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TITLE: Pharmacological inhibition of arachidonate 15-lipoxygenase (ALOX15) protects human spermatozoa against oxidative stress

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RUNNING TITLE: Protection of human spermatozoa against oxidative stress

SUMMARY SENTENCE: Pharmacological inhibition of ALOX15 reduced the level of oxidative stress experienced by human spermatozoa in response to exogenous ROS challenge.

KEYWORDS: Male fertility, oxidative stress, Arachidonate 15-Lipoxygenase, ALOX15, lipid peroxidation, reactive oxygen species, ROS

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ABSTRACT

One of the leading causes of male infertility is defective sperm function, a pathology that commonly arises from oxidative stress in the germline. Lipid peroxidation events in the sperm plasma membrane result in the generation of cytotoxic aldehydes such as 4-hydroxynonenal (4HNE), which accentuate the production of reactive oxygen species (ROS) and cause cellular damage. One of the key enzymes involved in the metabolism of polyunsaturated fatty acids to 4HNE in somatic cells is arachidonate 15-lipoxygenase (ALOX15). Although ALOX15 has yet to be characterized in human spermatozoa, our previous studies have revealed a strong link between ALOX15 activity and the levels of oxidative stress and 4HNE in mouse germ cell models. In view of these data, we sought to assess the function of ALOX15 in mature human spermatozoa and determine whether the pharmacological inhibition of this enzyme could influence the level of oxidative stress experienced by these cells. By driving oxidative stress *in vitro* with exogenous H_2O_2, our data reveal that 6,11-dihydro[1]benzothiopyrano[4,3-b]indole (PD146176; a selective ALOX15 inhibitor), was able to significantly reduce several deleterious, oxidative insults in spermatozoa. Indeed, PD146176 attenuated the production of ROS, as well as membrane lipid peroxidation and 4HNE production in human spermatozoa. Accordingly, ALOX15 inhibition also protected the functional competence of these cells to acrosome react and bind homologous human zonae pellucidae. Together, these results implicate ALOX15 in the propagation of an oxidative stress cascade within human spermatozoa and offer insight into potential therapeutic avenues to address male fertility that arises from oxidative stress.

INTRODUCTION

World-wide, as many as 15-20% of couples experience trouble conceiving [1], with male factor infertility contributing to at least 40-50% of these cases [2]. In developed countries it has been

estimated that approximately 1 in 20 men are sub-fertile [3]. While the numerous contributing factors responsible for these figures are highly complex, male infertility has long been known to be associated with defective sperm function, rather than simply low cell number [4]. In this context, oxidative stress caused by elevated levels of intracellular reactive oxygen species (ROS), is recognized as a major contributing factor to a loss of sperm cell function [5-10]. In a majority of somatic cells, sufficient cytosolic antioxidant mechanisms, mitigate the potential for ROS overproduction. In contrast, the highly specialized cytodifferentiation process of spermiogenesis sees the cytoplasmic content of the developing male germ cell severely depleted [11, 12]. The concomitant loss of antioxidant defenses during this differentiation process [13] and the abundance of poly-unsaturated fatty acids (PUFAs), which serve as major substrates for lipid peroxidation, place these cells at an increased risk of oxidative attack. The peroxidation cascades that ensue, produce a wide range of physiologically and pathologically important products including cytotoxic aldehydes, which initiate further production of ROS, precipitating a loss in function and cell death [5, 7, 10, 14, 15].

The metabolism of polyunsaturated fatty acids to lipid aldehydes can be mediated by both non-enzymatic and enzymatic means [16]. In regard to the latter, we have recently used a mouse germline model to establish a causative link between the generation of the lipid aldehyde, 4-hydroxynonenal (4HNE), and the enzymatic action of arachidonate 15-lipoxygenase (ALOX15) [17]; a member of the non-heme iron-containing lipoxygenase family. Furthermore, this study confirmed that the pharmacological inhibition of ALOX15 was able to reduce both ROS levels and 4HNE production in an immortalized spermatocyte cell line [GC-2spd(ts)] [17]. Such evidence builds on work conducted in somatic cells where lipoxygenases have been well-documented to act as catalysts for lipid peroxidation [16, 18, 19]. Although a thorough mechanistic understanding of the functions of ALOX15 is yet to be established, it's

enzymatic action is dependent on the central redox-active iron atom, thereby promoting lipid peroxidation via hydrogen extraction or oxygenation of lipid substrates [16, 19].

These observations are commensurate with compelling data obtained using transgenic mouse models, which have firmly implicated ALOX15 in the regulation of male fertility [20]. Specifically, Brütsch and colleagues (2015) exploited an elegant double knockout strategy to secure evidence that targeted deletion of the *Alox15* gene could rescue a range of adverse sequelae associated with the accompanying loss of a single glutathione peroxidase 4 (*Gpx4*) allele. In wild type animals, testicular GPX4 acts as antioxidant enzyme owing to its ability to catalyze the reduction of hydrogen peroxide (H_2O_2), organic hydroperoxides and lipid peroxides at the expense of reduced glutathione. Deletion of even a single *Gpx4* allele (i.e. genotype $Gpx4^{+/-}$), leads to notable defects in sperm function observed through reduced motility (total, rapid and progressive) and an attendant reduction in overall litter size for the animal [20]. In marked contrast, each of these functional attributes, as well as the overall fertility of the animals, were significantly improved in an equivalent transgenic mouse line that also harbored a complete ablation of the *Alox15* gene (i.e. genotype $Gpx4^{+/-}/Alox15^{-/-}$). Taken together, these data suggest that ALOX15 may hold a central role in the maintenance of redox status within the male reproductive system. While an equivalent role is yet to be explored in human spermatozoa, we postulate that ALOX15 may contribute to lipid peroxidation in these cells and that an elevation in its presence or activity may precipitate their oxidative demise. Given the prevalence of oxidative stress-induced dysfunction in the male germline, the present study sought to characterize the presence of ALOX15 in mature human spermatozoa and determine whether the selective inhibition of this lipoxygenase enzyme could reduce the levels of oxidative stress experienced by these cells.

MATERIALS AND METHODS

Ethical Approval

The experiments described in this study were conducted using human semen samples obtained with informed consent from a panel of healthy normozoospermic donors. Routine assessment of semen parameters were conducted in accordance with WHO criteria [21]. All studies were performed in accordance with the University of Newcastle's Human Ethics Committee guidelines (Approval No. H-2013-0319).

Reagents

Unless specified, chemical reagents were obtained from Sigma Aldrich (St. Louis, MO, USA) and were of research grade. PD146176 was obtained from Tocris Bioscience (Avonmouth, Bristol, UK). Mitosox^{TM} Red (MSR) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fluorescent probes, dihydroethidium (DHE), LIVE/DEAD, BODIPY-C_{11} and SYTOX Green were also purchased from Thermo Fisher Scientific. Primary antibodies raised against ALOX15 and CD59 were purchased from Abcam (Cambridge, MA, USA). Tris-HCl was purchased from ICN Biochemicals (Castle Hill, NSW, Australia), nitrocellulose and Percoll were from GE Healthcare (Chicago, IL, USA). Mowiol 4–88 was from Calbiochem (La Jolla, CA, USA), with paraformaldehyde was supplied by ProSciTech (Thuringowa, QLD, Australia). All antibodies and details for their use are included in Supplementary Table S1.

Preparation of human spermatozoa

Unless otherwise stated, all experimental procedures involving human spermatozoa were replicated at least three times, with each replicate consisting of spermatozoa obtained from at least one individual semen donor (i.e. at least n = 3 sperm donors). Enrichment of human spermatozoa was completed through the use of discontinuous Percoll gradients of (45% / 90%)

as previously described [5, 22]. Briefly, semen samples were layered on top of Percoll gradients and centrifuged at 500 × *g* for 30 min. To isolate the enriched sperm pellet, Percoll was removed and spermatozoa were re-suspended in a non-capacitating formulation of Biggers, Whitten and Whittingham (NC BWW) media [23] for centrifugation at 500 × *g* for 15 min. NC BWW was prepared without the inclusion of bicarbonate in order to restrict sperm progression into capacitation and consisted of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin and 20 mM HEPES buffer and 1 mg/ml polyvinyl alcohol (osmolarity of 290-310 mOsm/kg). Following centrifugation, spermatozoa were re-suspended in NC BWW at a concentration of 10×10^6 cells/ml.

Capacitation and acrosome reaction of human spermatozoa

In vitro induction of capacitation was implemented using a modified 'capacitating' Biggers, Whitten and Whittingham media [24]. In addition to the base ingredients utilized in the preparation of BWW, the capacitating BWW was supplemented with the additional reagents of 25 mM $NaHCO_3$, 3 mM pentoxifylline and 5 mM dibutyryl cyclic adenosine monophosphate (dbcAMP) as previously described [5]. Sperm populations (10×10^6 cells/ml) were incubated for 3 h in an atmosphere of 5% CO_2/95% air at 37 ˚C. Following capacitation, induction of the acrosome reaction was effected using calcium ionophore A23187 (2.5 µM). Capacitated sperm samples were incubated with A23187 for 30 min at 37 ˚C. Assessment of acrosome reaction rates in live spermatozoa was completed following an additional incubation (30 min, 37 ˚C) of samples in hypo-osmotic swelling media (HOS; 0.07% w/v sodium citrate; 1.3% w/v fructose). Spermatozoa were then air dried on 12-well Henley slides and permeabilized by immersion in ice-cold methanol. To assess the acrosome reaction, slides were stained with TRITC-labelled peanut agglutinin lectin (PNA, diluted 1µg/µl, 20 min, 37 ˚C). The acrosomal status of the

sperm populations was assessed using a Zeiss Axiovert fluorescence microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany), with curled tails and a loss of acrosomal PNA fluorescence indicative of viable sperm that have completed acrosomal exocytosis [25].

Immunocytochemistry

Non-capacitated and capacitated sperm populations were fixed using 4% paraformaldehyde and then washed with a 0.05 M glycine / phosphate buffered saline (PBS) solution. Cells were settled onto poly-L-lysine coverslips and permeabilized using 0.2% Triton X-100 as previously described [17]. Slides were then blocked in 3% bovine serum albumin (BSA) and primary antibody incubations were completed using anti-ALOX15 antibodies (5 µg/ml) overnight at 4 °C. Appropriate secondary antibodies were applied for 1 h at room temperature before counter staining slides with PNA-TRITC (1µg/µl) for 20 min. All coverslips were mounted onto glass slides using a 10% Mowiol anti-fade solution. ALOX15 labelling of spermatozoa was then examined using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss).

SDS-PAGE and immunoblotting

Following preparation of non-capacitated and capacitated sperm populations, sodium dodecyl sulfate (SDS) protein extraction was conducted for 5 min at 100 °C (0.375 M Tris pH 6.8, 2% w/v SDS, 10% w/v sucrose, protease inhibitor cocktail). Soluble protein was obtained following centrifugation (17, 000 × *g,* 15 min, 4 °C). SDS-PAGE and immunoblotting techniques were performed as previously described [17, 26]. Briefly, solubilized sperm proteins were diluted to appropriate concentrations in SDS-PAGE sample buffer (2% v/v mercaptoethanol, 2% w/v SDS and 10% w/v sucrose in 0.375 M Tris, pH 6.8 with bromophenol blue) then boiled for 5 min. Samples were loaded onto 4-20% Tris-Glycine gels and resolved (1 h, 150 V). Following gel electrophoresis, proteins were electrotransferred to nitrocellulose membranes (1 h, 350 mA)

prior to blocking of membranes in 5% skim milk powder/TBST or 3% BSA/TBST for 1 h at room temperature. Immunolabelling of target proteins was achieved by sequential incubation of membranes with anti-ALOX15 antibodies (diluted 1 µg/ml in 1% skim/TBST or 1% BSA/TBST solutions) overnight at 4°C followed by a goat-anti-rabbit HRP-conjugated secondary antibody (diluted 0.13 µg/ml in a 1% skim/TBST or 1% BSA/TBST solution) at room temperature for 1 h. Additionally, the detection of 4HNE-modified proteins in human sperm lysates was made possible through immunoblotting with an anti-4HNE antibody (Alpha Diagnostic International, San Antonio, TX, USA) (diluted 2 µg/ml in 1% BSA/TBST, overnight at 4°C) followed by a goat-anti-rabbit HRP-conjugated secondary antibody (used as above). Immunodetection was completed using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare) as per the manufacturer's instructions.

Surface labelling of human spermatozoa

The surface expression of ALOX15 was examined in non-capacitated and capacitated human sperm populations. Live populations of spermatozoa were labelled with the anti-ALOX15 antibody (5 µg/ml) for 30 min at 37 °C. The viability stain Live/Dead (diluted 1:10,000) was added in the final 10 min of the primary antibody incubation. Following three washes in BWW, samples were incubated with the secondary antibody goat-anti-rabbit Alexa-Fluor 488 (15 min, 37°C). A final BWW wash was completed and surface expression was assessed on the live sperm populations using the FACs Canto flow cytometer (BD Biosciences, San Jose, CA, USA). An anti-CD59 antibody was used as a positive control for surface labelling due to its previously documented presence on the surface of human spermatozoa [27]. CD59 staining was completed using an anti-CD59 antibody (30 min at 37 °C) in combination with a goat-anti-mouse Alexa-Fluor 488 (15 min, 37 °C).

Inhibition of ALOX15 in human spermatozoa and the induction of oxidative stress

To selectively inhibit ALOX15 catalytic activity in human spermatozoa, the indole based inhibitor 6,11-dihydro[1]benzothiopyrano[4,3-b]indole (PD146176) was used as previously described [17]. PD146176 is a non-competitive, ALOX15 specific inhibitor [28, 29], that has been demonstrated to significantly reduce 15-HPETE [30] and 13-HODE production [31]; both of which are major by-products of the ALOX15 metabolic pathway [32]. In pilot studies, we titrated effective doses of PD146176 based on the published IC_{50} value of 0.54 μM, and ensuring that sperm viability was not compromised in the dose-response range of 0.25 - 2.5 μM (please see Supplementary Figure S1). Following this, non-capacitated sperm samples (10×10^6 cells/ml) were pre-treated with PD146176 for 30 min at room temperature and then exposed to H_2O_2 as a means of inducing an oxidative insult. Hydrogen peroxide (Sigma; 349887) was diluted in BWW media from a 35% solution to a working concentration of 179 mM. This solution was then added to sperm populations of 10×10^6 cells/ml to the experiment-dependent final concentration outlined below (between 0.2 and 1 mM) and incubated for 1 hour at 37°C. Following incubation, human spermatozoa were washed free of H_2O_2 via three centrifugation events at 500 × g for 3 min where cells were thoroughly washed with fresh BWW media.

Notably, as documented below, the H_2O_2 treatment doses were varied between assays in accordance with previously established data concerning the efficacy of this insult to induce oxidative stress related changes in different functional parameters of human spermatozoa [33]. Thus, due to the differing sensitivity of the MitoSox™ Red (MSR), dihydroethidium (DHE), and BODIPY-C_{11} probes, the assessment of mitochondrial ROS was performed using 200 μM H_2O_2 to induce an MSR response, while the assessment of cellular ROS and lipid peroxidation were performed using 1 mM H_2O_2 to induce a dihydroethidium (DHE) and BODIPY-C_{11} responses. For the analysis of sperm function following ALOX15 inhibition, a concentration of 500 μM H_2O_2 was required before a marked change in sperm motility could be assessed. Taking

this into consideration, the levels of H_2O_2 employed to evaluate sperm capacitation parameters under conditions of oxidative stress, were reduced to 200 μM as this mild level of oxidative insult was permissive of the assessment of sperm-zona pellucida (ZP) interaction independent of any significant impact on sperm motility. Notwithstanding these experimental necessities, a consistent concentration of 0.25 μM PD146176 was co-administered alongside the varied H_2O_2 treatments in all assays.

Reactive oxygen species analysis

Mitochondrial and cellular ROS levels were established in ALOX15 inhibited sperm following exposure to H_2O_2. Mitochondrial ROS was examined using the MitoSox^{TM} Red probe (MSR), which was incubated with sperm populations at a final concentration of 20 μM for the duration of oxidative treatments with the inclusion of a SYTOX Green vitality stain (50 nM). Following H_2O_2 treatment, sperm samples were washed using NC BWW media and mitochondrial ROS levels were assessed by fluorescence microscopy, recording the percentage of cells with a positive, red fluorescent signal for MSR. Similarly, cellular ROS was assessed using the dihydroethidium (DHE) probe, which was used at a concentration of 2 μM (30 min, 37 ˚C) alongside SYTOX Green (50 nM). Cellular ROS assessment was then completed using fluorescence microscopy and recording those cells with red DHE incorporation as being positively labelled. Further, lipid peroxidation levels were measured using the BODIPY-C_{11} probe. This probe was pre-incubated with human sperm samples for 30 min at 37 ˚C prior to the induction to oxidative stress. Cells were then washed free from the probe and oxidative stress was induced as described previously. At the completion of the assay, lipid peroxidation levels were measured using the FACs Canto flow cytometer.

Zona pellucida binding assessment of human spermatozoa

Sperm-zona pellucida (ZP) binding was assessed using salt stored human zonae pellucidae from immature oocytes donated by IVF Australia (as described in [5]). Preservation of oocytes prior to analysis was achieved through immersion in a high salt storage medium comprising 1.5 M $MgCl_2$, 0.1% dextran, 0.01 mM HEPES buffer and 0.1% PVA at 4°C. Prior to use, oocytes were washed three times using PBS via serial aspiration. Oocytes were then placed within capacitating BWW droplets immersed in mineral oil and incubated in 5% CO_2/95% air/37°C for 30 min to enable the oocytes to equilibrate with the surrounding media. As homologous human zona pellucidae is an extremely limited resource, these experiments were performed as two independent replicates (n=2) using 4-6 oocytes in each treatment group. The treatment groups utilized for analysis included a non-capacitated negative control, a capacitated positive control, a H_2O_2 treated control and a PD146176 / H_2O_2 treated sperm sample. Following PD146176 / H_2O_2 treatment, spermatozoa were capacitated as previously described and the percentage of motile cells for each treatment group was recorded. Sperm populations were then diluted to a concentration of 1×10^6 cells/ml with a total of 2×10^5 cells (20 µl) being deposited into each oocyte-containing droplet. The human gametes were then co-incubated for 30 min at 5% CO_2/95% air/37 °C to allow sperm-ZP adhesion to occur. Following co-incubation, oocytes were washed in triplicate and mounted on glass slides using coverslips supported by 80% paraffin wax and 20% Vaseline gel at each corner to prevent oocyte compaction. Motile spermatozoa bound to zonae pellucidae were then counted, and representative images were taken for each treatment, using phase-contrast microscopy (Zeiss Axioplan 2).

Statistical Analysis

In this manuscript data are presented as mean values and accompanying standard error. JMP statistical software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analysis

using unpaired t-tests and the level of significance denoted by asterisks such that $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.005$ (***). All statistical testing was completed across three biological replicates (with each replicate comprising spermatozoa obtained from at least 1 donor) with the exception of sperm-zona pellucida interaction (individual sperm donors, $n = 2$). Further, the small sample size for zona binding experimentation required non-parametric measures for statistical testing using the Kolmogorov-Smirnov test for analysis.

RESULTS

Characterization of ALOX15 in human spermatozoa

The first aim of this study was to confirm the presence of ALOX15 in mature human sperm populations. This analysis was completed through the application of immunoblotting assays (Fig. 1), immunocytochemistry (Fig. 2A) and flow cytometry analysis to determine the subcellular localization of ALOX15 in human spermatozoa (Fig. 2C). Immunoblotting analysis with anti-ALOX15 antibodies confirmed that human spermatozoa do indeed harbor ALOX15 at the predicted molecular weight of ~75 kDa (i.e. 74,804 Da; Fig. 1A). Band densitometry analysis, normalized against that of the α-tubulin loading control, confirmed no overt changes in the abundance of this protein in populations of non-capacitated versus capacitated spermatozoa (Fig. 1B).

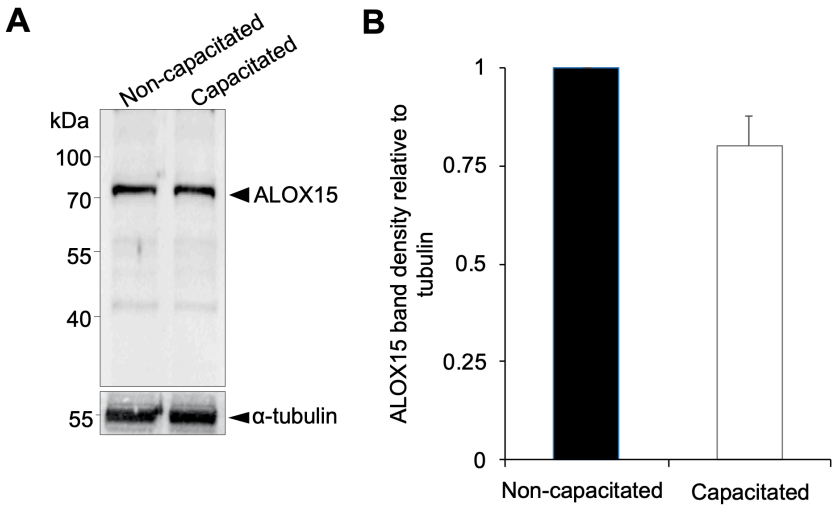


Figure 1: Detection of the ALOX15 enzyme in human spermatozoa. A) Proteins from non-capacitated and capacitated samples of pooled human sperm samples were solubilized by lysing the cells in an SDS based extraction buffer, prior to being resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were sequentially probed with anti-ALOX15 and an appropriate HRP-conjugated secondary antibody. After visualizing ALOX15 labeling, the membranes were stripped and re-probed with anti-α-tubulin antibodies to ensure equal protein loading. B) The intensity of protein labeling was determined using densitometric analyses, and the relative levels of ALOX15 were determined by normalization against the accompanying α-tubulin control. This experiment was replicated three times using individual human semen samples and representative immunoblots are included.

Distinct localization of ALOX15 was detected within the anterior region of the sperm head and throughout the mid- and principle- piece of the flagellum, with no changes in the localization of this protein occurring in response to the induction of capacitation (Fig. 2A). Notably, in fixed and permeabilized spermatozoa, ALOX15 displayed strong co-localization with PNA, a marker of the outer acrosomal membrane [34], thus confirming that this protein

resides within the peri-acrosomal region of the sperm head and consistent with previous analyses in the human testis [17] .

The use of a sensitive flow cytometry assay confirmed that ALOX15 is unlikely to be superficially exposed on the exterior leaflet of the membrane of live human spermatozoa (Fig. 2C). Indeed, in keeping with its cytosolic / inner membrane localization in somatic cells [35], this assay revealed that ALOX15 is inaccessible to surface labelling in human spermatozoa irrespective of their capacitation status (Fig. 2C). The specificity of this response was confirmed by labelling equivalent populations of spermatozoa with antibodies against CD59, a surface expressed antigen [27], employed as a positive control. Importantly in this regard, all ALOX15 labelling was eliminated upon substitution of the anti-ALOX15 antibody with buffer alone (i.e. secondary antibody only control, Fig. 2B, C).

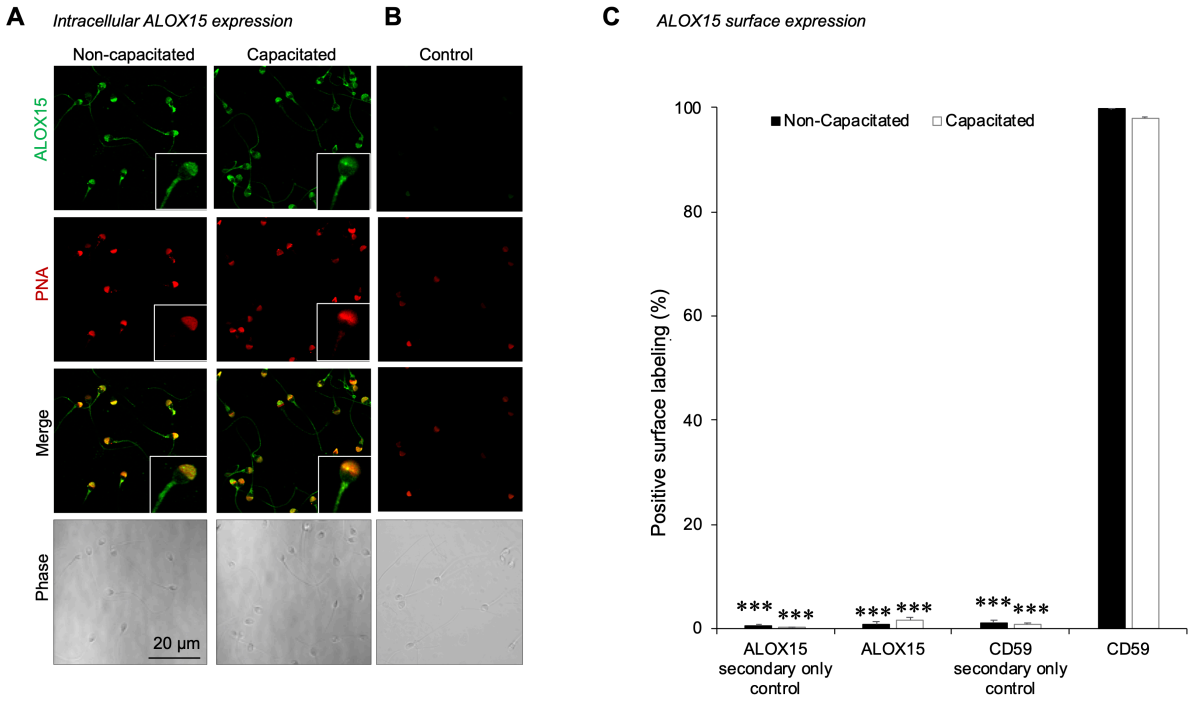


Figure 2: Localization of ALOX15 in human spermatozoa. A) Non-capacitated and capacitated human spermatozoa were fixed and permeabilized prior to sequential labeling with anti-ALOX15 antibodies and appropriate FITC-conjugated secondary antibodies (green). The cells were then counterstained with the acrosomal marker of TRITC-conjugated PNA (red). B)

This protocol was repeated in the absence of primary antibody to generate a secondary antibody only control. This experiment was replicated three times using human semen samples from different donors and representative images are presented. Insets in panel A depict higher magnification images of sperm labeling patterns. C) Alternatively, live populations of human spermatozoa were labeled with either anti-ALOX15 or anti-CD59 (a surface expressed antigen used as a positive control) antibodies and appropriate FITC conjugated secondary antibodies. The cells were then counterstained with the Live/Dead vitality stain prior to being subjected to flow cytometry. For this analysis, a minimum of 10,000 cells were assessed using the FACs Canto flow cytometer. This analysis was performed three times (n = 3 different sperm donors) and data are depicted as mean ± SEM. *** $p < 0.005$ compared to positive control (CD59) labelling.

The impact of ALOX15 inhibition on the generation of cellular ROS

Having established the presence of ALOX15 in human spermatozoa, we next sought to address the hypothesis that the activity of this lipoxygenase enzyme may be causally associated with oxidative stress and membrane lipid peroxidation in these cells. To dissect the involvement of ALOX15 in the oxidative stress cascade, human spermatozoa were co-incubated with PD146176, a selective ALOX15 inhibitor, alongside an exogenous H_2O_2 challenge. In pilot studies we titrated the dose of PD146176 to ensure it did not adversely impact sperm viability. The PD146176 concentrations (0.25 - 2.5 μM) selected for use in this study were based on a combination of the inhibitor's IC_{50} value of 0.54 μM [31], and on our previously published studies in the developing germ cells of the mouse [17]. As expected, none of the doses examined in this study precipitated any significant loss in spermatozoa viability during the 1 h treatment regimen (Supplementary Fig. S1). Based on these observations we selected a PD146176

concentration of 0.25 μM for all further analyses, including assessment of mitochondrial and cellular ROS production (using MSR and DHE probes, respectively) (Fig. 3A and B).

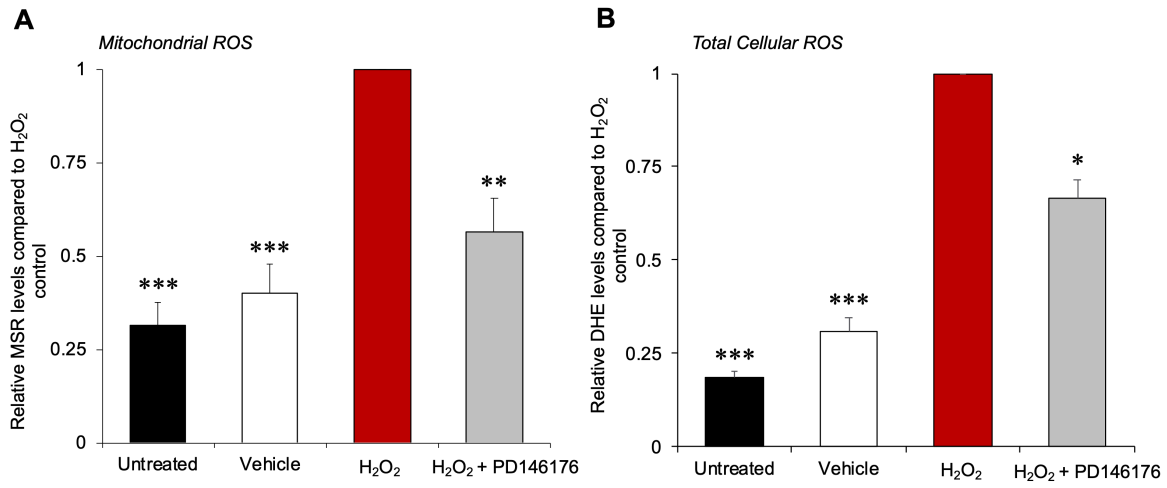


Figure 3: Assessment of ROS production following H_2O_2 ± PD146176 treatment of human spermatozoa. A) Human sperm samples were incubated with 200 μM H_2O_2 to induce oxidative stress alongside PD146176 treatment to achieve ALOX15 inhibition. The addition of MitoSox™ Red probe throughout treatments allowed visualization of mitochondrial superoxide production, as recorded using fluorescence microscopy. B) To assess cellular ROS using the fluorescent dihydroethidium probe (DHE) human sperm samples were incubated with 1 mM H_2O_2 to induce oxidative stress alongside PD146176 treatment to achieve ALOX15 inhibition. Due to some degree of variability in the basal levels of MSR and DHE positivity recorded between sperm samples, all data were normalized against the values obtained in the positive control samples (i.e. H_2O_2 treated spermatozoa). This analysis was performed in triplicate (n = 3 different sperm donors) and data are depicted as mean ± SEM, $p < 0.05$ *, $p < 0.01$ **, $p < 0.005$ *** compared to H_2O_2 control.

As anticipated, the addition of an exogenous H_2O_2 challenge initiated a significant ($p < 0.005$), 3.2-fold increase in the number of cells with a positive mitochondrial ROS (MSR) signal compared to the untreated control populations of spermatozoa (Fig. 3A). Notably however,

exposure to PD146176 for the duration of H_2O_2 treatment led to a significant ($p < 0.01$) attenuation of mitochondrial ROS. Indeed, this reduction amounted to an approximate halving of the number of spermatozoa producing excessive mitochondrial ROS. Importantly, these trends were mirrored in terms of cellular ROS production measured by DHE, with a 5.4-fold increase ($p < 0.005$) in the number of cells displaying a positive DHE signal following H_2O_2 exposure, and for this same population, a significant 1.5-fold reduction ($p < 0.05$) in the number of DHE positive spermatozoa upon the pharmacological inhibition of ALOX15 (Fig. 3B). Together, these data support the tenet that ALOX15 may be intrinsically linked to the propagation of oxidative stress in mature human spermatozoa.

Accordingly, we next elected to focus on the cumulative levels of membrane lipid peroxidation generated following the combined PD146176 / H_2O_2 treatment regimen (Fig. 4A). For this purpose, lipid peroxidation capacity was assessed using the BODIPY-C_{11} probe, which upon peroxidation, experiences a shift in fluorescence emission (i.e. from 590 nm to 510 nm), identifying lipid peroxidation-positive cells [36]. Measurement of BODIPY-C_{11} signals using flow cytometry revealed that H_2O_2 treatment resulted in a substantial, 12.3-fold increase in the number of spermatozoa affected by lipid peroxidation ($p < 0.005$). However, consistent with the ROS analysis reported above, the percentage of lipid peroxidation-positive spermatozoa was significantly reduced ($p < 0.01$) by approximately 1.6-fold when H_2O_2 treatment was performed in the presence of PD146176. However, as with both mitochondrial and cytosolic ROS levels, ALOX15 inhibition did not completely return the number of sperm harboring lipid peroxidation to levels that were comparable to that of the untreated control levels. In this regard, the addition of a PD146176-alone control ruled out any possibility that PD146176 may have any positive contribution to the levels of cellular lipid peroxidation (Supplementary Figure S2). As a final focus for these studies, the presence of 4HNE in human spermatozoa and the ability of this aldehyde to modify sperm proteins, was assessed by immunoblotting with a specific

anti-4HNE antibody. This analysis revealed a marked reduction in the presence of cellular 4HNE levels following ALOX15 inhibition (Figure 4B). These data were verified through band densitometry analysis relative to an anti-α-tubulin loading control, whereby a significant decrease in 4HNE was obtained in lysates of human sperm challenged with H_2O_2 in the presence of PD146176 (Figure 4C). Additionally, the influence of H_2O_2 on the expression of the ALOX15 protein itself was analysed through immunoblotting of human sperm lysates (Supplementary Figure 3A). As expected, no significant change in the expression profile of ALOX15 was detected between untreated and H_2O_2-exposed sperm cells (Supplementary Figure 3B).

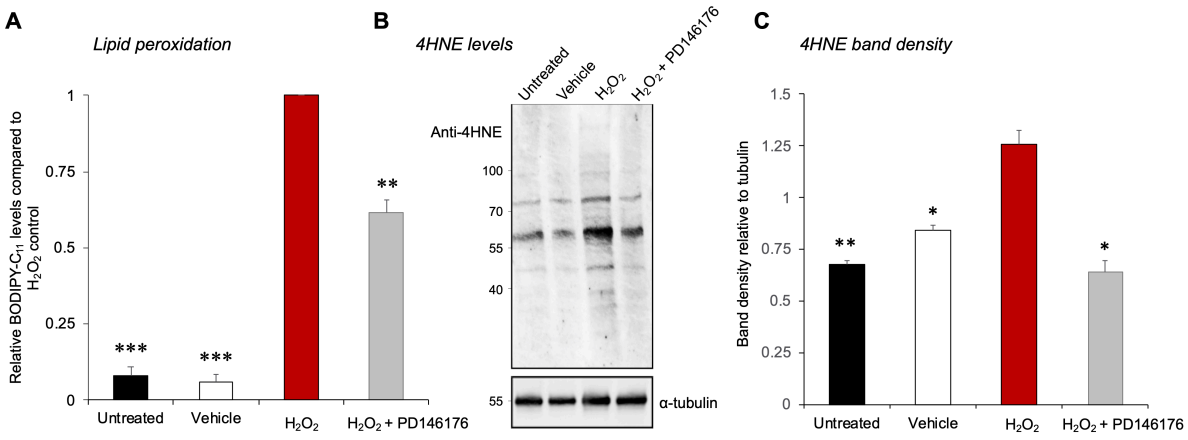


Figure 4: Assessment of lipid peroxidation following H_2O_2 ± PD146176 treatment of human spermatozoa. Sperm samples were incubated with 1mM H_2O_2 to induce oxidative stress and thereby promote lipid peroxidation alongside PD146176 treatment to achieve ALOX15 inhibition. A) Sperm samples were incubated with BODIPY-C_{11} prior to H_2O_2/PD146176 treatments. They were subsequently assessed for relative levels of lipid peroxidation (i.e. positive BODIPY-C_{11} labeling) using a flow cytometry. For this analysis, a minimum of 10,000 cells were assessed and due to donor variability all data were normalized to the H_2O_2 treated control. B) The presence of 4HNE in human spermatozoa following H_2O_2/PD146176 treatments was assessed by immunoblotting. Following treatment, samples

were lysed with SDS extraction buffer prior to being resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were sequentially probed with anti-4HNE and appropriate HRP-conjugated secondary antibodies. After visualizing labeled proteins, the membranes were stripped and re-probed with anti-α-tubulin antibodies to ensure equal protein loading. C) The intensity of protein labeling was determined using densitometric analyses, and the relative levels of 4HNE were determined by normalization against the accompanying α-tubulin bands. This analysis was performed in triplicate (n = 3 different sperm donors) and data are depicted as mean ± SEM, $p < 0.05$ *, $p < 0.01$ **, $p < 0.005$ *** compared to H_2O_2 control.

Assessment of the impact of ALOX15 inhibition on sperm function

In view of the favorable responses documented by attenuating the action of ALOX15 in human spermatozoa, we next elected to study the downstream implications of inhibiting ALOX15 activity on the functional parameters of spermatozoa. This study was underpinned by the hypothesis that a reduction in ROS generation and lipid peroxidation levels in human spermatozoa would counter the concomitant loss in the function of these cells, previously reported following oxidative challenge [5]. Initially, we focused on determining whether ROS-induced losses in sperm motility could be prevented through the inhibition of ALOX15. Analysis of overall sperm motility indicated a highly significant ($p < 0.01$), 1.4-fold decrease in the percentage of motile spermatozoa following H_2O_2 exposure, as measured following the induction of capacitation. Notably however, this detrimental effect was completely prevented, such that the percentage of motile cells was restored to statistically similar levels to that of untreated and vehicle controls, if the cells were supplemented with PD146176 for the duration of H_2O_2 treatment (Fig. 5A).

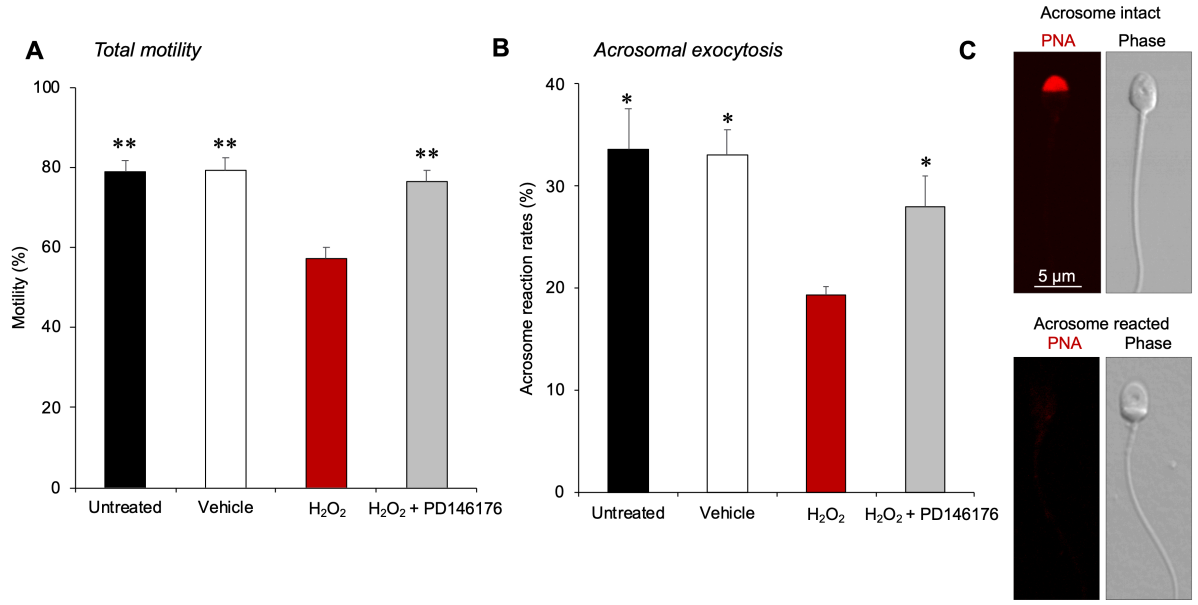


Figure 5: Motility assessment and acrosome reaction rates of human spermatozoa following $H_2O_2 \pm$ PD146176 treatment. A) Sperm samples were incubated with 500 µM H_2O_2 to induce oxidative stress with or without PD146176. Sperm motility was then assessed in a minimum of 100 cells per treatments group using light microscopy and these data are expressed as the percentage of motile cells. B) To induce the acrosome reaction, pooled human sperm samples exposed to 200 µM H_2O_2 were capacitated prior to incubation with a calcium ionophore, A23187. Following induction of the acrosome reaction, spermatozoa were incubated in hypo-osmotic swelling solution (HOS) to distinguish the live cell population. C) Representative PNA staining patterns utilized to determine the acrosomal status of human spermatozoa. These analyses were performed in triplicate (n = 3 different sperm donors) and data are depicted as mean ± SEM, $p < 0.05$ *, $p < 0.01$ **, $p < 0.005$ *** compared to H_2O_2 control.

Previously, we have demonstrated that the competence of sperm to both complete an acrosome reaction and engage in interactions with the outer vestments of the oocyte (i.e. the zone pellucida) are highly sensitive to oxidative stress; with even low levels of ROS able to compromise these important facets of human sperm function [5]. In view of these findings, we

sought to examine whether the inhibition of ALOX15 may afford a novel means by which to protect the functionality of spermatozoa against oxidative stress. For this purpose, we first evaluated the responsiveness of capacitated human spermatozoa to the induction of an acrosome reaction following exposure to a calcium ionophore agonist (i.e. A23187). Using fluorescently labelled PNA as a marker of acrosomal status (Fig. 5C), we were able to document a significant ($p < 0.05$) 1.7-fold reduction in the number of capacitated sperm having completed an acrosome reaction following the initiation of oxidative stress by H_2O_2 exposure (Fig. 5B). The application of PD146176 was however, able to restore acrosome reaction rates to levels that were indistinguishable from that of the capacitated control groups of spermatozoa.

In the final stage of this analysis, we repeated the oxidative stress induction regimen with a view to assessing its impact on sperm adhesion to homologous zonae pellucidae (ZP). In terms of the sequence for this assay, sperm were exposed to H_2O_2 ± PD146176 prior to capacitation, assessed for motility and placed in a droplet of salt stored oocytes. To negate any confounding influence on sperm motility, the concentration of H_2O_2 used in this assay was reduced to a level at which motility was unaffected (i.e. 200 μM; Supplementary Fig. 4). Following the co-incubation of the gametes, the number of motile sperm bound to the ZP was assessed revealing a significant reduction in ZP binding in the population exposed to H_2O_2 alone ($p < 0.05$) (Fig. 6A/6B). Excitingly however, supplementation with PD146176 during H_2O_2 treatment led to a complete rescue of sperm-ZP binding compared to the untreated capacitated control group (Fig. 6B, C).

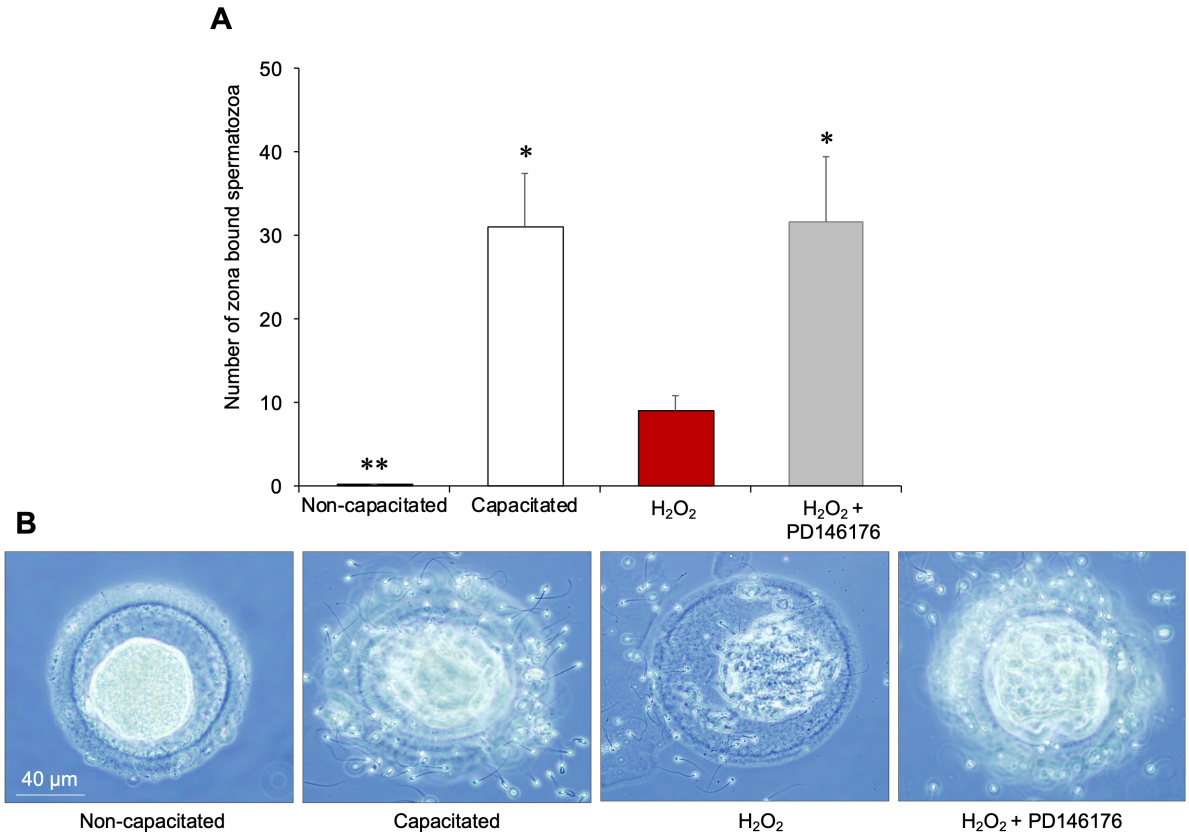


Figure 6: Zona pellucida binding assessment following H_2O_2 ± PD146176 treatment of human spermatozoa. Sperm samples were incubated with 200 µM H_2O_2 to induce oxidative stress with or without PD146176. Sperm samples were washed free of H_2O_2 and capacitated for 3 h. A) Sperm were co-incubated with oocytes for 30 min and the number of motile sperm bound to the zona pellucida was counted using phase contrast microscopy and images were captured on a Zeiss-Axiovert fluorescence microscope (B). As a cautionary note, the limited availability of human oocytes restricted the replication of these analyses to two separate experiments (each featuring n = 2 individual sperm donors and 4-6 oocytes per treatment group). Data are depicted as mean ± SEM, $p < 0.05$ *, $p < 0.01$ **, $p < 0.005$ *** compared to H_2O_2 control.

DISCUSSION

The results of this study provide the first characterization of ALOX15 in human spermatozoa and indicate that a disruption in ALOX15 activity reduces ROS production and membrane lipid peroxidation. Consequently, ALOX15 inhibition may afford a degree of protection for human spermatozoa against exogenously induced oxidative stress and thus maintain the integrity of the maturational events required for fertilization.

In non-stimulated somatic cells such as those of the hematopoietic lineage, lipoxygenase enzymes commonly reside within the cytoplasm. However, upon receipt of an appropriate oxidative stimulus, these enzymes endure a calcium-dependent translocation leading to their binding of the inner leaflet of the plasma membrane whereupon they fulfil their roles in the lipid peroxidation process [35]. This localization profile is consistent with our own data indicating that ALOX15 is likely to reside in the inner leaflet of the human sperm plasma membrane but is not exposed on the cell surface. Such a position ideally places ALOX15 to participate in the degradation of its lipid substrates in the sperm plasma membrane. In somatic cells, this catalytic reaction proceeds via alternate oxidation and reduction of the iron atom contained within the protein's catalytic site [16], thus facilitating the lipid peroxidation process and yielding a suite of secondary products including 4HNE. In line with this activity, the results outlined in this manuscript demonstrate a significant decrease in mitochondrial and cellular ROS in human spermatozoa exposed to PD146176 during oxidative challenge. Importantly, the attenuation of these ROS responses also led to a commensurate reduction in the overall degree of membrane lipid peroxidation experienced by spermatozoa. Downstream of lipid peroxidation we also recorded a significant decrease in 4HNE, a reactive aldehyde that has previously been linked to a severe reduction in sperm motility [37], sperm-egg recognition [5] and the ability to complete an acrosome reaction [5]. Consistent with these findings, we have found that the pharmacological inhibition of ALOX15 and resulting decreases in 4HNE during

conditions of cellular oxidative stress, lead to a significant improvement in all three of these functional parameters. These data therefore establish a critical link between 4HNE-mediated cellular dysfunction and the action of the ALOX15 enzyme in human spermatozoa. In view of this understanding, we propose a mechanistic model for the function of ALOX15 in human spermatozoa and its relationship with cellular ROS production (Figure 7).

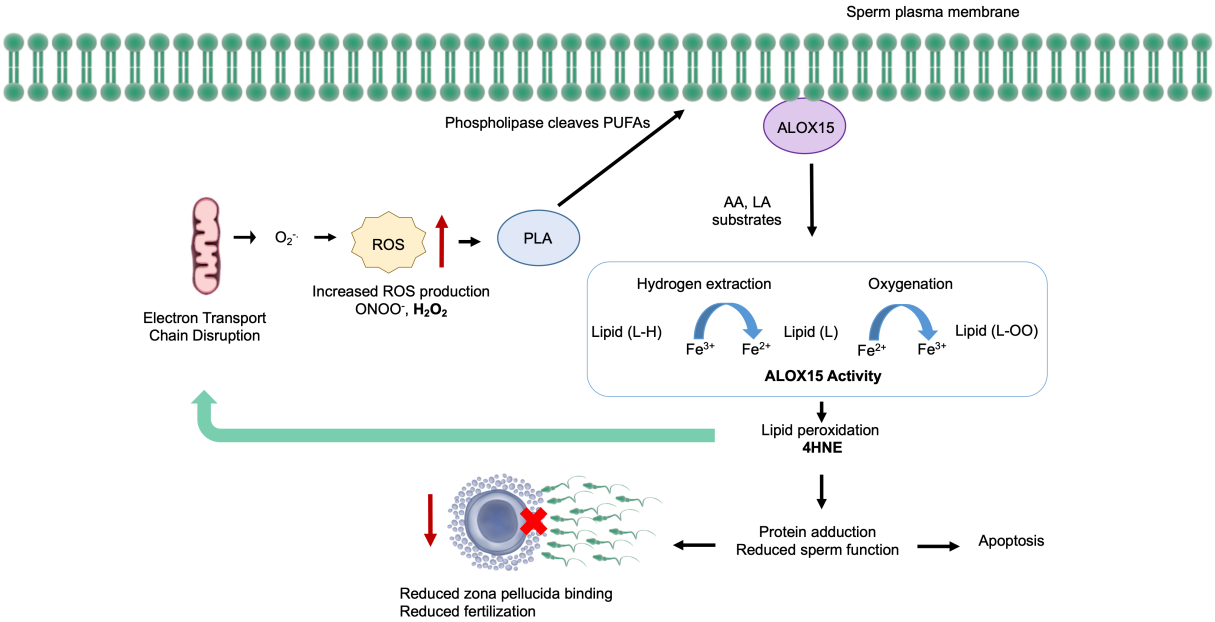


Figure 7: Proposed model for ALOX15 activity within ROS production cascades of human spermatozoa The propagation of oxidative stress in human spermatozoa is likely initiated through insults that elevate the production of superoxide ($O_2^{-\cdot}$) anions from within the mitochondrial electron transport chain (ETC). Highly reactive $O_2^{-\cdot}$ stimulates increased production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$), which can further disrupt the integrity of the ETC and accentuate ROS production. The ROS so produced may contribute to the activation of phospholipase proteins, which facilitate the dissociation of poly-unsaturated fatty acids (PUFAs) from the sperm plasma membrane. Upon activation of ALOX15 (presumably via Ca^{2+} dependent signaling), the enzyme may translocate to the inner leaflet of the plasma membrane, a position from which it can catalyze the degradation of PUFAs [i.e. arachidonic acid (AA) and linoleic acid (LA)] via

redox cycling of the iron atom held within its catalytic domain. ALOX15 activity culminates in 4HNE production, which facilitates further ETC disruption causing unchecked cascades of ROS production, lipid peroxidation, protein adduction, and DNA damage. These combined events compromise the physiological status of the sperm cell, leading to a reduction in sperm binding to zona pellucida and an attendant loss of fertility.

In line with our previous findings [6, 7, 10, 38, 39], this model indicates that the propagation of oxidative stress in human spermatozoa is likely initiated through insults that elevate the production of superoxide ($O_2^{-\cdot}$) anions from within the mitochondrial electron transport chain (ETC). Here we propose that the activation of ALOX15 under such conditions of oxidative stress catalyzes the degradation of PUFAs [i.e. arachidonic acid and linoleic acid] via redox cycling of the iron atom held within its catalytic domain. ALOX15 activity culminates in 4HNE production, which facilitates further ETC disruption causing unchecked cascades of ROS production, lipid peroxidation and protein adduction. These combined events compromise the physiological status of the sperm cell, leading to a reduction in sperm binding to zona pellucida.

The results described in this study on human spermatozoa are in agreement with our previously published work examining the role of ALOX15 in 4HNE-mediated protein damage in mouse GC-2 cells [17]. They are also congruous with the results obtained from elegant GPX4/ALOX15 double knockout mouse models published by Brütsch and colleagues, which firmly implicate ALOX15 in the subfertility observed in the GPX4 $^{+/-}$ mouse line [20]. Thus, the inhibition of ALOX15 in the testis could present a novel therapeutic strategy for the prevention of oxidative stress-mediated pathologies in the male germline. However, important considerations for this approach will involve research targeted towards understanding the physiological role of lipoxygenase enzymes in both the developing and mature germ cells, as

well as establishing the utility of PD146176 to reduce 4HNE production *in vivo* by first testing its ability to cross the blood-testis barrier.

In view of these potential limitations, preclinical experiments are currently underway investigating the utility of inhibiting ALOX15 enzymatic activity (through the application of PD146176) to combat neurodegenerative diseases such as Alzheimer's [40, 41]; a disease in which 4HNE has been implicated in the initiation of neuronal cell death and the generation of amyloid ß plaques [42-44]. Furthermore, *in vivo* experiments seeking to evaluate the effect of PD146176 in mouse models of Alzheimer's disease have revealed significant improvements in memory deficits, accompanied by reduced production and deposition of amyloid ß, in PD146176-treated mice compared to their untreated counterparts. These data reveal the distinct possibility of using PD146176 as a therapeutic strategy to reduce the progression of diseases involving oxidative stress and 4HNE production [45].

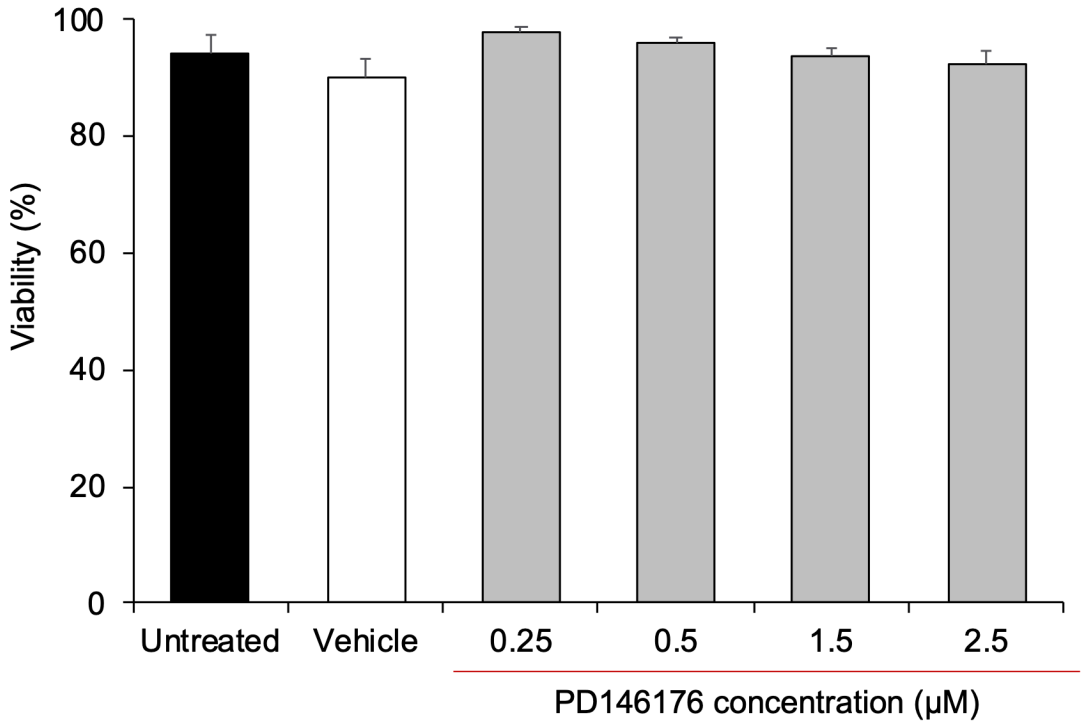
Overall, this project has confirmed the presence of ALOX15 in human spermatozoa and defined its specific cellular localization in the peri-acrosomal head, midpiece and tail regions of these specialized cells. Moreover, the pharmacological inhibition of ALOX15 proved effective in rescuing numerous sperm functional parameters that would otherwise be severely compromised by oxidative stress. Taken together, these data suggest that ALOX15 is, at least in part, responsible for the peroxidation of membrane lipids that leads to the production of deleterious aldehydes such as 4HNE in human spermatozoa. Given the extensive damage that 4HNE and ROS causes to both proteins and DNA in the male germline, the therapeutic benefits in reducing lipid peroxidation and the production of this aldehyde within spermatozoa, are clear. Considering the increasing numbers of men experiencing infertility tied to high levels of ROS and attendant poor DNA quality, further investigation into the role of the lipoxygenase protein family in regulating oxidative stress and sperm function may help to elucidate the complex

molecular mechanisms surrounding ROS-mediated male infertility and provide further therapeutic strategies.

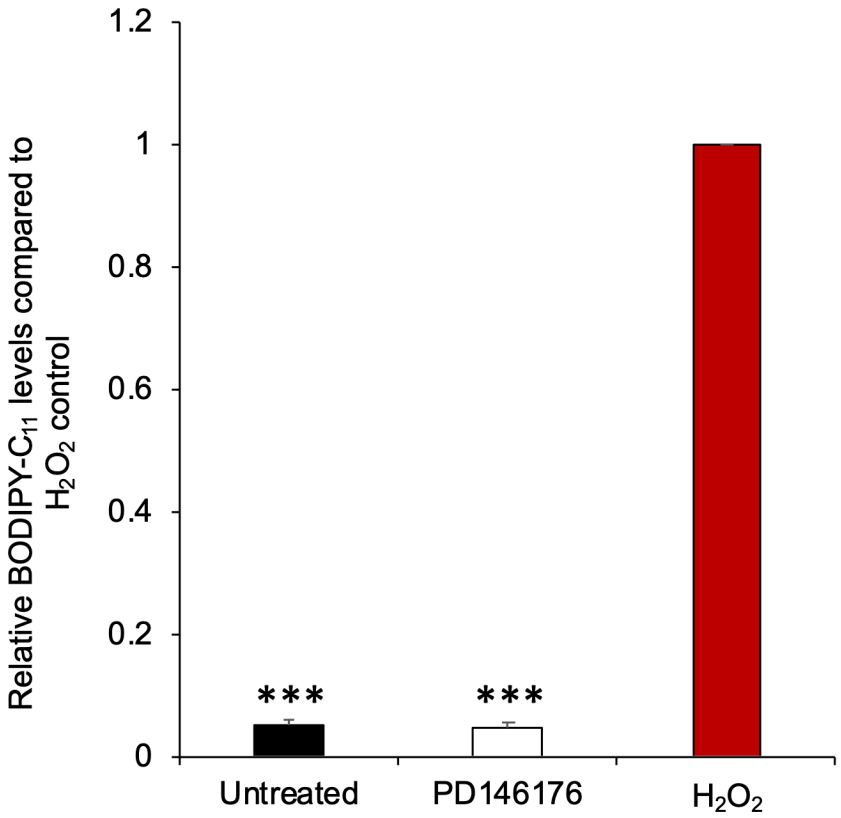
SUPPLEMENTARY FIGURES

Supplementary Table S1: Details of antibodies and molecular probes used throughout this study.

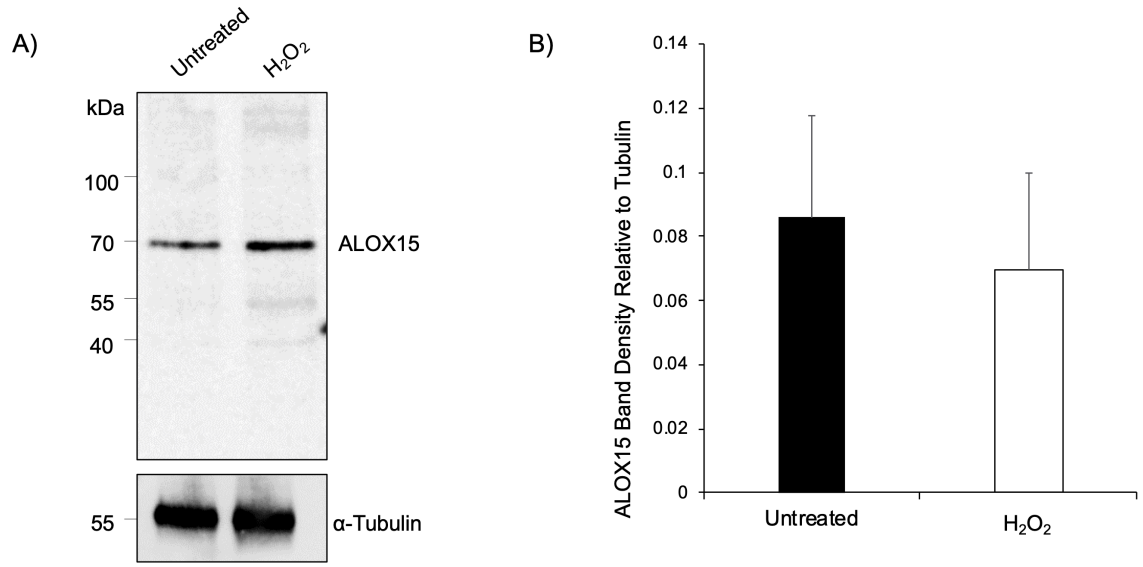
Antibody and probes (catalog number)	Final concentration (or dilution)			Supplier
	Fluorescence Assays	Immunoblot	Flow Cytometry	
Anti-ALOX15 (ab80221)	5 µg/ml	1 µg/ml	5 µg/ml	Abcam
TRITC-PNA (L3766)	10 µg/ml	-	-	Sigma
Anti-α-Tubulin (T5168)	-	4.3 µg/ml	-	Sigma
Anti-CD59 (ab9183)	-	-	20 µg/ml	Abcam
Anti-rabbit Alexa Fluor 488 (A11008)	10 µg/ml	-	5 µg/ml	Thermo Fisher Scientific
Anti-mouse Alexa Fluor 488 (A11005)	-	-	5 µg/ml	Thermo Fisher Scientific
Anti-rabbit HRP (DC03L)	-	0.13 µg/ml	-	Millipore
Anti-mouse HRP (sc-2005)	-	0.4 µg/ml	-	Santa Cruz Biotechnology
BODIPY-C_{11} (D3861)	-	-	5 µM	Thermo Fisher Scientific
Anti-4HNE (HNE 11-S)	-	2 µg/ml	-	Alpha Diagnostic International
MitoSox™ Red (MSR) (M36008)	20 µM	-	-	Thermo Fisher Scientific
Dihydroethidium (DHE) (D1168)	2 µM	-	-	Thermo Fisher Scientific



Supplementary Figure S1: Assessment of human sperm viability. Viability assessments were made using Eosin Y staining of human sperm populations following treatment of PD146176 at increasing concentrations (0.25 - 2.5 µM). Statistical analysis was completed using JMP software with error bars indicative of SEM, (n = 3 different sperm donors). No significant differences ($p > 0.05$) were observed between treatment groups.

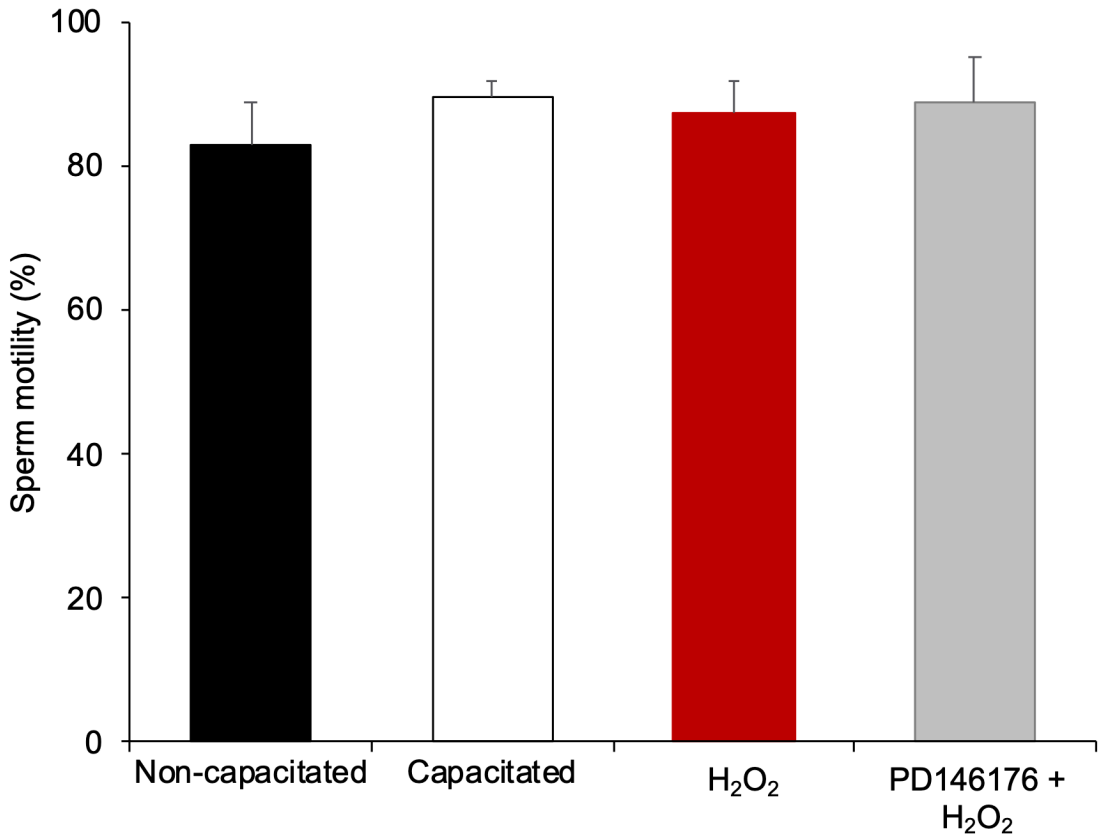


Supplementary Figure S2: Inhibitor-alone controls for BODIPY-C_{11} analysis. PD146176 was added to human spermatozoa at a concentration of 0.25µM and the levels of lipid peroxidation were examined relative to both untreated and H_2O_2 –exposed (1 mM) control samples. No significant difference in either sperm viability or the percentage of BODIPY-positive cells was observed between the untreated and inhibitor-alone sperm populations.



Supplementary Figure S3: Assessment of ALOX15 protein abundance

A) Proteins from untreated and H_2O_2-treated (1 mM) pooled human sperm samples were solubilized by lysing the cells in an SDS based extraction buffer, prior to being resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were sequentially probed with anti-ALOX15 and an appropriate HRP-conjugated secondary antibody. After visualizing ALOX15 labeling, the membranes were stripped and re-probed with anti-α-tubulin antibodies to ensure equal protein loading. B) Band densitometry was performed on 70 kDa bands ALOX15 bands relative to tubulin loading controls using Image J software.



Supplementary Figure S4: Percentage of motile spermatozoa prior to gamete co-incubation Following capacitation, sperm motility was assessed for each treatment group, to ensure zona binding competence was not influenced by a loss of cell motility. Total motility was recorded as the percentage of motile cells using light microscopy. Data are presented as mean ± SEM. No significant differences were found between treatment groups.

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Conflict of interest

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

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