité altérées. Les pourcentages de spermatozoïdes avec CDR, les niveaux de 4HNE et de nitrotyrosine dans le spermatozoïde entier, le nombre

d'accouplements infructueux et la taille des portées de souris mutantes contrôles et traitées étaient plus élevés que ceux de souris de type sauvage ayant subi le même traitement. Toutes les souris mutantes, à l'exception de souris D140A contrôles, avaient des niveaux plus élevés de nitrotyrosine dans leurs spermatozoïdes non perméabilisés ainsi que de pourcentages de spermatozoïdes ayant de niveaux d'oxydation de l'ADN élevés par rapport aux souris contrôles de type sauvage. Les mutants contrôles avaient des niveaux de 4HNE plus élevés dans leurs spermatozoïdes non perméabilisés par rapport aux souris contrôles de type sauvage. En conclusion, la peroxydase et l'iPLA$_2$ de la PRDX6 sont essentielles pour assurer la fertilité de souris mâles.

Lors de la préparation de cette thèse, j'ai contribué à la conception expérimentale, effectué toutes les expériences et analysé les données résultantes.

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1. Introduction

1.1 From spermatogenesis to fertilization: the spermatozoon's journey

1.1.1 Spermatogenesis

Spermatogenesis commences at puberty and is defined as the formation of spermatozoa from spermatogonia (germ cells) within the testis (Roosen-Runge et al., 1977). This process is divided into three steps, namely spermatocytogenesis, spermiogenesis, and spermiation (Valli et al., 2015). During spermatocytogenesis, spermatogonia proliferate through mitosis (Holstein et al., 2003), then differentiate into diplotene spermatocytes (Rowley & Heller, 1971; Feng et al., 2014). Both processes are regulated by spermatogonial cKIT receptor and cKIT ligand provided by Sertoli cells (Sandlow et al., 1996; Blume-Jensen et al., 2000). Maturation of diplotene spermatocytes into secondary spermatocytes requires meiosis I, a process that involves DNA replication and interaction between homologous chromosomes (Holstein et al., 2003; Feng et al., 2014). Thereafter, each secondary spermatocyte matures into 4 haploid spermatids through meiosis II (Feng et al., 2014; Matsumoto & Bremner, 2016). Energy required for the maturation of spermatocytes is provided by lactate, which is produced by Sertoli cells (Dias et al., 2014). Spermiogenesis involves the differentiation of haploid spermatids into spermatozoa and is initiated and regulated by the release of testosterone by Leydig cells (Clermont, 1963; Scialli, 1997). Spermiation involves the release of immotile testicular spermatozoa from Sertoli cells towards the efferent ducts that lead to the epididymis (Russell & Griswold, 1993). Additionally, during spermatogenesis, chromatin compaction occurs through the replacement of histones with protamines (Goldberg et al., 1977).

1.1.2 Structural components of spermatozoa

Spermatozoa is a compartmentalized cell that has a head and flagellum (Figure 1). The head of spermatozoa is composed of a nucleus that contains condensed chromatin, an acrosome that contains hydrolytic enzymes that facilitate sperm penetration through zona pellucida to promote fertilization of the oocyte, minimal amount of cytoplasm, and cytoskeletal structures involved in capacitation, notably actin (Yanagimachi, 1994; Jeon et al., 2001; Fukami et al., 2003; Breitbart et al., 2005; Eddy, 2006). Moreover, the head of spermatozoa also has an equatorial segment, which contains proteins involved in the fusion of the sperm plasma membrane with the oolema

(Yanagimachi, 1994). The post-acrosomal region of the sperm head also includes proteins involved in spermatozoa-oocyte fusion and is the primary site of the influx of calcium ions (Ca^{2+}) required for the induction of acrosome reaction, which is the release of hydrolytic enzymes that permit zona pellucida penetration (Kawai et al., 1989; Jeon et al., 2001; Fukami et al., 2003). The flagellum of spermatozoa is divided into the midpiece, principal piece and end piece (Figure 1) (Eddy, 2006). Within the midpiece, the mitochondrial sheath forms a helical structure that surrounds the 9 outer dense fibres and axoneme (Figure 1). It contains mitochondria that is responsible for the generation of energy through Kreb's cycle and oxidative phosphorylation in some species such as human and equine (Fernie et al., 2004; Eddy, 2006; Piomboni et al., 2012; Amaral et al., 2013; Darr et al., 2016; Davila et al., 2016). The principal piece is composed of an axoneme, outer dense fibres and fibrous sheath, while the end piece only consists of an axoneme (Figure 1). In the principal piece, the enzymes required for glycolysis are associated to the fibrous sheath (Eddy, 2006). Delimiting the entire spermatozoa structure is a plasma membrane composed of polyunsaturated fatty acids that causes the spermatozoa to be sensitive to lipid peroxidation (Jones et al., 1979; Alvarez & Aitken, 2012).

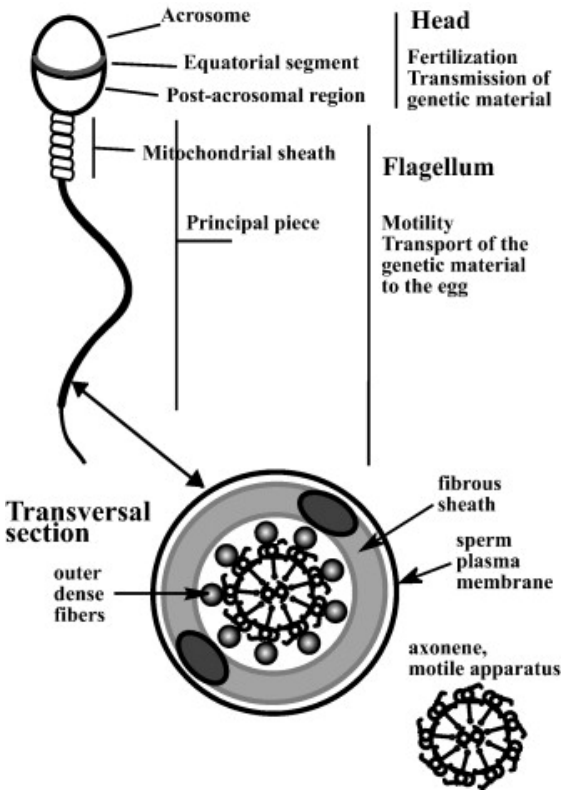

Figure 1: Structural morphology of human spermatozoa

Spermatozoa is composed of a head and flagellum. Acrosome, equatorial segment and post-acrosomal region are localized within the sperm head. The flagellum has a mitochondrial sheath, principal piece and end piece. The transverse view of the principal piece depicts an axoneme surrounded by 9 outer dense fibres, which are surrounded by a fibrous sheath. The figure was taken from de Lamirande & O'Flaherty (2008).

1.1.3 Sperm epididymal maturation

Sperm epididymal maturation involves the transit of spermatozoa along the epididymis, a tubule connecting the testis to vas deferens, and is required for the spermatozoon to acquire motility and the ability to fertilize oocytes (Sommer et al., 1996; Robaire et al., 2006). The most abundant cells of the epididymal epithelium are principal cells, which are important for the endocytosis of proteins from the epididymal fluid and the release of epididymosomes, which are vesicles that are derived from the epididymal epithelium and are important for sperm maturation as they supply enzymes involved in sperm motility, antioxidant protection, cell adhesion and oocyte recognition (Abe et al., 1983; Hermo et al., 1988; Saez et al., 2003; Robaire et al., 2006; Sullivan et al., 2007; Sullivan, 2008; Thimon et al., 2008; Cornwall, 2009; Sullivan, 2015; Liu &

O'Flaherty, 2017). Clear cells within the epididymal epithelium are also essential for sperm maturation since they are responsible for the removal of sperm cytoplasmic droplets through endocytosis (Hermo et al., 1988; Sullivan & Belleannée, 2018). This event allows the sperm cytoplasm, which contains both superoxide dismutase (SOD), an antioxidant enzyme that converts superoxide anion ($O_2^{\bullet-}$) into H_2O_2 (Griveau et al., 1995; Kehrer, 2000), and glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that provides NADPH required for ROS formation, to be removed during epididymal transit to prevent excessive ROS production in maturing spermatozoa (Aitken et al., 1994; Aitken et al., 1996). During sperm epididymal transit, glycocalyx, which is a thick layer of carbohydrates that surround sperm membrane and is important for sperm maturation, acquisition of motility and fertilizing competence (Schröter et al., 1999; Tollner et al., 2011; Tollner et al., 2012), is formed through the modification of glycans by glycosidases and glycosyl transferases provided by epididymal epithelial cells (Bernal et al., 1980; Tulsiani, 2006). Additionally, epididymis has DICER1, which is an enzyme that can modify various RNAs, notably microRNAs and non-coding RNAs (Li & Liu, 2011). Removal of DICER1 in mice leads to impaired synthesis of polyunsaturated fatty acids (PUFAs) and increased release of cholesterol into the epididymal lumen, therefore causing spermatozoa to be unable to achieve fertilizing competence due to inadequate sperm membrane lipid composition (Björkgren et al., 2012). Additionally, during sperm epididymal transit, disulfide bridges between thiol groups of protamines' cysteine (Cys) residues are formed to render chromatin more compact and to prevent the paternal genome from direct oxidation by reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) (Shalgi et al., 1989; Seligman & Shalgi, 1991; Kino & Sugiyama, 2000).

1.1.4 Sperm ejaculation

Upon ejaculation, mature spermatozoa enter in contact with fluids secreted by both seminal vesicles and the prostate (Robert & Gagnon, 1999; de Lamirande, 2007). Seminal plasma is rich in zinc ions (Zn^{2+}) and semenogelin, which is the protein needed to form the coagulum (Robert & Gagnon, 1999; Jonsson et al., 2005; de Lamirande, 2007). Zn^{2+} is required for DNA compaction and for the inhibition of prostate-specific antigen (PSA), a protease required for semenogelin removal to dissolve the coagulum (Robert & Gagnon, 1999; Jonsson et al., 2005; de Lamirande, 2007). Sperm capacitation is an event required for spermatozoa to achieve fertilizing

competence (Yanagimachi, 1994; de Lamirande & Gagnon, 1995; O'Flaherty et al., 2003; Florman & Ducibella, 2006; de Lamirande & O'Flaherty, 2012). Both Zn^{2+} and semenogelin inhibit sperm oxidase, an enzyme responsible for producing ROS required for capacitation, preventing premature capacitation (de Lamirande, 2007; de Lamirande & Lamothe, 2010). However, when spermatozoa reach the oviduct, Zn^{2+} and semenogelin are removed to permit sperm oxidase activation and the subsequent ROS production to initiate capacitation (O'Flaherty et al., 2004; O'Flaherty, de Lamirande, et al., 2006b).

1.1.5 Sperm capacitation, acrosome reaction

During sperm capacitation, which occurs within the female reproductive tract, specifically in the oviduct, and is a complex process characterized by changes in the physiological and biochemical properties of the spermatozoa required to successfully fertilize the oocyte, membrane fluidity increases due to cholesterol efflux, which enhances the permeability of sperm membrane to Ca^{2+} and bicarbonate (HCO_3^-) required to activate soluble adenylyl cyclase (sAC) (Austin & Bishop, 1958; Yanagimachi, 1994; Visconti et al., 1999; Flesch & Gadella, 2000; Gadella & Van Gestel, 2004; de Lamirande & O'Flaherty, 2012). sAC stimulates the production of cAMP necessary to activate protein kinase A (PKA), which phosphorylates substrates (O'Flaherty et al., 2004) involved in the phosphorylation of mitogen-activated kinase-like (MEK-like) proteins (O'Flaherty et al., 2005) and in the activation of tyrosine kinases (Leclerc et al., 1996). Phosphorylated MEK-like proteins and PKA promote activation of protein tyrosine kinases (PTKs), which phosphorylate protein tyrosine residues during capacitation (Visconti et al., 1995; Leclerc et al., 1996; O'Flaherty, de Lamirande, et al., 2006a). Capacitation also involves increased permeability of Ca^{2+} channels, enhanced intracellular pH, and hyperpolarization of the sperm plasma membrane (Zeng et al., 1995; Arnoult et al., 1999; Chávez et al., 2012; Orta et al., 2018). Energy and phosphate groups utilized for protein phosphorylation are provided by ATP produced through glycolysis and lactate dehydrogenase C4 (LDH-C4), an enzyme that converts NADH and lactate into pyruvate, which through glycolysis, is converted into acetyl-CoA that enters the Krebs cycle to generate reducing equivalents required for the production of ATP through mitochondrial oxidative phosphorylation (O'Flaherty et al., 2002; O'Flaherty, Beorlegui, et al., 2006; O'Flaherty, de Lamirande, et al., 2006a). Sperm capacitation also involves actin polymerization, a process that is required for the trafficking of phospholipase C (PLC) towards

the sperm membrane (Breitbart et al., 2005). PLC promotes Ca^{2+} influx to provide hyperactivated sperm motility and to promote acrosome reaction (Yanagimachi, 1994; Jeon et al., 2001; Fukami et al., 2003; Eddy, 2006).

1.2 Male infertility

1.2.1 Oxidative stress in male infertility

Currently, 1/6 of couples, which account for approximately 60-80 million couples, suffer from infertility worldwide (Comhaire & World Health Organization, 1987; Bushnik et al., 2012). Moreover, half of the infertility cases are caused by male infertility (Comhaire & World Health Organization, 1987; Bushnik et al., 2012). Treatment of male infertility is difficult because 60-75% of male infertility cases are unexplained (idiopathic infertility) (Wein A et al., 2012). Additionally, infertility can be caused and amplified by various medical conditions/factors, including varicocele, pyospermia, excessive exposure to pollutants, smoke, radiation, and drugs (Anderson & Williamson, 1988; Shy et al., 1988; Brennemann et al., 1997; Hasegawa et al., 1997; Smith et al., 2006; Harris et al., 2011; Said et al., 2012). Interestingly, these pathologies/factors contribute to oxidative stress caused by high levels of ROS and/or low levels of antioxidant enzymes (Anderson & Williamson, 1988; Brennemann et al., 1997; Hasegawa et al., 1997; Turner, 2001; Halliwell, 2006; Smith et al., 2006; Halliwell & Gutteridge, 2007; Agarwal et al., 2008; Said et al., 2012; Agarwal et al., 2014). Indeed, at least 30% of infertile men have high levels of seminal ROS (Iwasaki & Gagnon, 1992; Zini et al., 1993; Tremellen, 2008).

Varicocele is the widening of pampiniform venous plexus and is associated with elevated ROS production and impaired antioxidant activity within seminal plasma (Hendin et al., 1999; Alsaikhan et al., 2016). Pyospermia is a medical condition characterized by the presence of a high number of leukocytes ($>1x10^6$ leukocytes/mL) in the semen (Brunner et al., 2019). Pyospermia is associated with oxidative stress in semen since leukocytes are sources of ROS (Agarwal et al., 2014), and pyospermia patients have low SOD levels (Fridovich, 1997; Pasqualotto et al., 2008; Miriyala et al., 2011). Consequentially, spermatozoa from patients with pyospermia suffer from structural abnormalities, notably tail defects (Aziz et al., 2004; World Health, 2010). Additionally, patients who do not have pyospermia still have elevated ROS levels

within their semen, suggesting that spermatozoa from these patients are also vulnerable to oxidative stress (Agarwal et al., 2014).

Male infertility is driven, in part, by the capacity of oxidative stress to promote lipid peroxidation (Jones et al., 1979; Alvarez & Aitken, 2012), DNA damage in the form of DNA fragmentation and oxidation (Aitken et al., 1998; Barroso et al., 2000), decrease in mitochondrial membrane potential (Gallon et al., 2006; Koppers et al., 2008) and inhibition of enzymes involved in sperm motility (de Lamirande & Gagnon, 1992a, 1992b). For instance, compared to non-smokers, smokers have a higher concentration of leukocytes within their semen, which results in higher ROS levels associated with increased DNA oxidation and decreased protamination (Trummer et al., 2002; Hammadeh et al., 2010; Yamauchi et al., 2012; Zhang et al., 2013). Human spermatozoa lack enzymes involved in base excision repair, notably apurinic endonuclease 1, and the majority of damage within the paternal genome remains after oocyte fertilization; therefore, the presence of efficient antioxidant machinery is important to ensure male fertility (Yamauchi et al., 2012; Smith et al., 2013). Another factor that could promote oxidative stress is the presence of immature spermatozoa that produce high ROS levels, which can endanger healthy spermatozoa within the semen (Gil-Guzman et al., 2001; Ollero et al., 2001). Furthermore, aging is also associated with oxidative stress since spermatozoa from aging male rats have higher ROS levels and lower levels of antioxidant enzymes compared to younger counterparts (Weir & Robaire, 2007).

1.2.2 Negative effects of ROS within the spermatozoa

Spermatozoa are highly susceptible to oxidative stress due to the presence of polyunsaturated fatty acids (PUFAs) within their plasma membrane (Jones et al., 1979; Alvarez & Aitken, 2012). Lipid peroxidation involves the oxidation of PUFAs through a series of reactions between radicals, resulting in the production of lipid hydroperoxides (ROOH) (Yin et al., 2011; Saxena, 2014). Due to their instability, ROOH are degraded to form injurious products, notably aldehydes, ketones, carboxylic acids and 4-hydroxynonenal (4HNE), that lead to cellular toxicity (Benedetti et al., 1980; Saxena, 2014).

In the mitochondria, the process of oxidative phosphorylation, which is required to produce energy in the form of ATP, produces $O_2^{\bullet-}$ (Hatefi, 1985; Du et al., 1998), which can dismutate into H_2O_2 spontaneously or through the activity of SOD2, which is the SOD isoform localized within mitochondria (Griveau et al., 1995; Kehrer, 2000; Prasad et al., 2018). $O_2^{\bullet-}$ also reacts with nitric oxide ($NO^{\bullet}$) to form $ONOO^-$ (Griveau et al., 1995; Packer et al., 1996; Herrero et al., 2001). These ROS promote lipid peroxidation, and the 4HNE produced forms adducts with several proteins, including succinate dehydrogenase, a mitochondrial enzyme involved in the electron transport chain, to generate $O_2^{\bullet-}$ and restart the vicious cycle of ROS generation and lipid peroxidation (Aitken et al., 2012).

H_2O_2 and tertbutyl hydroperoxide (tBHP), an *in vivo* oxidative stress inducer, promote s-glutathionylation characterized as the reversible incorporation of glutathione into thiol groups to impair protein activity (Humphries et al., 2002; Morielli & O'Flaherty, 2015; Lee et al., 2017). $NO^{\bullet}$ and $ONOO^-$ promote tyrosine nitration, which induces irreversible inactivation of proteins (Gow et al., 1996; O'Flaherty & Matsushita-Fournier, 2017). ROS impair sperm motility by inducing s-glutathionylation and tyrosine nitration of tubulin, a structural protein found within sperm flagellum, and enzymes involved in energy generation, namely enzymes involved in glycolysis and Kreb's cycle (Fratelli et al., 2004; Landino et al., 2004; Morielli & O'Flaherty, 2015). Indeed, sperm motility is impaired in human spermatozoa treated with H_2O_2 or Da-NONOate ($NO^{\bullet}$ donor) at concentrations that do not impair sperm viability (Morielli & O'Flaherty, 2015).

Previous studies have shown that high ROS levels can inhibit PKA and protein kinase C (PKC) (Ward et al., 2000; Humphries et al., 2002). Indeed, during human sperm capacitation, phosphorylation of PKA substrates was prevented by oxidative stress generated by inhibition of peroxiredoxins (PRDXs) (Lee et al., 2017), which are antioxidant enzymes present within all of the sperm's subcellular compartments (O'Flaherty & Rico de Souza, 2010). H_2O_2- and Da-NONOate-treated human spermatozoa have lower levels of PKA- and PKC-dependent tyrosine phosphorylation compared to untreated spermatozoa, confirming that ROS promote impairment of sperm capacitation (Morielli & O'Flaherty, 2015). 4HNE also has the capacity to inhibit both PKC and phosphorylation-induced activation of extracellular signal-regulated kinase (ERK) in

rat hepatocytes (Pronzato et al., 1990; Chiarpotto et al., 1999; Sampey et al., 2007). Impairment of sperm capacitation could also be due to the prevention of actin polymerization since actin can be oxidized by lipid peroxides within the brain and can undergo s-glutathionylation (Dalle-Donne et al., 2005; Bizzozero et al., 2007).

ROS also promote lipid peroxidation of epididymal epithelium since tBHP-treated rats have higher 4HNE levels within their cauda epididymis at 3 and 6 weeks post-treatment (Wu et al., 2020). ROS can also directly oxidize DNA bases, with guanosine being the most affected to form 8-hydroxy-2'-deoxyguanosine (8-OHdG), while 4HNE forms adducts with DNA promoting mutations in the paternal genome (Kino & Sugiyama, 2000; Zhong & Yin, 2015). ROS are also involved in preventing compaction of chromatin as evidenced by the low protamination levels observed in smokers, which then result in impaired protection of the paternal genome from ROS-induced DNA damage (Trummer et al., 2002; Hammadeh et al., 2010; Yamauchi et al., 2012; Zhang et al., 2013). Currently, it is unknown whether ROS prevent the production of protamines or impair the replacement of histones with protamines during spermatogenesis however, DNA oxidation and fragmentation levels are negatively correlated with protamination levels (Ozkosem et al., 2016). Also, smaller litter sizes are associated with impaired compaction of chromatin and increased levels of DNA fragmentation and oxidation in mouse spermatozoa (Ozkosem et al., 2016).

1.2.3 Positive effects of ROS within spermatozoa

Despite their injurious effects on many events required for maintaining fertility, ROS, at low levels, are important for cell signalling events such as sperm capacitation (O'Flaherty, de Lamirande, et al., 2006b; de Lamirande & O'Flaherty, 2012). During the early stage of capacitation, H_2O_2 and $O_2^{\bullet-}$ produced by sperm oxidase (de Lamirande et al., 1997; O'Flaherty et al., 2003) and $NO^{\bullet}$ produced by nitric oxide synthase (NOS) (Herrero et al., 1997; Herrero et al., 1999; Belén Herrero et al., 2000) activate sAC to stimulate PKA activity (Figure 2) (Tan et al., 1995; Persad et al., 1997). H_2O_2 activates PKC pathway (O'Flaherty, de Lamirande, et al., 2006b), while $O_2^{\bullet-}$ and $NO^{\bullet}$ stimulate ERK pathway during sperm capacitation (Figure 2) (de Lamirande & Gagnon, 2002; Thundathil et al., 2003). Phosphorylated PKA substrates (O'Flaherty, de Lamirande, et al., 2006b) and rapidly accelerated fibrosarcoma (RAF) activated

by both PKC and ERK pathways phosphorylate MEK-like proteins to ultimately induce phosphorylation of protein tyrosine residues by PTKs during late capacitation (Figure 2) (Gopalakrishna & Anderson, 1989; O'Flaherty, de Lamirande, et al., 2006b). To maintain phosphorylation status of proteins, $O_2^{\bullet -}$ and $NO^{\bullet}$ inhibit protein phosphatases (PP), while H_2O_2 and $NO^{\bullet}$ inhibit protein tyrosine phosphatases (PTP) (Figure 2) (Hecht & Zick, 1992; Sommer et al., 2002; Chiarugi & Cirri, 2003; Salmeen & Barford, 2005). Additionally, H_2O_2 produced through sperm oxidase activation is utilized by glutathione peroxidases (GPXs) as hydrogen receivers to promote chromatin compaction during epididymal maturation of spermatozoa (Aitken & Vernet, 1998).

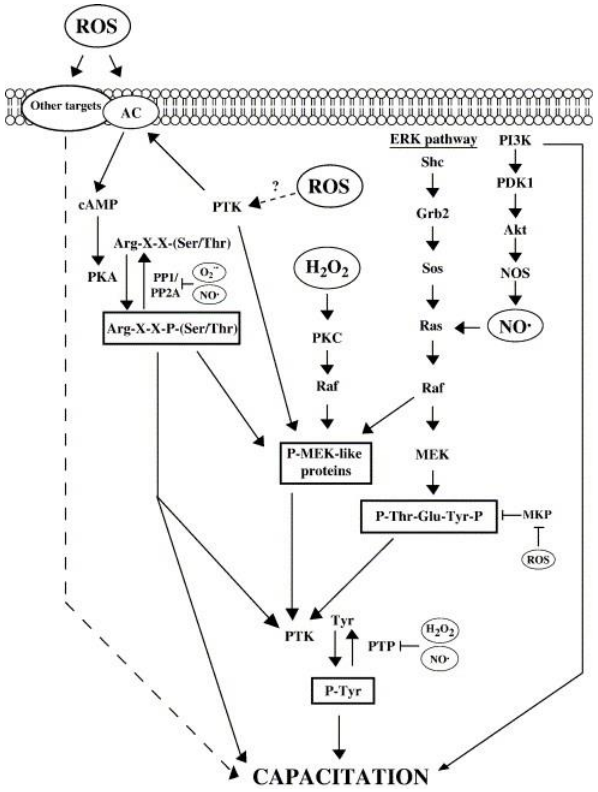

Figure 2: Schematic representation of the mechanisms involved in sperm capacitation

ROS promote sAC activation to induce activation of PKA required to phosphorylated PKA substrates within their Arg-X-X-(Ser/Thr) motifs. ROS, namely H_2O_2 and $NO^{\bullet}$, regulate capacitation by stimulating PKC and ERK pathways respectively to promote RAF activation. Phosphorylated PKA substrates and RAF phosphorylate MEK-like proteins, which activate PTKs that phosphorylate protein tyrosine residues during late capacitation. ROS regulates capacitation since $O_2^{\bullet -}$ and $NO^{\bullet}$ inhibit PP, while H_2O_2 and $NO^{\bullet}$ inhibit PTP. The figure was taken from O'Flaherty, de Lamirande et al., (2006a).

1.3 Antioxidant system of human spermatozoa

1.3.1 ROS scavengers within spermatozoa

Antioxidant enzymes present within spermatozoa include SOD, which consists of 3 isoforms: copper/zinc SOD (Cu/Zn SOD; SOD1) localized within cytosol, manganese SOD (MnSOD; SOD2) localized within the mitochondria and SOD3 (secretory isoform) (Prasad et al., 2018). As previously mentioned, SODs convert $O_2^{\bullet-}$ into H_2O_2 (Griveau et al., 1995; Kehrer, 2000). SOD1 and SOD3 are responsible for 75% and 25% of enzymatic activity within the seminal plasma, respectively (Peeker et al., 1997). In somatic cells, the H_2O_2 produced by SOD activity can then be scavenged by catalase present in peroxisomes (Chance et al., 1979; Schrader & Fahimi, 2004; Balaban et al., 2005; Kehrer et al., 2010; Ighodaro & Akinloye, 2018). However, spermatozoa lack catalase since the peroxisomes are eliminated from spermatids during spermatogenesis (Luers et al., 2006; Nenicu et al., 2007). Another group of antioxidant enzymes present in somatic cells are glutathione peroxidases (GPXs), a group of selenium-dependent (GPX1, GPX2, GPX3, and GPX4) or selenium-independent (GPX5, GPX6, GPX7, GPX8, and GPX9) enzymes that can scavenge H_2O_2 and $ONOO^-$ (Ursini et al., 1985; Takahashi et al., 1987; Ghyselinck et al., 1989; Chu et al., 1993; Sies et al., 1997; Herbette et al., 2007). Mitochondrial GPX4 (mGPX4) reacts with lipid hydroperoxides to form the mitochondrial sheath during spermiogenesis (Ursini et al., 1999; Foresta et al., 2002), while nuclear GPX4 (nGPX4) prevents the oxidation of protamines' thiol groups to prevent DNA damage (Conrad et al., 2005). Peroxiredoxins are antioxidant enzymes that are present throughout the entire sperm cell, can reduce oxidized phospholipids, and can scavenge $ONOO^-$, inorganic and organic hydroperoxides (Zhang et al., 1997; Peshenko & Shichi, 2001; Dubuisson et al., 2004; O'Flaherty & Rico de Souza, 2010; O'Flaherty, 2014b).

1.3.2 Vulnerability of spermatozoa to oxidative stress

Spermatozoa is highly susceptible to oxidative stress since cytosolic SOD1, which is the primary sperm SOD isoform, exists at minimal levels due to the removal of peroxisomes during spermatogenesis (Zini et al., 1993; Aitken et al., 1994; Aitken et al., 1996). The protection provided by other SOD isoforms is negligible since mitochondrial SOD2 and extracellular SOD3 levels in human spermatozoa are insignificant (Peeker et al., 1997). Contrary to somatic cells, which possess high levels of catalase (Chance et al., 1979; Schrader & Fahimi, 2004),

spermatozoa lack catalase since during spermatogenesis, catalase-containing peroxisomes are removed (Luers et al., 2006; Nenicu et al., 2007). Furthermore, treatment of human spermatozoa with sodium azide, a catalase inhibitor, failed to increase sperm H_2O_2 and lipid peroxidation levels, suggesting that catalase is absent in human spermatozoa (O'Flaherty, 2014a). Human spermatozoa, testes and seminal plasma do not contain GPX2, GPX3, GPX4, or GPX5 (Williams et al., 1998; Chabory et al., 2010). GPX1 can scavenge H_2O_2 by using reduced glutathione (GSH) (Lubos et al., 2011); however GPX1$^{-/-}$ male mice remain fertile (Ho et al., 1997). Indeed, no significant increase in lipid peroxidation levels was observed for human spermatozoa treated with H_2O_2 coupled with inhibitors that impair GPX1 activity, such as carmustine (glutathione reductase inhibitor) or diethyl maleate (GSH scavenger), compared to untreated controls (O'Flaherty, 2014a). These findings confirm that maintenance of fertility in men doesn't require GPX1 (Ho et al., 1997; O'Flaherty, 2014a). Additionally, GPX4 isoforms are not essential to protect mature spermatozoa against oxidative stress since mGPX4 lacks antioxidant activity in mature human spermatozoa as it is an insoluble structural protein forming part of the mitochondrial sheath (Ursini et al., 1999; Foresta et al., 2002), and despite having higher levels of free thiols within their sperm head compared to wild-type, nGPX4$^{-/-}$ mice remain fertile (Conrad et al., 2005).

1.3.3 Characterization of Peroxiredoxins (PRDXs)

Peroxiredoxins are selenium-independent and acidic antioxidant enzymes that are present in all eukaryotes and prokaryotes (Rhee et al., 2001; Hofmann et al., 2002; Wood, Poole, et al., 2003; Rhee et al., 2005). PRDXs are classified as either 1-Cys or 2-Cys PRDXs, depending on having one and two cysteine residues in their active site, respectively (Rhee et al., 2001). PRDXs have a peroxidase activity that scavenges H_2O_2, organic hydroperoxides and $ONOO^-$ (Fisher, 2017). 2-Cys PRDXs include PRDXs1-5 (Rhee et al., 2001), and after reacting with ROS, they become oxidized and inactive (Figure 3). To re-activate 2-Cys PRDXs, they must be reduced by the thioredoxin-thioredoxin reductase (TRX/TRD) system, which requires NADPH provided by glucose-6-phosphate dehydrogenase (G6PDH), NADP isocitrate dehydrogenase (NADP-ICDH) and malic enzyme (ME) in spermatozoa (Figure 3) (Fernandez & O'Flaherty, 2018). In somatic cells, hyperoxidized 2-Cys PRDXs are reactivated by sulfinic acid reductases, such as sulfiredoxin (SRX), which is absent within spermatozoa, and sestrins (Figure 3) (Chang et al.,

2004; Jönsson & Lowther, 2007; Baek et al., 2012; Abbas et al., 2013; O'Flaherty, 2019). PRDX6 is the only 1-Cys PRDX, and when oxidized in somatic cells, its reactivation requires GSH and glutathione-S-transferase pi (GSTpi); however, it remains unclear whether GSTpi is present within human spermatozoa (Figure 4) (Ralat et al., 2006; Ralat et al., 2008; Fernandez & O'Flaherty, 2018). Contrary to hyperoxidized 2-Cys PRDXs, hyperoxidized PRDX6 can no longer be reactivated (Figure 4) (Manevich et al., 2009; O'Flaherty, 2019). During oxidative stress, hyperoxidized PRDXs form high molecular mass complexes with each other, therefore marking the transition of PRDXs from serving as ROS scavengers to serving as chaperones to prevent protein misfolding and/or unfolding (Figures 3, 4) (Jang et al., 2004; Rhee et al., 2005; Chae et al., 2012; O'Flaherty, 2019).

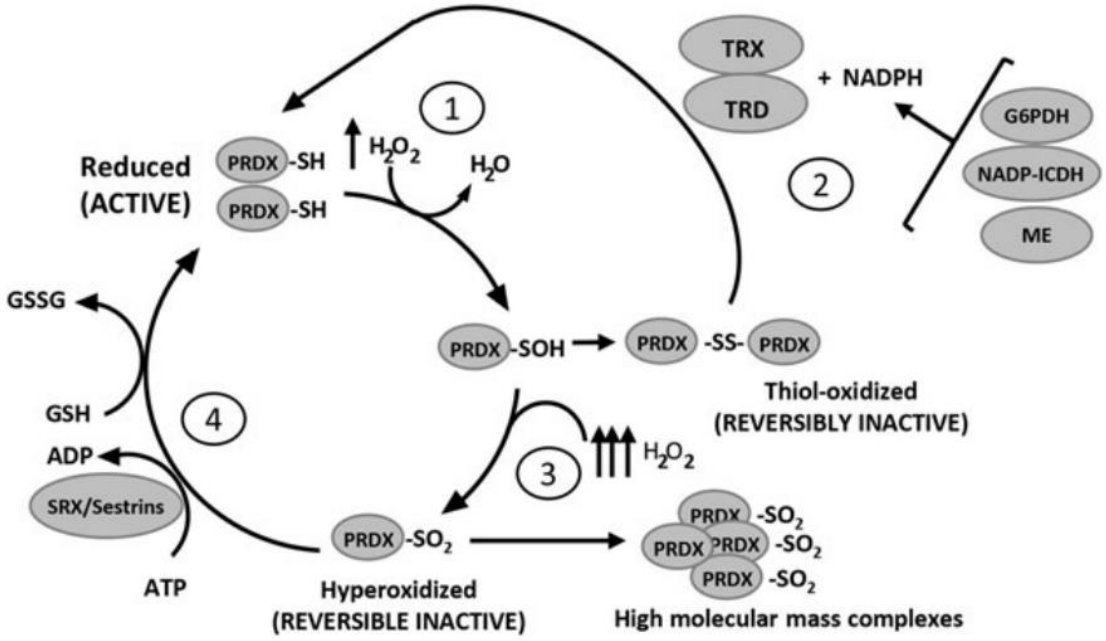

Figure 3: Schematic representation of the reactivation system of 2-Cys PRDXs

(1) 2-Cys PRDXs become inactivated through H_2O_2-mediated thiol oxidation. (2) NADPH required to reactivate oxidized 2-Cys PRDXs are provided by G6PDH, NADP-ICDH and ME. (3) Increasing H_2O_2 levels results in hyperoxidized 2-Cys PRDXs, which form high molecular mass complexes to serve as chaperones that prevent protein misfolding/unfolding. (4) SRX and sestrins utilize ATP to reactivate hyperoxidized 2-Cys PRDXs, then GSH is used to form reduced 2-Cys PRDXs. The figure was taken from Scarlata & O'Flaherty (2020).

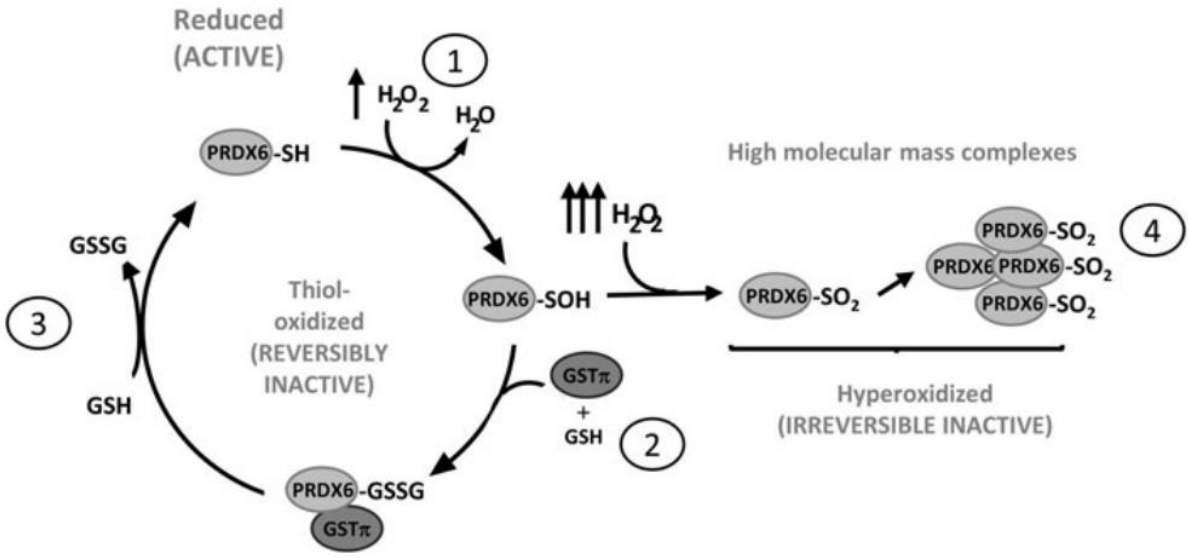

Figure 4: Schematic representation of the reactivation system of PRDX6

(1) PRDX6 becomes inactivated through H_2O_2-mediated thiol oxidation. (2) GSTpi utilizes reduced GSH to reactivate oxidized PRDX6. (3) Another reduced GSH is used to fully reactivate oxidized PRDX6. (4) High H_2O_2 levels result in the formation of hyperoxidized PRDX6 that forms high molecular mass complexes with other hyperoxidized PRDXs since its reactivation can no longer occur. The figure was taken from Scarlata & O'Flaherty (2020).

Physiologically, PRDXs oxidation leads to an increase in ROS levels important for cell signalling, then GST/GSTpi and TRX/TRD system reduce oxidized PRDX6 and 2-Cys PRDXs, respectively, to decrease ROS levels (Wood, Poole, et al., 2003; Wood, Schröder, et al., 2003; O'Flaherty, 2014a). However, PRDXs hyperoxidation and cellular toxicity occur when deficient reducing equivalents are present due to the inactivity of enzymes involved in PRDXs reactivation (O'Flaherty, 2014a). Furthermore, NADPH deficiency also indirectly affects PRDX6 reactivation since the TRX/TRD system can also increase reduced GSH, which normally exists in low levels within spermatozoa (Li, 1975; Sun et al., 2001). During oxidative stress, spermatozoa cannot produce sufficient NADPH required to reactivate oxidized 2-Cys PRDXs and PRDX6 since elevated H_2O_2 levels lead to G6PDH inhibition (Griveau et al., 1995).

Peroxiredoxins are essential players in the antioxidant protection and redox signalling in different cell types, as evidenced by the fact that PRDX1 absence promotes higher susceptibility

to tumours, shorter lifespan, and elevated levels of DNA damage in mice (Neumann et al., 2003; Egler et al., 2005). Moreover, PRDX2 removal leads to faster senescence in embryonic fibroblasts displaying oxidative stress (Han et al., 2005). PRDXs are also involved in male reproduction since PRDX2 forms a complex with GPX4 to construct the mitochondrial sheath during spermiogenesis (Manandhar et al., 2009). Additionally, PRDX4 is important for acrosome formation in rats and for the prevention of testicular atrophy and DNA damage in mice spermatozoa (Sasagawa et al., 2001; Iuchi et al., 2009). PRDX5 is hypothesized to be important for the ability of spermatozoa to bind to zona pellucida since it is localized within the membrane of boar spermatozoa (van Gestel et al., 2007).

Among the members of the PRDXs family, PRDX6 is the only one that possesses Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) and lysophosphatidylcholine acyltransferase (LPCAT) that respectively removes oxidized phospholipids and reintegrates reduced fatty acids into sperm's plasma membrane (Fisher, 2017). Peroxidase activity of PRDX6 requires cysteine 47 (C47) residue localized within the pocket of the protein (Figure 5). In contrast, $iPLA_2$ activity of PRDX6 requires a catalytic triad formed by serine 32, aspartic acid 140 and histidine 26 (S32, D140, H26, respectively) localized on the protein surface (Figure 5) (Manevich et al., 2009). Head of oxidized phospholipids binds to the active site of $iPLA_2$, while the sn-2 fatty acyl chain binds to the active site of PRDX6 peroxidase (Manevich et al., 2007; Manevich et al., 2009). Moreover, PRDX6 is the sole PRDX found in all of the sperm's subcellular compartments, notably the head, mitochondria and flagellum, and can react with H_2O_2 at sufficiently low concentrations to promote capacitation (O'Flaherty & Rico de Souza, 2010). Studies using the PRDX6 knockout mice model have demonstrated that *Prdx6*$^{-/-}$ male mice are infertile and possess spermatozoa with low quality and significant DNA damage (Ozkosem et al., 2015, 2016; Moawad et al., 2017; Scarlata et al., 2020).

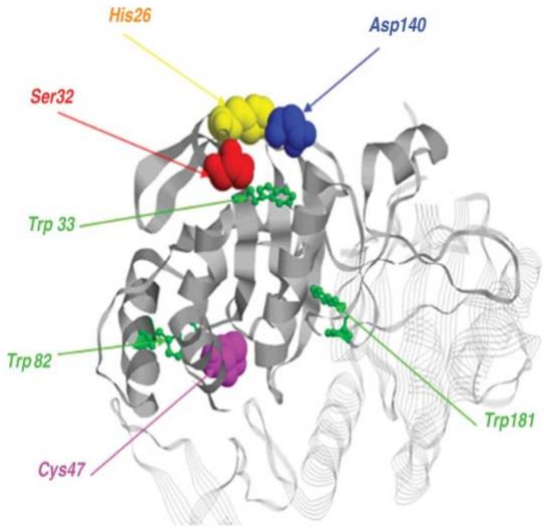

Figure 5: Schematic representation of PRDX6 structure

PRDX6 is shown as a homodimer composed of a dark gray monomer and a strand-like monomer. S32 (serine 32), H26 (histidine 26) and D140 (aspartic acid 140) assemble to form a catalytic triad responsible for $iPLA_2$ activity. C47 (cysteine 47) serves as the catalytic center required for peroxidase activity. The figure was taken from Manevich et al., (2007).

1.3.4 PRDX6: primary protector of spermatozoa against oxidative stress

PRDXs are important for maintaining fertility in humans since infertile men with clinical varicocele or idiopathic infertility have low levels of PRDXs (Gong et al., 2012). Moreover, 14% and 90% of idiopathic infertile men have high levels of hyperoxidized (inactive) PRDX1 and PRDX6, respectively (Gong et al., 2012). Compared to fertile individuals, infertile patients have lower sperm motility and higher levels of lipid peroxidation and DNA damage in their spermatozoa (Gong et al., 2012). PRDXs are also important for maintaining fertility in rodents since spermatozoa from tBHP-treated Sprague-Dawley rats have high levels of hyperoxidized PRDX1 and PRDX6, lower motility and higher DNA oxidation levels compared to spermatozoa from untreated rats (Liu & O'Flaherty, 2017). Interestingly, spermatozoa from treated rats have increased amounts of PRDX1 and PRDX6 provided by the epididymal epithelium through the secretion of epididymosomes (Liu & O'Flaherty, 2017). However, as previously mentioned, these PRDXs were heavily oxidized and therefore inactive and incapable of protecting spermatozoa against oxidative stress (Liu & O'Flaherty, 2017). Hence, despite epididymis' attempt to protect spermatozoa from oxidative stress, PRDX1 and PRDX6 hyperoxidation (Liu

& O'Flaherty, 2017) led to similar comorbidities observed in the spermatozoa of infertile men (Gong et al., 2012), suggesting that both PRDXs are important to support male fertility.

Previous studies involving PRDXs knockout mice models have shown that compared to 2-Cys PRDXs, PRDX6 is more important for the maintenance of male fertility as evidenced by the preservation of fertility and the absence of anomalies within the testis and spermatozoa of mice lacking functional genes that encode for PRDX1, PRDX2 or PRDX3 (Lee et al., 2003; Neumann et al., 2003; Li et al., 2007; Iuchi et al., 2009). Additionally, PRDX5$^{-/-}$ mice are also fertile (Argyropoulou et al., 2016). PRDX4$^{-/-}$ mice have higher levels of oxidative stress within their spermatozoa and testes and decreased sperm viability due to testicular atrophy; however, their fertility is not affected (Sasagawa et al., 2001; Iuchi et al., 2009). Similar to nGPX4$^{-/-}$ mice, *Prdx6*$^{-/-}$ mice also have elevated free thiols levels within their sperm head (Ozkosem et al., 2016); however, *Prdx6*$^{-/-}$ male mice are infertile (Ozkosem et al., 2015, 2016; Moawad et al., 2017; Scarlata et al., 2020). *Prdx6*$^{-/-}$ males also have higher levels of DNA damage, lipid peroxidation and protein oxidation, as well as impaired sperm viability and motility compared to wild-type mice (Ozkosem et al., 2016). Considering that only *Prdx6*$^{-/-}$ mice are infertile among all PRDXs knockout models, PRDX6 is the primary antioxidant enzyme involved in preventing sperm oxidative stress and thus, ensuring male fertility (Ozkosem et al., 2015, 2016; Moawad et al., 2017; Scarlata et al., 2020).

The importance of PRDX6 as the primary antioxidant enzyme that protects spermatozoa against oxidative stress was further supported by the lower cleavage rates during *in vitro* fertilization obtained when *Prdx6*$^{-/-}$ spermatozoa or wild-type spermatozoa treated with 1-Hexadecyl-3-(trifluoroethyl)-sn-glycero-2-phosphomethanol (MJ33), an iPLA$_2$ inhibitor, were used compared to when untreated wild-type spermatozoa was used (Moawad et al., 2017). In human spermatozoa, PRDX6 has a stronger capacity to prevent lipid peroxidation than 2-Cys PRDXs, as evidenced by the higher sperm 4HNE levels observed when PRDX6 peroxidase or iPLA$_2$ were inactive (Figure 6) compared to when 2-Cys PRDXs were inactive (Fernandez & O'Flaherty, 2018). Consequentially, due to stronger 4HNE-mediated disruption of mitochondrial activity (Aitken et al., 2012), inhibition of PRDX6 peroxidase or iPLA$_2$ in human spermatozoa resulted in higher sperm mitochondrial $O_2^{\bullet-}$ levels and associated oxidative stress (Figure 6)

compared to when 2-Cys PRDXs were inhibited (Fernandez & O'Flaherty, 2018). Additionally, among the inhibitors of PRDXs and their reactivation enzymes, only ezatiostat, an inhibitor of GSTpi, promoted the increase of $ONOO^-$ levels in human spermatozoa treated with Da-NONOate and antimycin A, which is an inhibitor of complex III within mitochondria that prevents electron transfer, therefore, leading to an increase in $O_2^{\bullet-}$ levels (Alexandre & Lehninger, 1984; Campo et al., 1992; Maguire et al., 1992; Xia et al., 1997; Pham et al., 2000; Aon et al., 2003), suggesting that PRDX6 peroxidase has a major role in scavenging $ONOO^-$ in human spermatozoa (Figure 6) (Fernandez & O'Flaherty, 2018). Consequently, due to the resulting elevated levels of $O_2^{\bullet-}$ and 4HNE, $iPLA_2$ inactivity led to a stronger increase in DNA oxidation levels (Figure 6) and a decrease in sperm viability compared to 2-Cys PRDXs inactivity (Fernandez & O'Flaherty, 2018).

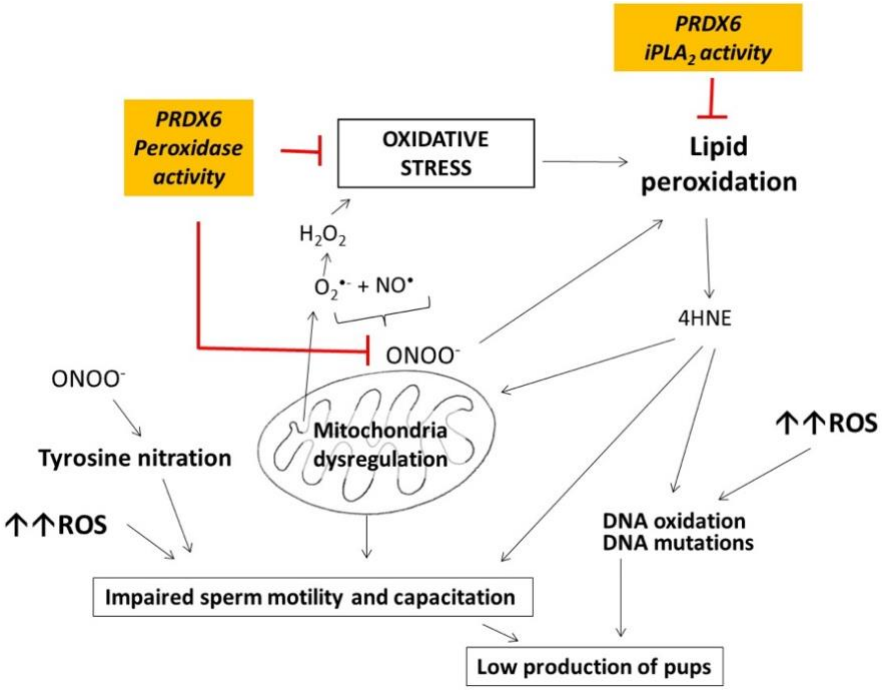

Figure 6: Schematic depicting the involvement of PRDX6 in regulating parameters of human spermatozoa associated with male fertility

Inhibition of PRDX6 peroxidase and $iPLA_2$ using ezatiostat and MJ33 respectively leads to increased lipid peroxidation, therefore resulting in the increase in sperm 4HNE levels. 4HNE promotes DNA damage, impairment of sperm motility and capacitation, and disruption of mitochondrial activity to generate $O_2^{\bullet-}$, which can dismutate into H_2O_2 or react with $NO^{\bullet}$ to form $ONOO^-$. H_2O_2 and $ONOO^-$, as ROS, further promote lipid peroxidation. Modified from Fernandez & O'Flaherty (2018).

2. Research Rationale

2.1 Involvement of PRDX6 in sperm motility

Compared to wild-type mice spermatozoa, *Prdx6*$^{-/-}$ spermatozoa had lower total and progressive motility, suggesting that PRDX6 is important for maintaining sperm motility (Moawad et al., 2017). Due to oxidative stress associated with aging, the decrease in motility of *Prdx6*$^{-/-}$ spermatozoa was aggravated (Ozkosem et al., 2015). In Sprague-Dawley rats, oxidative stress caused by tBHP resulted in impairment of epididymal sperm motility associated with PRDX6 hyperoxidation, confirming that inactivation of PRDX6 results in the impairment of sperm motility (Liu & O'Flaherty, 2017). iPLA$_2$ is essential for the maintenance of sperm motility since mice spermatozoa treated with MJ33 had lower motility compared to untreated controls (Moawad et al., 2017). Among all ROS involved in sperm oxidative stress, $ONOO^-$ is potentially the primary ROS responsible for disruption of sperm motility since treatment of *Prdx6*$^{-/-}$ male mice with γ-tocopherol, which is a better scavenger of $ONOO^-$ compared to α-tocopherol (Jiang et al., 2001; Galli et al., 2004), resulted in the improvement of sperm motility and restoration of fertility in *Prdx6*$^{-/-}$ male mice (Scarlata et al., 2020). PRDX6 is also vital for human sperm motility since thiol-oxidized PRDX6 levels within spermatozoa of idiopathic infertility or varicocele patients were negatively correlated with sperm motility (Gong et al., 2012). Overall, these results suggest that the ROS-scavenging capacity of PRDX6 is necessary to prevent the occurrence of events that impair sperm motility, namely tyrosine nitration or s-glutathionylation of tubulin, and enzymes involved in energy generation (Fratelli et al., 2004; Landino et al., 2004; Morielli & O'Flaherty, 2015).

2.2 Involvement of PRDX6 in sperm capacitation

PRDX6 ensures maintenance of sperm capacitation since *Prdx6*$^{-/-}$ mice had a lower percentage of capacitated spermatozoa compared to wild-type mice (Ozkosem et al., 2016). iPLA$_2$ activity may account for PRDX6's ability to maintain sperm capacitation since iPLA$_2$ inhibition was sufficient to impair capacitation to similar levels experienced by spermatozoa from *Prdx6*$^{-/-}$ mice (Ozkosem et al., 2016). In accordance with this result, administration of penicillamine (PEN), a ROS scavenger, was unable to increase the levels of phosphotyrosine and phosphorylated PKA substrates to normal levels when iPLA$_2$ of human spermatozoa was inhibited, suggesting that iPLA$_2$ activity is necessary for sperm capacitation to occur (Lee et al., 2017). Additionally,

iPLA$_2$ inhibition impaired actin polymerization (Lee et al., 2017). Collectively, these results suggest that the ROS-scavenging capacity of PRDX6 is potentially involved in preventing ROS- and 4HNE-mediated inhibition of capacitation-associated kinases (Pronzato et al., 1990; Chiarpotto et al., 1999; Sampey et al., 2007), and actin s-glutathionylation (Dalle-Donne et al., 2005; Bizzozero et al., 2007).

2.3 Involvement of PRDX6 in mice's reproductive outcomes

PRDX6 is essential for *in vivo* fertilization since *Prdx6*$^{-/-}$ mice had lower pups/male and litters/male compared to wild-type mice, which were worsened by oxidative stress associated with aging (Ozkosem et al., 2015). The importance of PRDX6 in preventing oxidative stress is highlighted by the fact that *Prdx6*$^{-/-}$ male mice produced smaller litter sizes compared to wild-type mice in response to tBHP treatment (Ozkosem et al., 2016). Among all ROS involved in sperm oxidative stress, $ONOO^-$ is potentially the primary ROS responsible for the impairment of mice's reproductive outcomes since when *Prdx6*$^{-/-}$ males were treated with γ-tocopherol, their litters sizes increased similarly to that of wild-type males fed with control diet, indicating that high levels of $ONOO^-$ is a major cause of male infertility in this mouse model (Scarlata et al., 2020). *Prdx6*$^{-/-}$ spermatozoa and MJ33-treated wild-type spermatozoa also suffer from impaired capacitation and lower membrane fluidity compared to spermatozoa from wild-type mice (Ozkosem et al., 2016); therefore, it is possible that sperm binding to the zona pellucida and oolemma is impaired when PRDX6 iPLA$_2$ is inactive or absent. Indeed, the absence of either PRDX6 or its iPLA$_2$ activity decreased the recurrence of acrosome reaction and prevents binding of spermatozoa to both zona pellucida and oolemma (Moawad et al., 2017). These abnormalities may also account for the impairment of *in vitro* fertilization since the usage of *Prdx6*$^{-/-}$ spermatozoa led to lower *in vitro* fertilization rates, decreased formation of male/female pronuclei, and inability to produce viable blastocysts (Moawad et al., 2017).

2.4 Involvement of PRDX6 in sperm epididymal maturation

PRDX6 absence leads to impaired sperm cytoplasm removal since *Prdx6*$^{-/-}$ mice had higher percentages of spermatozoa with cytoplasmic droplet retention (CDR) compared to wild-type mice, and this abnormal phenotype was worsened by oxidative stress associated with aging (Ozkosem et al., 2015). $ONOO^-$ is potentially the primary ROS responsible for preventing sperm

cytoplasm removal since γ-tocopherol-treated *Prdx6*$^{-/-}$ mice had similar percentages of spermatozoa with CDR compared to wild-type mice fed with the control diet (Scarlata et al., 2020). In addition to its ability to prevent lipid peroxidation of sperm membrane (Ozkosem et al., 2016), PRDX6 also prevents lipid peroxidation of the epididymal epithelium as evidenced by the elevated 4HNE levels within cauda epididymis of tBHP-treated rats that are associated with PRDX6 hyperoxidation (Liu & O'Flaherty, 2017; Wu et al., 2020). Collectively, these results suggest that lipid peroxidation of epididymal epithelium occurs in *Prdx6*$^{-/-}$ mice (Liu & O'Flaherty, 2017; Wu et al., 2020) and may be involved in the impairment of molecular mechanisms required for sperm cytoplasm absorption during epididymal maturation.

2.5 Involvement of PRDX6 in the prevention of sperm DNA oxidation

PRDX6 ensures the protection of human sperm DNA from damage since thiol-oxidized PRDX6 levels in idiopathic infertility or varicocele patients are positively correlated with DNA fragmentation levels (Gong et al., 2012). In mice, PRDX6 serves the same function since its absence leads to a decrease in protamination of chromatin, therefore exposing DNA bases to oxidation and making DNA strands more susceptible to fragmentation (Ozkosem et al., 2015). Similarly, in tBHP-treated Sprague-Dawley rats, the increase in epididymal sperm DNA oxidation levels associated with PRDX6 hyperoxidation further confirms that PRDX6 must be active to prevent sperm DNA damage (Liu & O'Flaherty, 2017). PRDX6 can withstand the long-term effects of oxidative stress through its ROS-scavenging capacity, as evidenced by the long-term decrease in sperm DNA oxidation and fragmentation levels and the long-term increase in protamination levels observed in tBHP-treated wild-type mice (Ozkosem et al., 2016). $ONOO^-$ is potentially the primary ROS responsible for promoting sperm DNA damage since γ-tocopherol-treated *Prdx6*$^{-/-}$ mice had similar DNA oxidation levels compared to wild-type mice fed with the control diet (Scarlata et al., 2020).

3. Hypothesis and Objective

Since *Prdx6*$^{-/-}$ mice are infertile (Ozkosem et al., 2015, 2016; Moawad et al., 2017; Scarlata et al., 2020) and PRDX6 has peroxidase and $iPLA_2$ activities (Fisher, 2017), we aimed to assess the involvement of both PRDX6 enzymatic activities on the maintenance of male mouse fertility. I hypothesized that the absence of either of these PRDX6 enzymatic activities would impair male mouse fertility. The objective was to investigate changes in sperm parameters associated with male fertility for knock-in strains lacking either PRDX6 enzymatic activities and were challenged with an *in vivo* oxidative stress induced by tBHP treatment.

4. Materials and Methods

4.1 Materials

Mouse monoclonal anti-3-nitrotyrosine and rabbit polyclonal anti-4HNE antibodies were purchased from Abcam (Toronto, Ontario, Canada). The goat polyclonal anti-4HNE antibody was purchased from MilliporeSigma (Burlington, Massachusetts, USA). Mouse monoclonal anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody was purchased from StressMarq Biosciences (Victoria, British Columbia, Canada). Donkey polyclonal anti-rabbit (H+L) and goat polyclonal anti-mouse (H+L) antibodies, both conjugated with horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pennsylvania, USA). Goat polyclonal anti-mouse (H+L) and donkey polyclonal anti-goat (H+L) antibodies, both conjugated with AlexaFluor 555, and Giemsa stain, were purchased from Thermo Fisher Scientific (Saint-Laurent, Québec, Canada). Other chemicals used were of at least reagent grade and purchased from Sigma-Aldrich (St Louis, Missouri, USA).

4.2 Animals

Two-month-old male C57BL/6J (wild-type; purchased from Charles River Laboratories, Laval, Quebec, Canada), *Prdx6*$^{-/-}$, C47S (peroxidase-deficient) and D140A (iPLA$_2$-deficient) knock-in strains were used in this study. *Prdx6*$^{-/-}$ mice were generated by the laboratory of Dr. Ye Shih Ho (Wayne State University) and Dr. Aron Fisher (University of Pennsylvania) through the construction of a vector, which promotes targeted modification of PRDX6's exon 1 and 2 and is transfected into mouse embryonic stem cells (Mo et al., 2003). C47S (cysteine to serine transition at codon 47 of exon 2) and D140A (aspartate to alanine transition at codon 140 of exon 4) mice were generated through homologous recombination using targeting vectors (Li et al., 2015). The progeny with black coat color (C57BL/6J mice) and the desired genetic modifications were backcrossed until full homozygosity was achieved, which was confirmed by Jackson Laboratory's microsatellite analysis (Liu et al., 2010). *Prdx6*$^{-/-}$, C47S and D140A mice to start our colonies were generously donated by Dr. Aron Fisher (University of Pennsylvania). C57BL/6J mice (wild-type) were used as controls. The four mouse colonies were maintained at the Research Institute of McGill University Health Centre. Mice were given food and water *ad libitum* and were exposed to 14 hours of light and 10 hours of dark. Regulations implemented by the Canadian Council for Animal Care and approved by the Animal Care Committees of McGill

University and the Research Institute of McGill University Health Centre were followed (animal protocol #5656).

Mice from each strain were intraperitoneally injected daily with either saline or 60 μM tBHP/100 g of body weight for 9 consecutive days to determine the effects of tBHP-induced oxidative stress on epididymal spermatozoa (Rajesh Kumar et al., 2002). Twenty-four hours after tBHP treatment, mice were euthanized, and then epididymides were extracted and put in phosphate-buffered saline (PBS) composed of 1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. 27G needle was used to puncture cauda epididymides five times to allow spermatozoa to swim out into the medium for 10 mins at 37ºC. Spermatozoa were then diluted to 5×10^6 cells/ml using Biggers, Whitten and Whittingham medium (BWW medium; 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, 20 mM HEPES, pH 7.4).

4.3 Thermoresistance test to assess sperm motility

Cauda epididymis spermatozoa from the four mice strains were incubated in PBS, which is a solution that lacks nutrients required for sperm survival (Bedard et al., 2011), at 37ºC. Sperm aliquots were taken at various time points (0, 30, 60, 90, 120 min) to determine sperm motility using a computer-assisted sperm analysis (CASA) system with Sperm Vision HR software version 1.01 (Minitube, Ingersoll, ON, Canada).

4.4 Assessment of sperm capacitation

Cauda epididymis spermatozoa in BWW medium (pH 7.4) were incubated with (capacitated) or without (non-capacitated) 5 mg/ml bovine serum albumin (BSA) and 20 mM HCO_3^- (capacitation inducers) for 60 min at 37ºC (Ozkosem et al., 2016). Then, sperm suspensions were centrifuged at 600 x g for 5 min at 20ºC. The supernatant was discarded, and the pellet was resuspended in BWW medium and incubated with 5 μM progesterone for 30 min at 37ºC to induce the acrosome reaction. After incubation, sperm samples were centrifuged at 600 x g for 5 min at 20ºC and fixed with 100% ethanol. Sperm samples were smeared onto slides. Samples were incubated with Giemsa stain (1:10 solution) diluted in ddH_2O for 1.5 hours at 20ºC. Slides were washed with ddH_2O. 30 μL of ddH_2O was added on each slide to rehydrate samples prior to

the addition of the coverslip. Acrosomes were labelled with Giemsa stain displaying a purple colour. Spermatozoa without labelled acrosome (Giemsa stain-negative) were considered acrosome-reacted. At least 200 spermatozoa per sample were analyzed using a Carl Zeiss Axiophot microscope (Oberkochen, Germany) at 1000X magnification.

4.5 Assessment of sperm epididymal maturation

The presence of the cytoplasmic droplet in cauda epididymis spermatozoa was considered to assess sperm epididymal maturation. Fields obtained through CASA system during sperm motility assessment were saved, then two hundred spermatozoa per sample were visually inspected for the presence of the cytoplasmic droplet. Percentages of spermatozoa with cytoplasmic droplet retention were recorded for each group.

4.6 Quantification of reproductive outcomes

After treatment, control and tBHP-treated males from the four strains were mated with age-matched wild-type females (ratio 1:1). Mice reproductive outcomes (litter sizes and the number of unsuccessful mating) were quantified after three consecutive matings. Female mice were checked daily for the presence of a vaginal plug to confirm the mating. Males were euthanized after the last mating and then organs were harvested.

4.7 SDS-PAGE and immunoblotting to determine 4HNE and nitrotyrosine levels in spermatozoa

Sperm suspensions in BWW medium were supplemented with sample buffer containing dithiothreitol (DTT). Samples were heated at 100ºC for 5 min, then centrifuged at 21,000 RCF for 5 min at 20ºC. Supernatant aliquots were loaded into 10% polyacrylamide gels. SDS-PAGE electrophoresis was conducted using electrophoresis buffer 1x (25 mM Tris base, 182 mM glycine, 0.1% SDS). Transfer buffer 1x (25 mM Tris base, 192 mM glycine, 20% methanol) was used for protein transfer into nitrocellulose membranes. Membranes were blocked using 5% skim milk in 2 mM Tris (pH 7.8)-buffered saline and 0.1% Tween 20 (TTBS 1X) for 1 hour at 20ºC. Membranes were washed with TTBS 1X and then incubated with either 1:500 anti-4HNE or 1:1000 anti-nitrotyrosine antibodies overnight at 4ºC. Following incubation, membranes were washed 5 times (10 min each) with TTBS 1X. Then, membranes were incubated with a

horseradish peroxidase-conjugated donkey anti-rabbit or goat anti-mouse antibody (1:5,000) diluted in TTBS 1X for 1 hour at 20ºC, for 4HNE and nitrotyrosine, respectively. Membranes were washed and then incubated with enhanced chemiluminescence (ECL) solution for 3 min at 20ºC to detect positive immunoreactive bands using radiographic films.

Equal loading controls were performed by stripping the membranes using stripping buffer for 5 min at 20ºC. Membranes were washed, blocked using 5% skim milk for 1 hour at 20ºC, washed, and then incubated with the anti-α-tubulin antibody (1:10,000 dilution). Membranes were washed and then incubated with goat anti-mouse antibody diluted in TTBS 1X for 45 min at 20ºC. Membranes were washed and then incubated with ECL solution for 3 min at 20ºC. Films were developed to detect positive immunoreactive tubulin bands. Densitometry analysis was done using ImageJ version 1.53 (National Institutes of Health, Bethesda, Maryland, USA). The relative intensity of each band was obtained and normalized to the respective intensity of α-tubulin band.

4.8 Immunocytochemistry to determine 4HNE and nitrotyrosine levels in spermatozoa

Levels of 4HNE and nitrotyrosine in the sperm plasma membrane were determined in non-permeabilized spermatozoa. Sperm samples were smeared onto slides. Slides were dried at 20ºC. Slides were rehydrated with PBS + 0.1% Triton (PBS-T) for 5 min and then incubated in fresh PBS-T for 25 min at 20ºC. Slides were dried briefly. A circle was drawn onto each slide using ImmedgePen. 1% horse serum in PBS-T (for 4HNE) and 5% goat serum in PBS-T (for nitrotyrosine) was used for blocking for 30 min at 20ºC. Slides were washed with PBS-T and then incubated with anti-4HNE (1:100 dilution in 1% horse serum in PBS-T) or anti-nitrotyrosine (1:100 dilution in 1% goat serum in PBS-T) antibody overnight at 4ºC. Slides were washed and then incubated with a donkey anti-goat antibody (1:2000 dilution in PBS-T + 1% BSA) for 4HNE, or with a goat anti-mouse antibody (1:1000 dilution in PBS-T + 1% BSA) for nitrotyrosine, for 1 hour at 20ºC. Slides were washed. ProLong Antifade with DAPI was added prior to the coverslip application. Negative control was treated with PBS-T rather than the primary antibody. Leica DFC450C microscope at 40X magnification with Leica Application Suite X (LASX) software (Leica Microsystems, Wetzlar, Germany) was used to take images for

each sample at the same exposure time. ImageJ was used to subtract background fluorescence and to quantify average relative fluorescence intensity (RFI) for each sample. More than 200 spermatozoa were included in the analysis for each sample. To localize intracellular 4HNE and nitrotyrosine fluorescence signal in spermatozoa, spermatozoa were permeabilized using 100% methanol for 10 min at -20ºC prior to rehydration with PBS-T. Carl Zeiss Axiophot (Oberkochen, Germany) at 1000X magnification with Northern Eclipse V8 software (Empix Imaging Inc., Mississauga, Ontario, Canada) was used for localization of 4HNE fluorescence signal within permeabilized spermatozoa.

4.9 Immunocytochemistry to determine DNA oxidation levels in spermatozoa

Sperm DNA oxidation was assessed by determining the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Scarlata et al., 2020). Sperm samples were smeared onto slides. Slides were dried at 20ºC. Slides were treated with 20 mM H_2O_2, washed with PBS-T and then incubated with resuspension buffer (50 mM Tris-HCl, pH 7.4 + 1% SDS + 40 mM DTT + 1 mM EDTA) for 5 min at 20ºC. Slides were washed and then dried briefly. A circle was drawn onto each slide using ImmedgePen. 5% goat serum in PBS-T was used for blocking for 1 hour at 20ºC. Slides were washed and then incubated with anti-8OHdG antibody (1:100 dilution in 1% goat serum in PBS-T) overnight at 4ºC. Slides were washed and then incubated with a goat anti-mouse antibody (1:2000 dilution in PBS-T + 1% BSA) for 1 hour at 20ºC. Slides were washed. ProLong Antifade with DAPI was added prior to the coverslip application. Negative control was treated with PBS-T rather than the primary antibody. Leica DFC450C microscope at 40X magnification with Leica Application Suite X (LASX) software (Leica Microsystems, Wetzlar, Germany) was used to take images for each sample at the same exposure time. ImageJ was used to subtract background fluorescence and to quantify the average RFI for each sample. More than 200 spermatozoa were included in the analysis for each sample. Results of DNA oxidation were expressed as percentages of spermatozoa with a strong signal for 8OHdG.

4.10 Statistical analysis

All results are presented as mean ± SEM where the medians are included in the dot plot graphs. The normality of the data and homogeneity of variances were determined using Shapiro–Wilk and Bartlett tests, respectively. We used the two-way ANOVA and Bonferroni tests (to assess

changes due to genotype and treatment) and chi-square tests as appropriate using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) to determine statistical differences among groups.

5. Results

5.1 Effect of the absence of PRDX6 peroxidase and iPLA$_2$ and oxidative stress on male mice fertility

We wanted to know the consequences of PRDX6 peroxidase or iPLA$_2$ absence in male mice coupled with *in vivo* oxidative stress generated by tBHP treatment. As previously reported (Ozkosem et al., 2016), tBHP treatment promoted a higher number of unsuccessful matings and smaller litter sizes in *Prdx6*$^{-/-}$ males, compared to wild-type males (Table 1 and Figure 7). Wild-type control males had the lowest number of unsuccessful matings. When wild-type males were treated with tBHP, the numbers of unsuccessful mating were similar to those observed in control *Prdx6*$^{-/-}$, C47S and D140A males. tBHP treatment promoted a trend of increase in the number of unsuccessful matings for all strains; however, no significant differences were recorded among them.

Number of unsuccessful matings

Strain	Control	tBHP-treated
Wild-type	0$^{\&}$	5
C47S	5	10
D140A	5	10
***Prdx6*$^{-/-}$**	6	11

Table 1: **PRDX6 peroxidase or iPLA$_2$ absence leads to impaired mating of mice.**
The number of unsuccessful matings was recorded for control and treated mice strains after gestation. Control and treated males were intraperitoneally injected with saline and tBHP respectively for 9 days, then bred with age-matched wild-type female mice thrice. Unsuccessful matings are defined as matings that produced no pups. n = 15 matings for each group. $^{\&}$ means lower than all others. Chi-Square test, $p \leq 0.05$.

Control wild-type males produced the largest litters among all groups (Figure 7). Treated wild-type males had similar litter sizes compared to control mutant males. No significant differences in the average litter sizes were observed among control and treated mutant males. Treated mutant males produced significantly smaller litters compared to their respective controls.

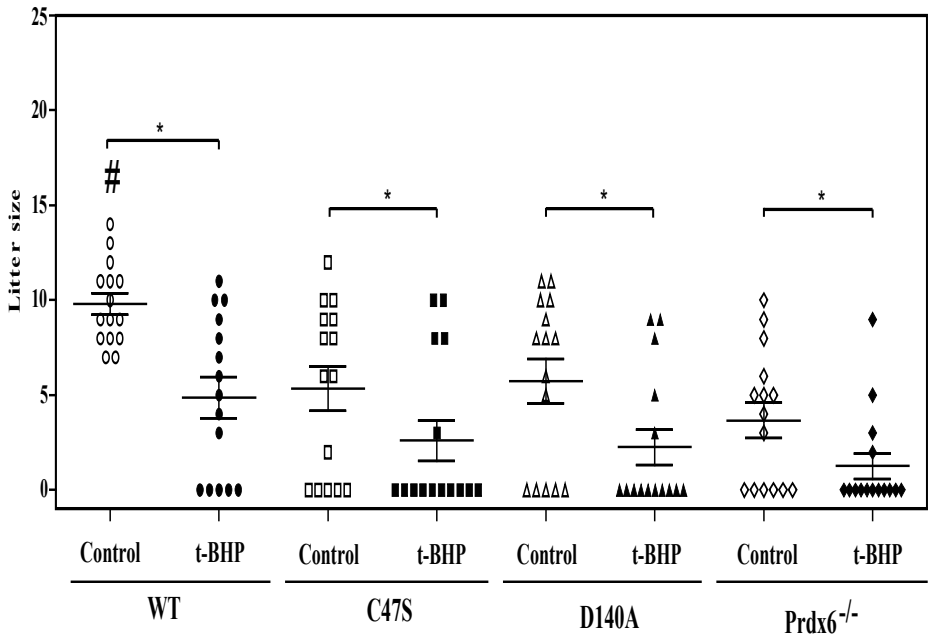

Figure 7: PRDX6 peroxidase or iPLA$_2$ absence leads to decreased litter sizes.

Average number of litter sizes produced by 5 males from each mouse strain after 3 consecutive matings. # means higher than all other groups. WT: wild-type strain. * means significant differences within the same strain. ANOVA and Tukey tests; $p<0.05$.

5.2 Effect of the absence of PRDX6 peroxidase and iPLA$_2$ on sperm epididymal maturation

Retention of the cytoplasmic droplet by spermatozoon is a marker for impaired epididymal function (Dadoune, 1988); therefore, elevated percentage of spermatozoa with cytoplasmic droplet retention (CDR) reflects the impairment of sperm epididymal maturation (Syntin & Robaire, 2001). Control wild-type males had the lowest percentages of spermatozoa with CDR among all groups (Figure 8). Percentages of spermatozoa with CDR in treated wild-type males were similar to that observed in control mutant males. No significant differences in percentages of spermatozoa with CDR were observed among control, and among treated mutant males. The tBHP treatment induced a significant increase in percentages of spermatozoa with CDR for all strains.

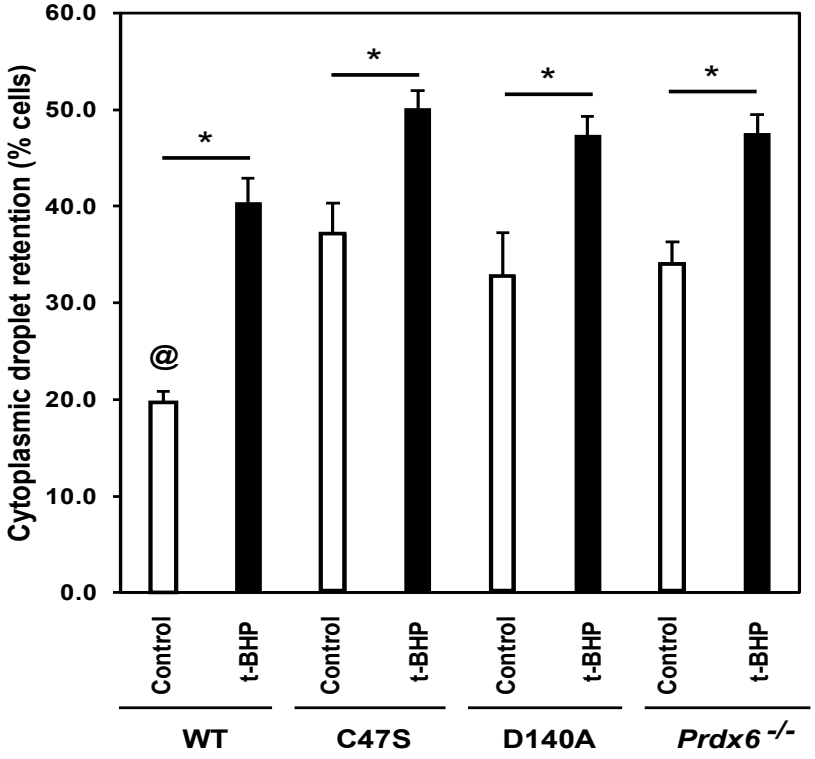

Figure 8: Removal of sperm cytoplasm is impaired in C47S and D140A mutant mice.

Fields provided by CASA during sperm motility assessment were used to quantify percentages of spermatozoa (>200 spermatozoa/sample) that had a visible cytoplasmic droplet within their structure. n = 5 mice for each group. WT: wild-type strain. * means significant differences within same strain. @ means smaller than all others. Two-way ANOVA and Bonferroni posthoc tests; $p<0.05$.

5.3 Effect of the absence of PRDX6 peroxidase and iPLA$_2$ on lipid peroxidation levels in spermatozoa

We determined lipid peroxidation in spermatozoa by assessing the levels of 4HNE, a subproduct of lipid peroxidation (Benedetti et al., 1980; Saxena, 2014), using immunoblotting. Control wild-type males had the lowest sperm 4HNE levels among all groups (Figure 9). The tBHP treatment significantly increased sperm 4HNE levels for all strains. Sperm 4HNE levels of treated wild-type males were similar compared to that of control mutant males. No significant differences in sperm 4HNE levels were observed among control and among treated mutant strains.

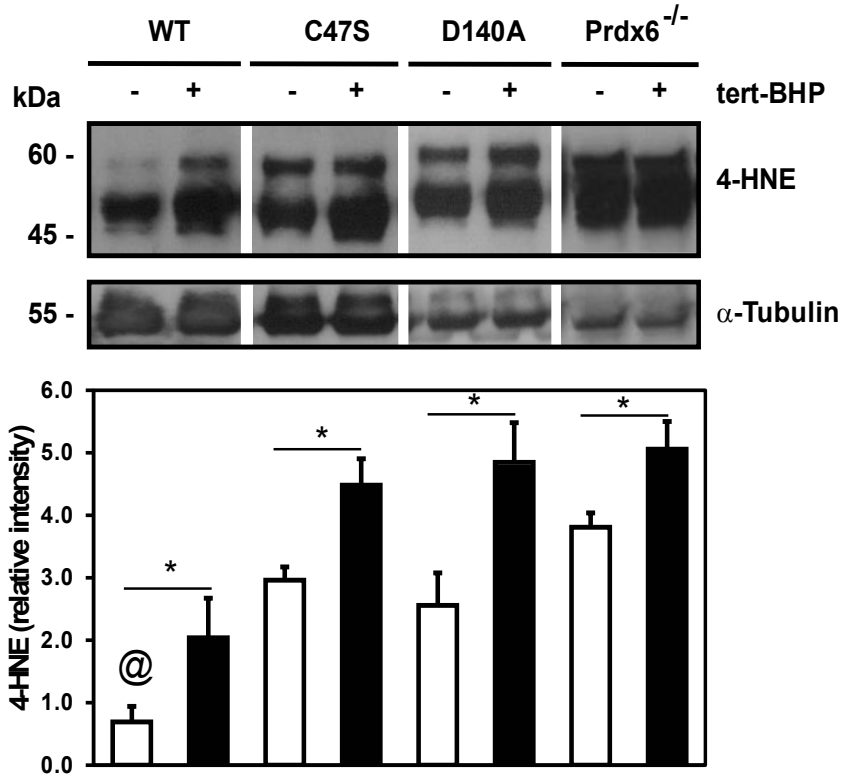

Figure 9: PRDX6 peroxidase or iPLA$_2$ absence leads to increased formation of 4HNE adducts.

Representative immunoblot depicting the levels of sperm 4HNE for each group. Densitometry analysis was conducted using ImageJ to measure the relative intensity of 4HNE signal for each strain. Each sperm sample was done by pooling epididymal spermatozoa from 3 different males. A total of 4 of these samples per group were used for the analysis. @ means smaller than all others. WT: wild-type strain. * means significant differences within the same strain. Two-way ANOVA and Bonferroni posthoc tests; $p<0.05$.

We used non-permeabilized spermatozoa for immunocytochemistry to determine the localization of sperm membrane proteins that are adducted by 4HNE, as well as their levels for each group. Strong labelling is present within the sperm head, particularly within the acrosome and nucleus (Figure 10A). Labelling is also observed within the entire flagellum, notably within midpiece, principal piece and end piece. Permeabilized spermatozoa were also used to localize the intracellular proteins that 4HNE adducts. A similar pattern of 4HNE labelling was observed in permeabilized spermatozoa (Figure 11).

Spermatozoa from mutant strains, irrespective of treatment, had significantly higher 4HNE levels compared to that of control wild-type males (Figure 10B). Spermatozoa from treated *Prdx6*$^{-/-}$ males had the highest 4HNE levels among all groups. Spermatozoa from treated wild-type males had elevated levels of 4HNE that were similar to those recorded in spermatozoa from control and treated C47S and D140A males, and control *Prdx6*$^{-/-}$ males. The tBHP treatment significantly increased 4HNE levels of spermatozoa from wild-type and *Prdx6*$^{-/-}$ males.

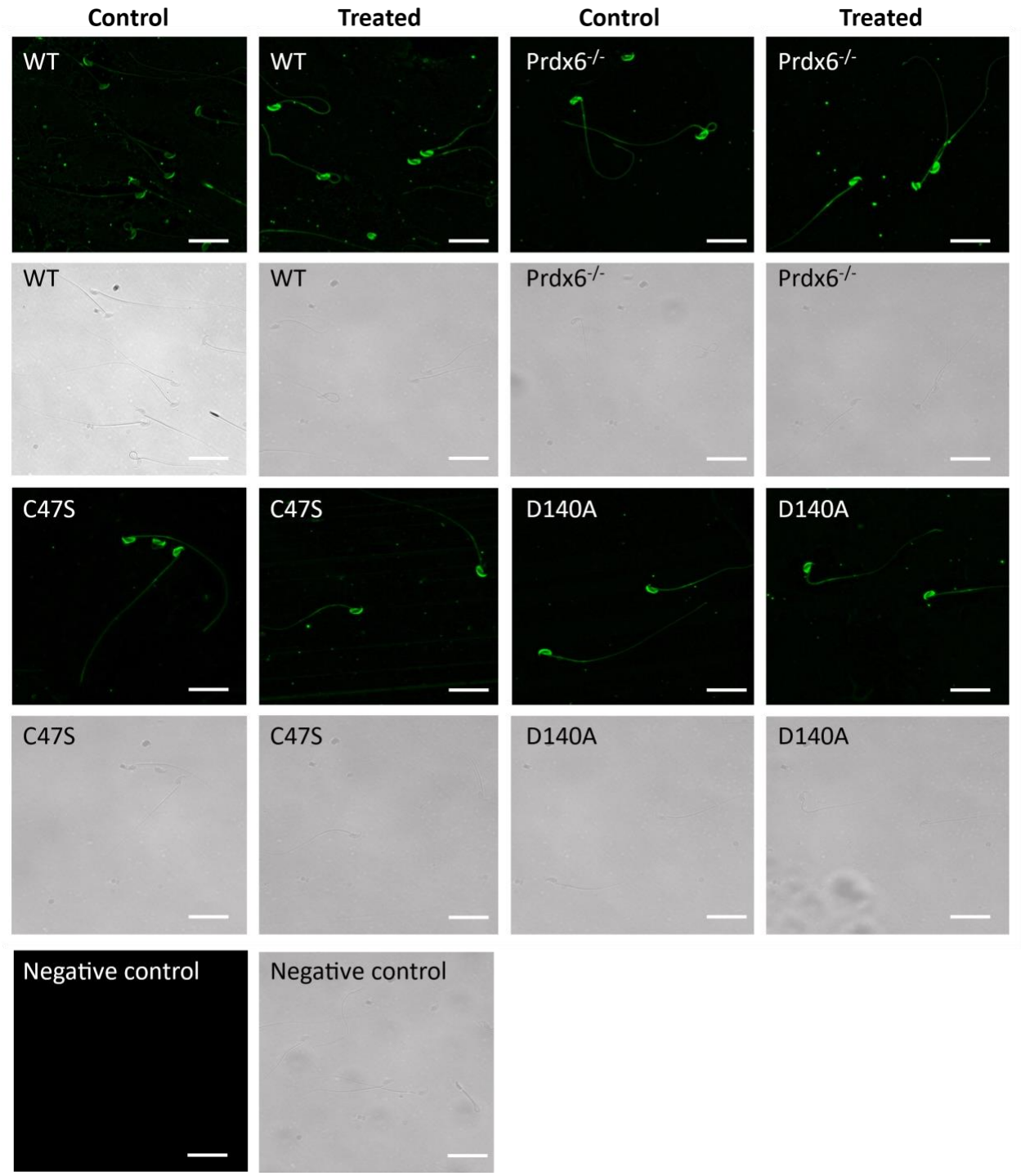

Figure 10A: Representative pictures depicting the intensity of 4HNE labelling in non-permeabilized spermatozoa from each group.

Fluorescence and brightfield images of non-permeabilized spermatozoa showing 4HNE labelling. WT: wild-type strain. White bar = 25 microns.

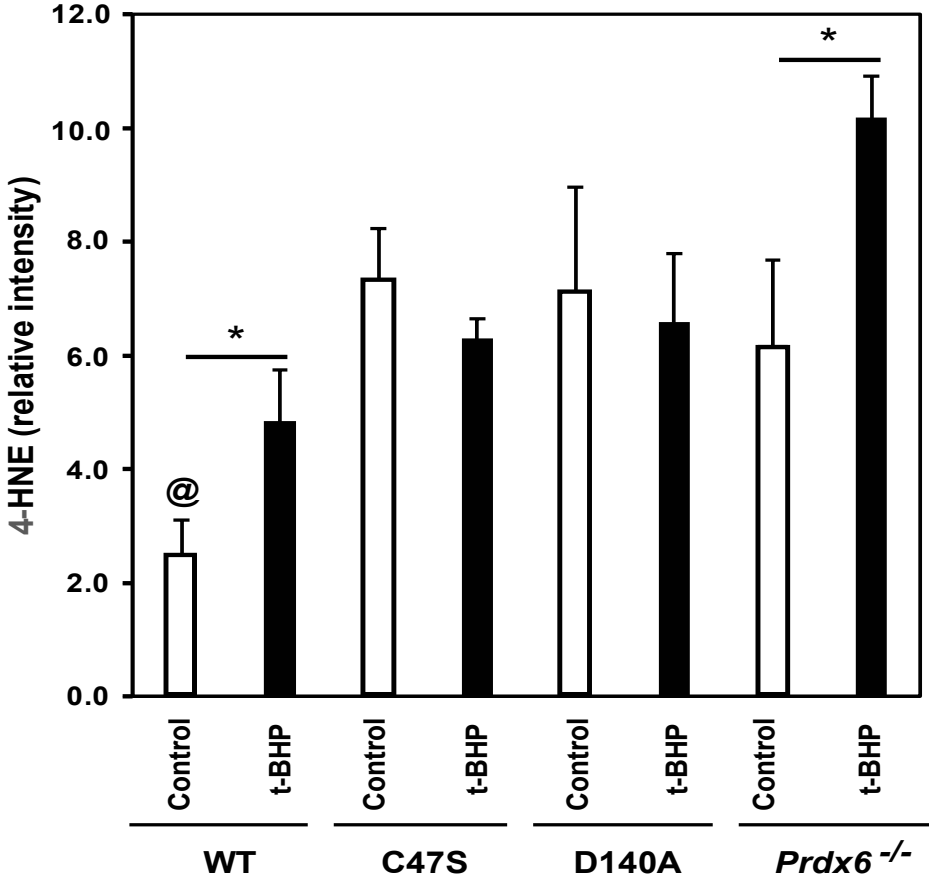

Figure 10B: PRDX6 peroxidase or iPLA$_2$ absence leads to increased lipid peroxidation. Average RFI of 4HNE signal was quantified in non-permeabilized epididymal spermatozoa from control and treated mice strains. More than 200 spermatozoa per sample were analyzed using ImageJ. n = 4 mice for each strain. WT: wild-type strain. * means significant differences within same strain. @ means lowest among all others. Two-way ANOVA and Bonferroni posthoc tests; $p<0.05$.

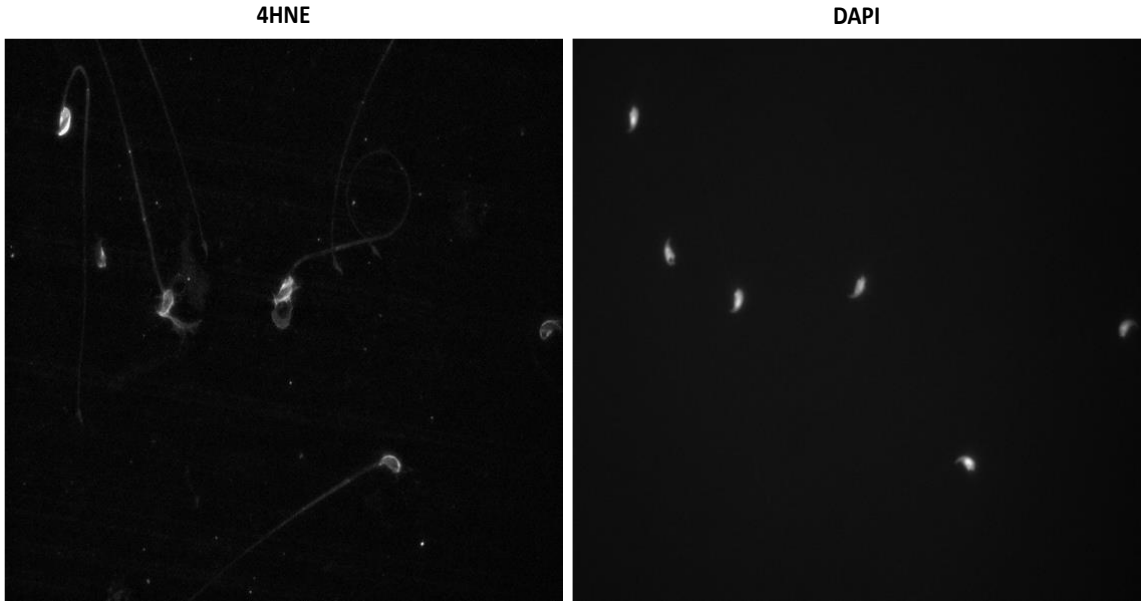

Figure 11: 4HNE labelling is observed throughout the entire structure of permeabilized spermatozoa.

Wild-type epididymal spermatozoa were permeabilized using 100% methanol for 10 min at -20ºC before being rehydrated with PBS-T. Fluorescence and DAPI image of permeabilized spermatozoa showing 4HNE labelling.

5.4 Effect of the absence of PRDX6 peroxidase and $iPLA_2$ on tyrosine nitration levels in spermatozoa

We previously demonstrated that tyrosine nitration is induced by $ONOO^-$ in mouse spermatozoa (Scarlata et al., 2020) and can be promoted by $NO^\bullet$ donors in human spermatozoa (Morielli & O'Flaherty, 2015). The immunoblotting result revealed that spermatozoa from control wild-type males had the lowest nitrotyrosine levels among all groups (Figure 12). The tBHP treatment significantly increased nitrotyrosine levels in spermatozoa from each strain. Spermatozoa from control mutant males had lower nitrotyrosine levels compared to spermatozoa from their respective treated males. Spermatozoa from control D140A males had similar nitrotyrosine levels compared to spermatozoa from treated wild-type males.

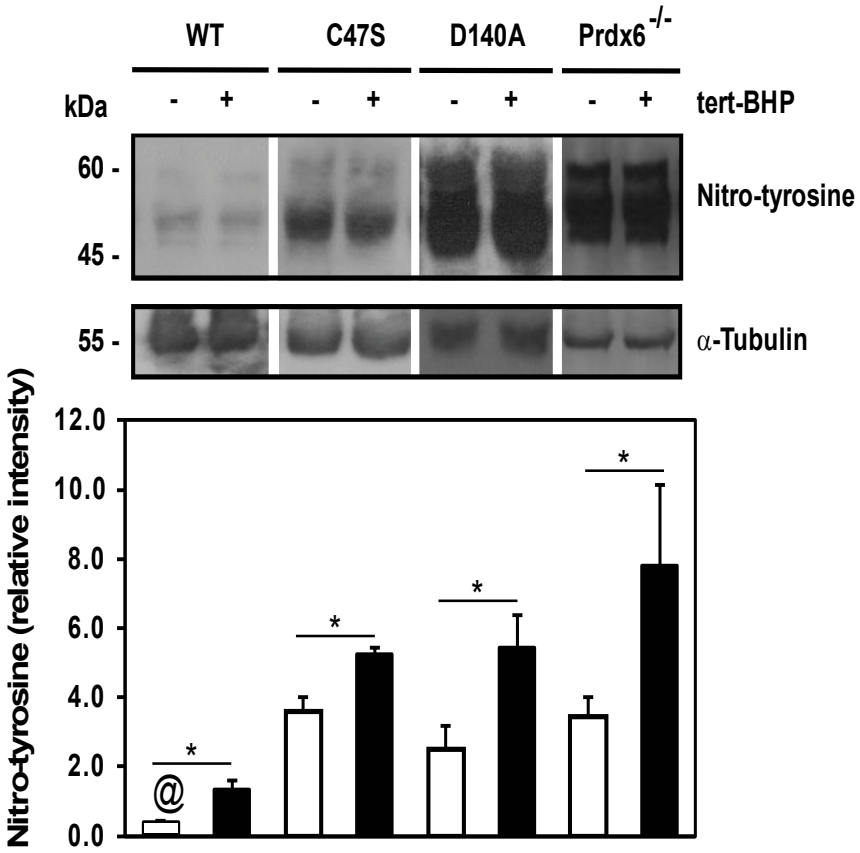

Figure 12: PRDX6 peroxidase or iPLA$_2$ absence leads to increased tyrosine nitration of sperm proteins.

Representative immunoblot depicting the levels of sperm nitrotyrosine for each group. Densitometry analysis was conducted using ImageJ to measure the relative intensity of nitrotyrosine signal for each strain. Each sperm sample was done by pooling epididymal spermatozoa from 3 different males. A total of 4 of these samples per group were used for the analysis. @ means lowest among all others. WT: wild-type strain. * means significant differences in the same strain. Two-way ANOVA and Bonferroni posthoc tests; $p<0.05$.

By using non-permeabilized spermatozoa, we also quantified the levels of sperm membrane proteins that underwent tyrosine nitration. Non-permeabilized spermatozoa have strong labelling within the midpiece, moderate labelling within the principal piece and end piece, and weak labelling within the head (Figure 13A). Spermatozoa from treated *Prdx6*$^{-/-}$ mice had the highest nitrotyrosine levels among all groups (Figure 13B). Control and treated wild-type males had similar nitrotyrosine levels in their spermatozoa. Spermatozoa from control and treated C47S males had higher nitrotyrosine levels compared to spermatozoa from wild-type males. Interestingly, spermatozoa from control D140A males had similar nitrotyrosine levels compared to spermatozoa from wild-type males. However, the tBHP treatment promoted the increase of nitrotyrosine levels in spermatozoa from D140A males to similar levels as that of spermatozoa from C47S males. Spermatozoa from control *Prdx6*$^{-/-}$ males had similar nitrotyrosine levels compared to spermatozoa from C47S and treated D140A males.

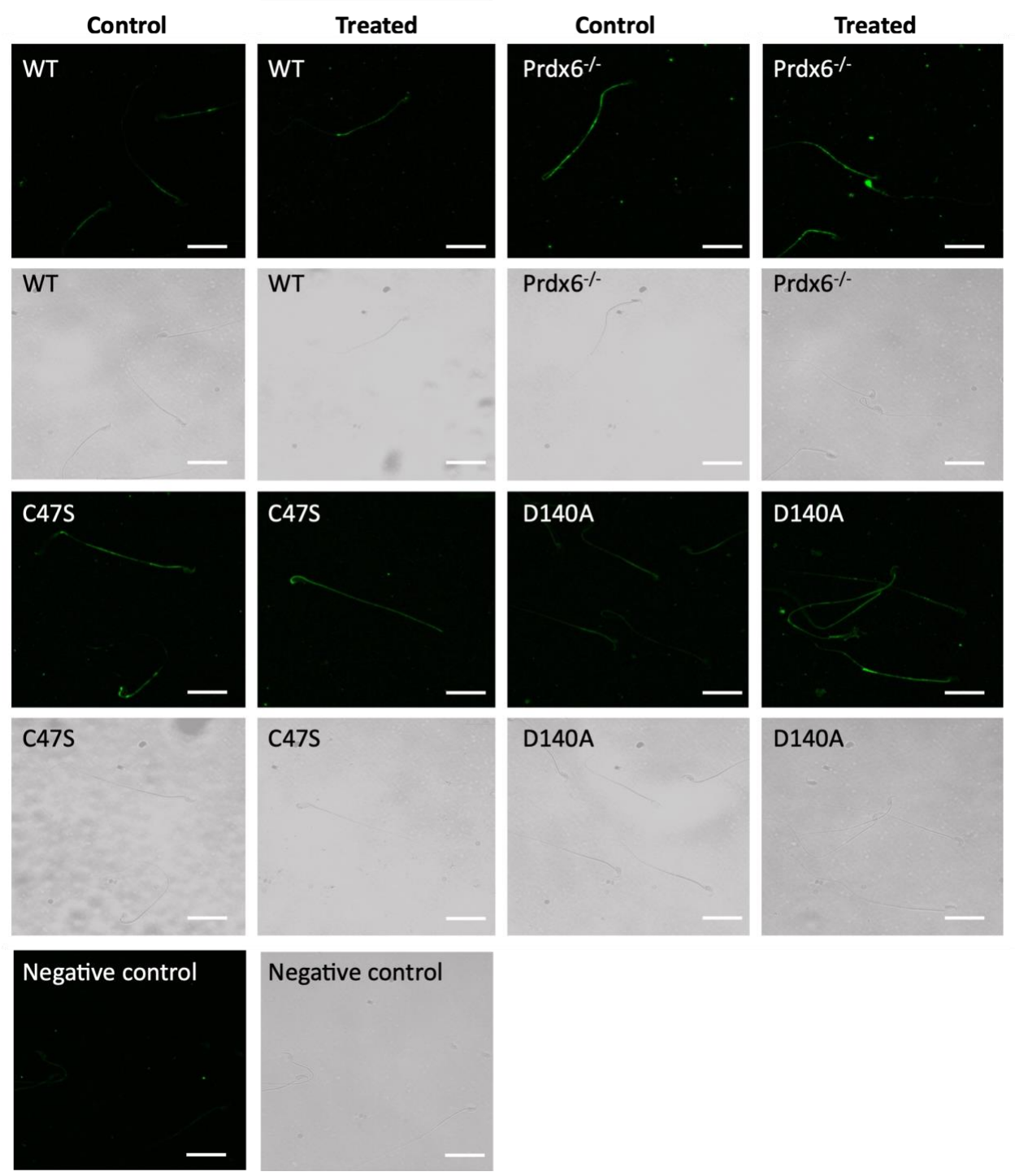

Figure 13A: Representative pictures depicting the intensity of nitrotyrosine labelling in non-permeabilized spermatozoa from each group.

Fluorescence and brightfield images of non-permeabilized spermatozoa showing nitrotyrosine labeling. WT: wild-type strain. White bar = 25 microns.

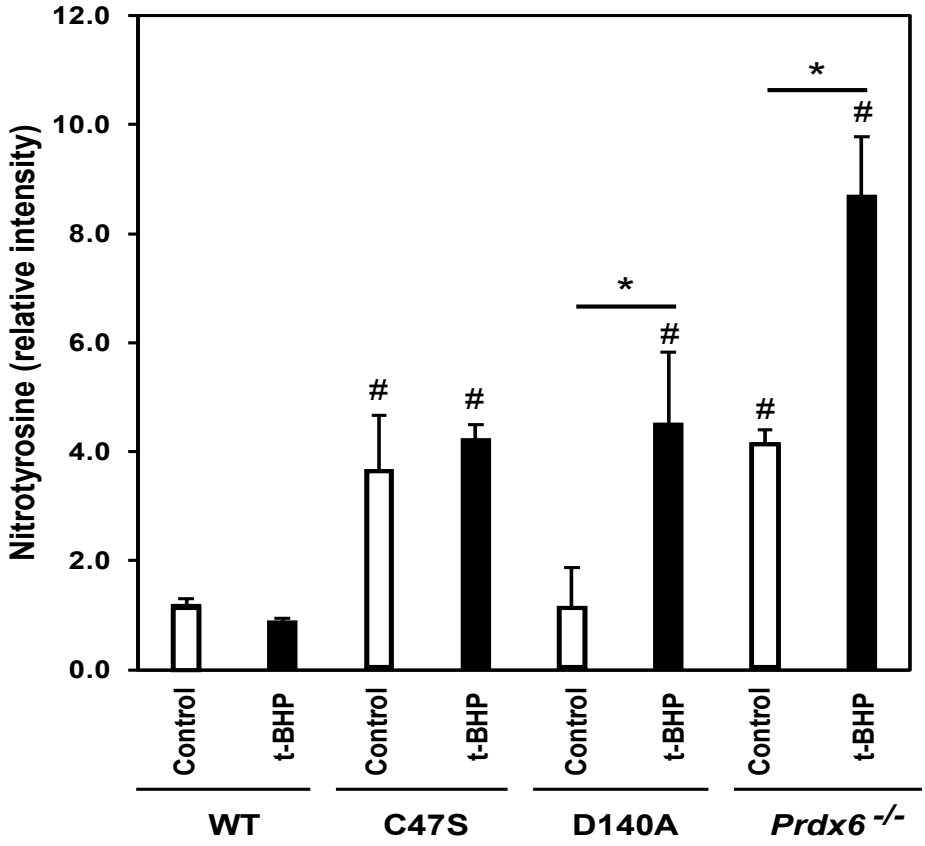

Figure 13B: PRDX6 peroxidase or iPLA$_2$ absence leads to increased nitrotyrosine levels of non-permeabilized spermatozoa in response to oxidative stress.

Average RFI of nitrotyrosine signal was quantified in non-permeabilized epididymal spermatozoa from control and treated mice strains. > 200 spermatozoa per sample were analyzed using ImageJ. n = 4 mice for each strain. # means higher than control wild-type mice. WT: wild-type strain. * means significant differences within same strain. Two-way ANOVA and Bonferroni posthoc tests; $p<0.05$.

5.5 Effect of the absence of PRDX6 peroxidase and iPLA$_2$ on sperm DNA oxidation

Since *Prdx6*$^{-/-}$ spermatozoa show high levels of DNA oxidation (Ozkosem et al., 2015, 2016), we wanted to know whether the absence of either PRDX6 peroxidase or iPLA$_2$ is responsible for this abnormality. Spermatozoa from control wild-type and control D140A males had the lowest percentages of DNA oxidation among all groups (Figure 14). The tBHP treatment significantly increased percentages of spermatozoa with positive DNA oxidation labelling for wild-type, D140A and *Prdx6*$^{-/-}$ mice. Spermatozoa from control and treated C47S males had similar DNA oxidation percentages compared to spermatozoa from treated D140A and control *Prdx6*$^{-/-}$ males.

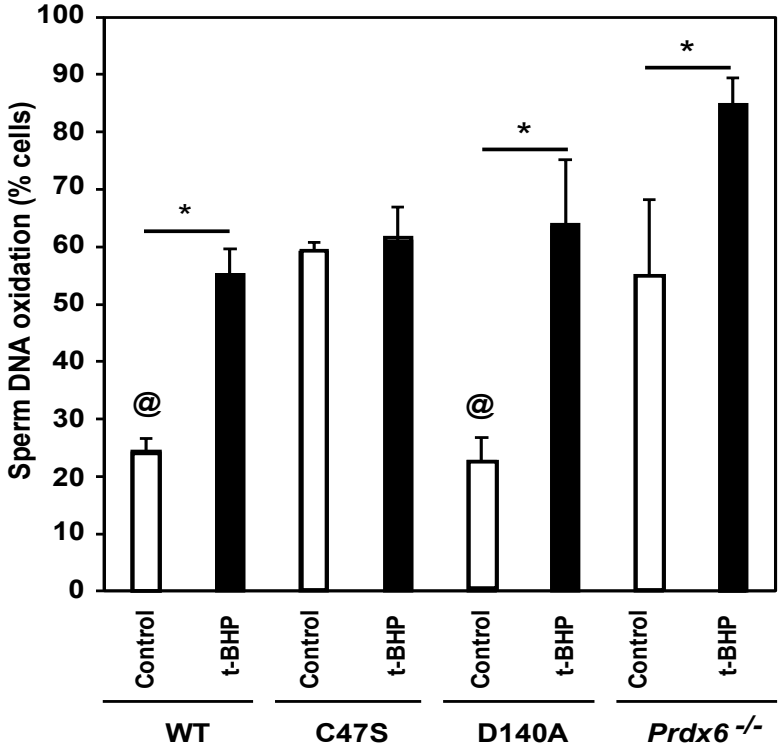

Figure 14: PRDX6 peroxidase or iPLA$_2$ absence leads to increased vulnerability of mice spermatozoa to DNA oxidation.

RFI of DNA oxidation signal was quantified for > 200 permeabilized epididymal spermatozoa for each sample. Percentages of spermatozoa that reached the threshold RFI established for positive DNA oxidation labelling were recorded for each sample. n = 3 mice for each strain. @ means lower than all other groups. WT: wild-type strain. * means significant differences within the same strain. Two-way ANOVA and Bonferroni posthoc tests; $p<0.05$.

5.6 Effect of the absence of PRDX6 peroxidase and iPLA$_2$ on sperm motility

Thermoresistance test using PBS facilitates assessment of sperm's ability to maintain their motility *in vitro* using a medium lacking nutrients required for sperm survival (Bedard et al., 2011). Thus, we can compare the ability of spermatozoa from each control strain to maintain their motility when subjected to an unfavourable environment. Total and progressive sperm motility of mutant males were lower at zero-time (baseline) compared to that of wild-type males (Figure 15). Total and progressive sperm motility of mutant strains followed a decreasing trend during 2-hour incubation in PBS and were significantly lower at all time points compared to that of wild-type males. Total and progressive sperm motility of wild-type males followed an increasing trend from baseline until 60 min, a decreasing trend from 60 min until 90 min, then slightly increased from 90 min until the end of incubation. No significant differences in total and progressive sperm motility were observed between the three mutant strains at all times.

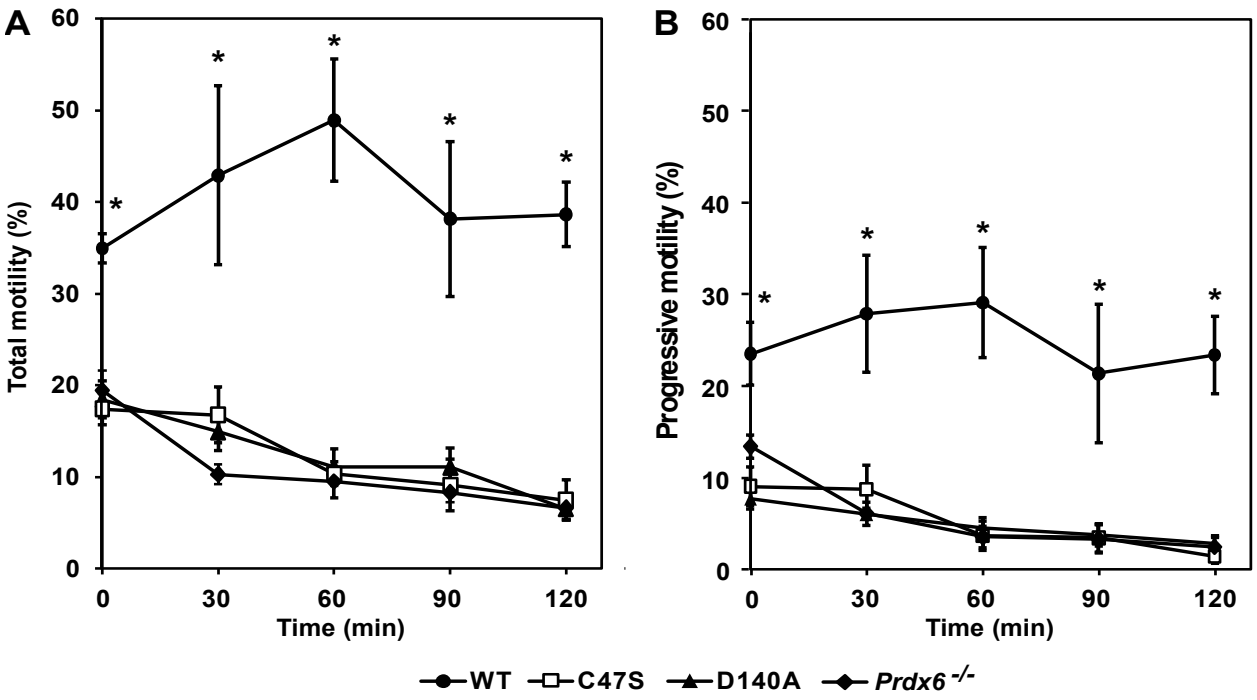

Figure 15: PRDX6 peroxidase or iPLA$_2$ absence leads to decreased sperm motility.

Temporal change in the total and progressive sperm motility for each control strain throughout 2-hour incubation in PBS at 37ºC. WT: wild-type strain. * means higher than all others at the same time. Two-way ANOVA and Tukey tests; n = 5 mice for each strain, $p<0.05$.

5.7 Effect of the absence of PRDX6 peroxidase and iPLA$_2$ on sperm capacitation

Since capacitation is essential for sperm to acquire fertilizing competence (de Lamirande & Gagnon, 1995; O'Flaherty et al., 2003; Florman & Ducibella, 2006), we compared the recurrence of sperm capacitation among the mouse strains by quantifying the percentage of acrosome reaction (%AR) after progesterone treatment. Capacitated wild-type spermatozoa had the highest %AR among all groups (Figure 16). Spermatozoa from mutant strains incubated under capacitating conditions had significantly lower %AR compared to that of capacitated wild-type spermatozoa. Capacitated spermatozoa from C47S males had significantly higher %AR compared to that of non-capacitated counterparts. However, this percentage was lower than that recorded for wild-type capacitated spermatozoa.

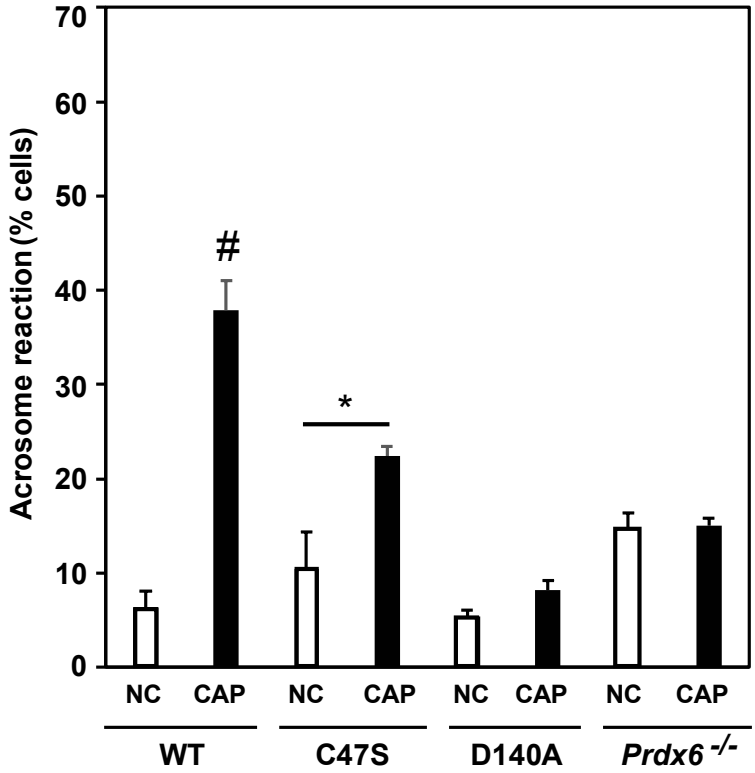

Figure 16: PRDX6 peroxidase or iPLA$_2$ absence leads to impaired sperm capacitation. Percentages of acrosome-reaction (%AR) of non-capacitated (NC) and BSA/HCO_3^--treated (CAP; capacitated) spermatozoa from each control strain were recorded after progesterone treatment. %AR was equal to the percentage of spermatozoa lacking the acrosome (Giemsa stain-negative) among >200 spermatozoa/sample assessed using bright field microscopy. n = 5 mice for each strain. # means higher than all others. WT: wild-type strain. * means significant difference within the same strain. Two-way ANOVA and Tukey tests; $p<0.05$.

6. Discussion

Results presented in this thesis confirm the importance of PRDX6 in ensuring male mice fertility (Ozkosem et al., 2015, 2016; Moawad et al., 2017; Scarlata et al., 2020) and suggest, for the first time, that PRDX6 peroxidase and $iPLA_2$ are both essential to ensure male mice fertility. Oxidative stress induced by the tBHP treatment caused the pup production of wild-type males to decrease to similar levels as that of control *Prdx6*$^{-/-}$ males (Ozkosem et al., 2016). Interestingly, we found that the absence of either PRDX6 peroxidase or $iPLA_2$ activities promoted the same abnormal reproductive outcome (reduction in litter sizes and high number of unsuccessful matings) (Figure 7 and Table 1), indicating that the two PRDX6 enzymatic activities are essential to maintain male mouse fertility.

Treated C47S and D140A males suffer from impaired epididymal sperm maturation, as evidenced by their higher percentages of spermatozoa with CDR compared to treated wild-type males (Figure 8). We observed that PRDX6 peroxidase and $iPLA_2$ prevent sperm lipid peroxidation in males treated with tBHP (Figures 9 and 10B). Previous studies involving rats have demonstrated that tBHP treatment leads to an increase in PRDX6 hyperoxidation in spermatozoa (Liu & O'Flaherty, 2017) and to an increase in 4HNE levels that is associated with a significant increase of PRDX6, and a trend of increase in catalase and PRDX1 levels within the epididymis (Wu et al., 2020). Interestingly, no significant increase in the expression of antioxidant enzymes was recorded within the testes of treated males (Wu et al., 2020). These findings highlight the primary role of PRDX6 in the antioxidant protection of the epididymis (Liu & O'Flaherty, 2017; Wu et al., 2020). The fact that high percentages of spermatozoa from C47S and D140A males showed retention of cytoplasmic droplets (Figure 8) suggests that the function of epididymis becomes impaired when either PRDX6 peroxidase or $iPLA_2$ is absent. Therefore, it is possible that lipid peroxidation of the epididymal epithelium, which may potentially occur in both C47S and D140A males, promotes inactivation of clear cells to prevent sperm cytoplasm absorption (Hermo et al., 1988; Sullivan & Belleannée, 2018), however further studies should be conducted to confirm this hypothesis. Impairment of epididymal function due to PRDX6 peroxidase or $iPLA_2$ absence may also suggest that activity of principal cells involved in the secretion of epididymosomes could have been affected (Sullivan, 2008), therefore potentially preventing the acquisition of antioxidant enzymes that could prevent sperm oxidative

stress (O'Flaherty, 2019). Increased retention of cytoplasmic droplets may have also contributed to the impairment of sperm motility (Figure 15), and capacitation (Figure 16) observed in C47S and D140A males since impaired cytoplasm removal leads to accumulation of ROS generated through SOD and G6PDH activity (Aitken et al., 1994; Griveau et al., 1995; Aitken et al., 1996; Kehrer, 2000). Lack of antioxidant protection due to the absence of either PRDX6 peroxidase or $iPLA_2$ may have also been worsened since impaired integrity of epididymal epithelium due to lipid peroxidation may have prevented binding of epididymosomes to sperm membrane, resulting in spermatozoa's inability to acquire antioxidant protection that could mitigate oxidative stress (O'Flaherty, 2019).

C47S males, irrespective of treatment, and treated D140A males had higher percentages of spermatozoa with positive DNA oxidation labeling compared to control wild-type males (Figure 14), and similar percentages compared to *Prdx6*$^{-/-}$ or treated wild-type males, suggesting that PRDX6 peroxidase and $iPLA_2$ are required to protect DNA bases from ROS-induced oxidation. Indeed, inhibition of either PRDX6 peroxidase or $iPLA_2$ led to a significant increase in human sperm DNA oxidation levels (Fernandez & O'Flaherty, 2018). For all groups, except for treated wild-type males, a trend of increase in sperm nitrotyrosine levels (Figure 13B) was similar to that of percentages of spermatozoa with positive DNA oxidation labeling (Figure 14). Moreover, in agreement with this result, when *Prdx6*$^{-/-}$ males, which normally have elevated levels of sperm DNA oxidation, were fed with γ-tocopherol-enriched diet, their sperm DNA oxidation levels decreased to similar levels recorded in wild-type males fed with control diet (Scarlata et al., 2020). Considering that γ-tocopherol is a more efficient scavenger of $ONOO^-$ compared to α-tocopherol (Jiang et al., 2001; Galli et al., 2004), these results suggest that $ONOO^-$ is the primary ROS responsible for sperm DNA oxidation (Scarlata et al., 2020). Treated wild-type males were more vulnerable to sperm DNA oxidation compared to control wild-type males (Figure 14), which was confirmed by a previous study (Ozkosem et al., 2016), despite having low nitrotyrosine levels (Figures 12 and 13B). Due to oxidative stress experienced by treated wild-type males, as evidenced by their elevated sperm 4HNE levels (Figures 9 and 10B), it's reasonable to believe that other ROS, apart from $ONOO^-$, generated through 4HNE-mediated disruption of mitochondrial activity (Aitken et al., 2012) may have contributed to its increase in percentages of spermatozoa with positive DNA oxidation labeling (Figure 14). Indeed, elevated

sperm DNA oxidation levels have been associated with abnormal mitochondrial function resulting in the production of high levels of $O_2^{\bullet-}$ that can dismutate to H_2O_2 through SOD activity (Griveau et al., 1995; Kehrer, 2000; Fernandez & O'Flaherty, 2018).

Previous *in vitro* study has shown that the pharmacological inhibition of either PRDX6 peroxidase or $iPLA_2$ activities results in high levels of lipid peroxidation in human spermatozoa (Fernandez & O'Flaherty, 2018). The absence of the *PRDX6* gene also leads to higher sperm lipid peroxidation levels in mouse spermatozoa (Ozkosem et al., 2016). In accordance with these results, spermatozoa from control and treated *Prdx6*$^{-/-}$ males have higher 4HNE levels in both non-permeabilized (Figure 10B) and whole spermatozoa (Figure 9) compared to wild-type counterparts, confirming that PRDX6 prevents sperm lipid peroxidation. Additionally, PRDX6 prevents lipid peroxidation through its two enzymatic activities, as evidenced by the higher sperm 4HNE levels of control knock-in strains compared to that of control wild-type males (Figures 9 and 10B). Interestingly, treated wild-type males had higher 4HNE levels within both whole spermatozoa (Figure 9) and non-permeabilized spermatozoa (Figure 10B) compared to control wild-type males, suggesting that wild-type mice suffer from sperm oxidative stress in response to tBHP treatment, a result that was confirmed by a previous study (Ozkosem et al., 2016). Considering that non-permeabilized spermatozoa from both control and treated groups of both knock-in strains had similar 4HNE levels compared to that of treated wild-type males (Figure 10B), it is possible that during tBHP-induced oxidative stress, wild-type spermatozoa suffer from lipid peroxidation because of the inactivation of $iPLA_2$ and PRDX6 peroxidase activities.

The absence of PRDX6 peroxidase or $iPLA_2$ impairs spermatozoa's ability to withstand oxidative stress, as evidenced by the higher formation of 4HNE adducts within whole spermatozoa of treated knock-in strains compared to that of treated wild-type males (Figure 9). 4HNE labelling is present throughout all of permeabilized spermatozoa's subcellular compartments (Figure 11); therefore, formation of 4HNE adducts occurs throughout all subcellular structures of spermatozoa. Several proteins have been shown to be adducted by 4HNE, notably enzymes involved in the electron transport chain (succinate dehydrogenase) (Aitken et al., 2012), tubulin, glycolytic enzymes (phosphofructokinase, aldolase-A), and

enzymes involved in Kreb's cycle (malate dehydrogenase 2) (Baker et al., 2015). Adduction of these proteins by 4HNE may be responsible for the impairment of sperm motility observed in all mutant males (Figure 15). A-kinase anchoring protein 4 (AKAP4), a protein that is associated with fibrous sheath and is important for sperm motility (Turner et al., 1998; Miki et al., 2002), also forms adducts with 4HNE (Nixon et al., 2019). Potential sperm membrane proteins that may form adducts with 4HNE are voltage-dependent anion-selective channel protein 2 (VDAC2) and Na^+/K^+ transporting ATPase subunit alpha-4 (ATP1A4) (Baker et al., 2015). VDAC2 is a channel that transports Ca^{2+} into mitochondria (Yeste et al., 2015) and is suggested to be present within either the acrosomal membrane or plasma membrane of both mouse and human spermatozoa (Triphan et al., 2008; Liu et al., 2011). VDAC2 inhibition results in decreased Ca^{2+} influx required for acrosome reaction (Liu et al., 2011). ATP1A4 is localized within the sperm membrane (Kaplan, 2002) and is involved in the maintenance of ion concentrations to preserve the resting potential of the sperm membrane (Feraille & Doucet, 2001). Rat spermatozoa that have inactive ATP1A4 display impaired motility, depolarization of plasma membrane, and lower intracellular pH (Jimenez et al., 2010). Furthermore, compared to fertile men, asthenozoospermic patients have lower sperm VDAC levels, while patients suffering from unilateral varicocele have lower sperm ATP1A4 levels (Agarwal et al., 2015; Hashemitabar et al., 2015).

As previously mentioned, PRDX6 peroxidase is the main $ONOO^-$ scavenger in human spermatozoa (Fernandez & O'Flaherty, 2018). In accordance with this result, the intensity of nitrotyrosine labelling within non-permeabilized spermatozoa, which is found in the midpiece and principal piece of mouse (Figure 13A) and human spermatozoa (Morielli & O'Flaherty, 2015), is similar for control C47S and control *Prdx6*$^{-/-}$ males (Figure 13B), suggesting that PRDX6 peroxidase could also be the main $ONOO^-$ scavenger in mouse spermatozoa. Consequentially, spermatozoa from control D140A males have similar nitrotyrosine levels compared to that of treated wild-type males (Figures 12 and 13B), implying that the presence of PRDX6 peroxidase is sufficient to prevent the increase in $ONOO^-$ levels. Noteworthy, despite PRDX6 peroxidase absence, levels of tyrosine-nitrated proteins within sperm membrane did not increase further in C47S males in response to tBHP treatment (Figure 13B), suggesting that removal of oxidized phospholipids by $iPLA_2$ may have been accelerated to prevent production of 4HNE, which can disrupt mitochondrial activity to generate $O_2^{\bullet-}$ that can react with $NO^\bullet$ to form

$ONOO^-$ (Griveau et al., 1995; Packer et al., 1996; Herrero et al., 2001; Aitken et al., 2012). Conversely, despite the presence of PRDX6 peroxidase, levels of tyrosine nitrated sperm proteins increased significantly in response to tBHP treatment for D140A males (Figures 12 and 13B). The high affinity of PRDX6 peroxidase to H_2O_2 even at low concentrations required for capacitation may explain this result (O'Flaherty & Rico de Souza, 2010), therefore during tBHP-induced oxidative stress, H_2O_2 produced may have potentially oxidized and inactivated PRDX6 peroxidase, resulting in the inability to scavenge $ONOO^-$ and the subsequent increase in nitrotyrosine levels. Accumulation of oxidized phospholipids due to $iPLA_2$ absence (Fisher, 2017) may have also contributed to the increase in sperm $ONOO^-$ levels, leading to the increase in nitrotyrosine levels observed in spermatozoa of treated D140A males.

The absence of PRDX6 peroxidase or $iPLA_2$ increases the vulnerability of sperm membrane proteins to tyrosine nitration, as evidenced by the higher nitrotyrosine levels of non-permeabilized spermatozoa from treated knock-in strains compared to that of treated wild-type males (Figure 13B). Within the sperm membrane, a potential target for tyrosine nitration is matrix metallopeptidase 9 (MMP9), a protein localized within the inner acrosomal membrane and is hypothesized to be important for sperm penetration through zona pellucida (Ferrer et al., 2012). Tyrosine nitration of MMP9 results in the inability of spermatozoa to bind to the zona pellucida and penetrate it to fertilize the oocyte (Wang et al., 2011). Additionally, the previous study has shown that inhibition of protein disulfide-isomerase A3 (ERp57), an enzyme that can catalyze the reduction of disulfide bonds (Frickel et al., 2004) and is present within the acrosome of rat spermatids and the mouse sperm membrane (Ohtani et al., 1993; Ellerman et al., 2006), leads to impaired sperm membrane fusion with oolemma (Inoue et al., 2005). It is possible that ERp57 also undergoes tyrosine nitration in mouse spermatozoa since this enzyme can be tyrosine nitrated as it was reported in a mouse model of amyotrophic lateral sclerosis (ALS) (Casoni et al., 2005). Similarly, the absence of either PRDX6 enzymatic activities leads to increased vulnerability of intracellular proteins to tyrosine nitration, as demonstrated by the higher nitrotyrosine levels within whole spermatozoa of control and treated knock-in strains compared to that of their wild-type counterparts (Figure 12). Permeabilized spermatozoa from both mice (Scarlata et al., 2020) and humans (Morielli & O'Flaherty, 2015) have nitrotyrosine labelling within their head, midpiece and principal piece. Considering that in humans, proteins that

undergo tyrosine nitration are mainly found within Triton-insoluble fraction, it is possible that enzymes involved in glycolysis and Kreb's cycle account for the nitrotyrosine signal observed in both species (Morielli & O'Flaherty, 2015). Indeed, along with tubulin, these enzymes undergo tyrosine nitration during oxidative stress (Fratelli et al., 2004; Landino et al., 2004; Morielli & O'Flaherty, 2015). A potential tyrosine nitration target is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme that is localized within the fibrous sheath of sperm flagellum (Westhoff & Kamp, 1997) and is thiol oxidized by H_2O_2 during oxidative stress (Elkina et al., 2011). Considering that PRDX1, PRDX5 and PRDX6 are also found within Triton-insoluble fraction, it's likely that these antioxidant enzymes also undergo tyrosine nitration, therefore resulting in increased sperm oxidative stress (O'Flaherty & Rico de Souza, 2010).

As previously mentioned, $ONOO^-$ is involved in the impairment of mouse sperm motility (Scarlata et al., 2020), and PRDX6 peroxidase is the main $ONOO^-$ scavenger in human spermatozoa (Fernandez & O'Flaherty, 2018). Considering that tubulin and enzymes involved in energy generation undergo tyrosine nitration during oxidative stress (Fratelli et al., 2004; Landino et al., 2004; Morielli & O'Flaherty, 2015), it is reasonable to believe that $ONOO^-$ produced through 4HNE-mediated disruption of mitochondrial activity (Griveau et al., 1995; Packer et al., 1996; Herrero et al., 2001; Aitken et al., 2012) promotes tyrosine nitration of these enzymes to impair sperm motility. For C47S males, it's possible that PRDX6 peroxidase absence led to the inability to scavenge $ONOO^-$, therefore causing sperm motility to be impaired (Figure 15). For D140A males, it's plausible that sperm oxidative stress generated by incubation in PBS may have resulted in H_2O_2-induced PRDX6 peroxidase inhibition and subsequent disruption of sperm motility due to inability to scavenge $ONOO^-$. Additionally, elevated lipid peroxidation levels in both C47S and D140A males (Figures 9 and 10B) may have also contributed to the impairment of sperm motility since the number of polyunsaturated fatty acids in the sperm membrane is positively correlated with sperm motility (Roqueta-Rivera et al., 2010; Safarinejad et al., 2010; Am-in et al., 2011; Martínez-Soto et al., 2013).

PRDX6 peroxidase or $iPLA_2$ inhibition impairs sperm capacitation since the %AR of capacitated spermatozoa from both C47S and D140A males was lower compared to that of wild-type males

(Figure 16). Interestingly, a small but significant increase in %AR was observed for spermatozoa from C47S males (Figure 16), suggesting that compared to PRDX6 peroxidase, $iPLA_2$ is more important for the maintenance of sperm capacitation. This result is further supported by the fact that capacitation of MJ33-treated wild-type spermatozoa was impaired to a similar level as that of *Prdx6*$^{-/-}$ spermatozoa (Ozkosem et al., 2016), confirming that $iPLA_2$ activity is essential for the maintenance of sperm capacitation. Results of this thesis demonstrate that elevated sperm 4HNE levels of control C47S and D140A males (Figures 9 and 10B) were associated with the impairment of sperm capacitation (Figure 16), suggesting that 4HNE may be the culprit for the low %AR observed in these strains. Several kinases such as PKA (Leclerc et al., 1996; O'Flaherty et al., 2004, 2005), PKC (O'Flaherty, de Lamirande, et al., 2006b) and ERK (de Lamirande & Gagnon, 2002; Thundathil et al., 2003) are important to ensure sperm capacitation. According to this observation, it's plausible that 4HNE impairs sperm capacitation by inactivating these kinases since high 4HNE levels promote inhibition of PKC and ERK activation in rat hepatocytes (Pronzato et al., 1990; Chiarpotto et al., 1999; Sampey et al., 2007). Furthermore, it's also reasonable to think that ROS produced by 4HNE-mediated disruption of mitochondrial activity (Aitken et al., 2012) also impair capacitation-associated phosphorylation pathways since elevated ROS levels can inhibit PKA and PKC (Ward et al., 2000; Humphries et al., 2002). Indeed, levels of phosphotyrosine and phosphorylated PKA substrates were lower, while lipid peroxidation levels were higher for MJ33-treated capacitated wild-type spermatozoa compared to that of untreated capacitated wild-type spermatozoa (Lee et al., 2017), suggesting that 4HNE and ROS are involved in the disruption of capacitation-associated phosphorylation pathways. Elevated ROS and 4HNE levels may also impair capacitation by preventing actin polymerization, an event required for acrosome reaction to occur (Brener et al., 2003; Breitbart et al., 2005), since actin can undergo oxidation by lipid peroxides and s-glutathionylation (Dalle-Donne et al., 2005; Bizzozero et al., 2007).

Despite a recent study indicating that $ONOO^-$ is the primary ROS involved in inducing infertility (Scarlata et al., 2020), control D140A males, which possess low sperm nitrotyrosine levels (Figures 12 and 13B), remain infertile (Figure 7 and Table 1) due to elevated 4HNE (Figures 9 and 10B), sperm DNA oxidation levels (Figure 14) and percentages of spermatozoa with CDR (Figure 8) and impaired sperm motility (Figure 15) and capacitation (Figure 16), suggesting that

infertility is multi-causal and that oxidative stress can be established by a net increase in one particular ROS or a combination of them.

Indicators of poor sperm quality manifested in C47S and D140A males, notably elevated sperm 4HNE, nitrotyrosine, DNA oxidation levels, percentages of spermatozoa with CDR, and impairment of sperm motility and capacitation collectively contributed to their infertility (Figure 7 and Table 1). Another factor that may have promoted infertility in both knock-in strains is the presence of abnormalities within the sperm membrane, as evidenced by the lower sperm membrane fluidity (Ozkosem et al., 2016), and impairment of sperm's binding to both zona pellucida and oolemma caused by PRDX6 absence (Moawad et al., 2017). The inability for spermatozoa to bind to both zona pellucida and oolemma may be driven by ROS-induced oxidation/modification of important proteins involved in these processes, notably tyrosine nitration of MMP9, which, as previously mentioned, prevents oocyte fertilization due to premature induction of capacitation (Wang et al., 2011). Considering that presence of ERp57 is important to allow sperm membrane fusion with oolemma, potential tyrosine nitration of ERp57 may also lead to the inability for spermatozoon to fertilize the oocyte (Inoue et al., 2005). Izumo sperm-Egg fusion 1 (IZUMO1), a receptor localized within sperm membrane and is important for sperm membrane fusion with oolemma, could be another ROS target since IZUMO1$^{-/-}$ animals are infertile (Inoue et al., 2005; Inoue et al., 2015; Young et al., 2016). Further research should be conducted to verify if the activity of these proteins is indeed impaired in both C47S and D140A mice.

The previous study has demonstrated that spermatogenesis normally occurs in *Prdx6*$^{-/-}$ males (Moawad et al., 2017). Their spermatozoa have identical morphology and acrosome structure compared to wild-type spermatozoa, suggesting that PRDX6 absence doesn't affect the testis (Moawad et al., 2017). Hence, results presented in this thesis indicate that the lack of antioxidant protection in *Prdx6*$^{-/-}$, C47S and D140A mice lead to defects within spermatozoa that occur post-spermatogenesis and within the epididymal epithelium, culminating in the impairment of mice's reproductive outcomes. A table summarizing the involvement of both PRDX6 peroxidase and iPLA$_2$ in the regulation of all parameters measured is provided in the next page (Table 2).

Parameter	Peroxidase	iPLA$_2$
Sperm motility	Yes	Yes
Sperm capacitation	Yes	Yes (predominant)
Reproductive outcomes (without tBHP)	Yes	Yes
Reproductive outcomes (with tBHP)	Yes	Yes
Percentages of sperm with cytoplasmic droplet retention (without tBHP)	Yes	Yes
Percentages of sperm with cytoplasmic droplet retention (with tBHP)	Yes	Yes
Whole sperm 4HNE levels (without tBHP)	Yes	Yes
Whole sperm 4HNE levels (with tBHP)	Yes	Yes
Levels of 4HNE-adducted sperm membrane proteins (without tBHP)	Yes	Yes
Levels of 4HNE-adducted sperm membrane proteins (with tBHP)	Yes	Yes
Whole sperm nitrotyrosine levels (without tBHP)	Yes (predominant)	Yes
Whole sperm nitrotyrosine levels (with tBHP)	Yes	Yes
Levels of tyrosine-nitrated sperm membrane proteins (without tBHP)	Yes	No
Levels of tyrosine-nitrated sperm membrane proteins (with tBHP)	Yes	Yes
Sperm DNA oxidation levels (without tBHP)	Yes	No
Sperm DNA oxidation levels (with tBHP)	Yes	Yes

Table 2: PRDX6 peroxidase and iPLA$_2$ are involved in the regulation of parameters that are associated with male fertility

The words 'Yes' and 'No' imply that the absence of the corresponding PRDX6 enzymatic activity led to significant and insignificant effect on the research parameter compared to wild-type counterpart respectively. The word 'predominant' implies that the absence of the corresponding PRDX6 enzymatic activity has a stronger effect compared to the absence of the other enzymatic activity.

7. Conclusion

Infertility observed in C47S and D140A males is due to impaired sperm motility, capacitation, epididymal maturation, and elevated sperm lipid peroxidation, nitrotyrosine and DNA oxidation levels. tBHP treatment worsened infertility of C47S and D140A mice, suggesting that these strains lack antioxidant protection required to prevent sperm oxidative stress. PRDX6 peroxidase and $iPLA_2$ are independently required to support male mice fertility.

Results presented here could aid in designing novel diagnostic tools that could determine the exact causes of male infertility. Assays aimed to measure PRDX6 peroxidase, and $iPLA_2$ activities could provide insights into whether men's infertility is caused by PRDX6 inactivity and/or PRDX6 gene mutations. Moreover, considering that oxidative stress can be primarily caused by a specific ROS, notably $ONOO^-$ (Scarlata et al., 2020), assays that can measure sperm nitrotyrosine levels could be used as guidance by physicians to prescribe γ-tocopherol, which if proven to be effective in infertile men, may potentially be a suitable drug for targeted antioxidant therapy against $ONOO^-$-induced infertility (Jiang et al., 2001; Galli et al., 2004; Scarlata et al., 2020).

8. Future Directions

Results of this thesis suggest that PRDX6 peroxidase and $iPLA_2$ ensure male mouse fertility; therefore, research aimed to determine single nucleotide polymorphisms (SNPs) present within the genes that encode for these enzymatic activities in infertile men but absent in fertile men would be beneficial in identifying genetic modifications that trigger male infertility. Additionally, clinical trials aimed to determine the efficacy of γ-tocopherol (Jiang et al., 2001; Galli et al., 2004; Scarlata et al., 2020) in mitigating oxidative stress in infertile men should also be conducted. Pre-clinical research that involves the administration of drugs that can upregulate the activity of either PRDX6 peroxidase or $iPLA_2$ in mice could aid in uncovering potential systematic and local side effects that fertility-inducing drugs may cause. To fully understand the mechanisms that promote male infertility, the identity of proteins/enzymes that are affected by ROS generated by PRDX6 peroxidase or $iPLA_2$ absence should also be determined to identify novel markers of male infertility. These studies would provide information regarding the consequences of oxidative stress on molecular mechanisms necessary to ensure male fertility.

9. References

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10. Appendix

McGill

May 11, 2021

Animal Certificate

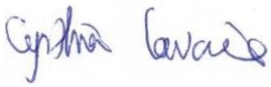
This is to certify that **Dr. Cristian O'Flaherty, RI-MUHC Glen site,** currently holds an approved **Animal Use Protocol # 2009-5656** with McGill University and its Affiliated Hospital's Research Institutes for the following project:

Animal Use Protocol Title: Regulation of redox signaling in male reproduction

Start date: April 1, 2021

Expiration date: March 31, 2022

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.



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