The impact of oxidative stress on chaperone-mediated human sperm–egg interaction

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STUDY QUESTION: How does oxidative stress impact upon human sperm–egg interaction and in particular the formation of zona pellucida-receptor complexes on the sperm surface?

SUMMARY ANSWER: Oxidative stress during human sperm capacitation resulted in the chemical alkylation of the molecular chaperone heat shock protein A2 (HSPA2), a concomitant reduction in surface expression of the zona pellucida-receptor arylsulphatase A (ARSA) and a severe loss of zona pellucida binding ability.

WHAT IS KNOWN ALREADY: An inability to bind to the zona pellucida is commonly encountered in the defective spermatozoa generated by male infertility patients; however, the underlying mechanisms remain unresolved. Recent studies have revealed that zona pellucida binding is mediated by molecular chaperones, particularly HSPA2, that facilitate the formation of multimeric zona pellucida-receptor complexes on the surface of mammalian spermatozoa during capacitation.

STUDY DESIGN, SIZE, DURATION: Spermatozoa were collected from healthy normozoospermic donors (n = 15). Low levels of oxidative stress were induced in populations of non-capacitated spermatozoa by a 1 h treatment with 4-hydroxynonenal (4HNE) or hydrogen peroxide (H₂O₂) and then these insults were removed and cells were capacitated for 3 h.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Motility, membrane fluidity, protein tyrosine phosphorylation and lipid raft distribution were evaluated after sperm capacitation to determine the impact of oxidative stress on this process. The surface expression of ARSA and sperm adhesion molecule 1 (SPAM1) was observed using fluorescence microscopy, and the ability of treated cells to interact with homologous human zona pellucidae was assessed through gamete co-incubation. Proximity ligation was used to evaluate the state of the HSPA2-laden zona pellucida-receptor complex and an immunoprecipitation approach was taken to establish the chemical alkylation of HSPA2 by the cytotoxic lipid aldehyde 4HNE. The validity of these findings was then tested through treatment of oxidatively stressed cells with the nucleophile penicillamine in order to scavenge lipid aldehydes and limit their ability to interact with HSPA2. All experiments were performed on samples pooled from two or more donors per replicate, with a minimum of three replicates.

MAIN RESULTS AND THE ROLE OF CHANCE: The oxidative treatments employed in this study did not influence sperm motility or capacitation-associated changes in membrane fluidity, tyrosine phosphorylation and lipid raft redistribution. However, they did significantly impair zona pellucida binding compared with the capacitated control (P < 0.01). The reduction in zona pellucida binding was associated with the impaired surface expression (P < 0.02) of a zona pellucida-receptor complex comprising HSPA2, SPAM1 and ARSA. Proximity ligation and immunoprecipitation assays demonstrated that impaired zona pellucida binding was, in turn, associated with the chemical alkylation of HSPA2 with 4HNE and the concomitant disruption of this zona pellucida-receptor complex. The use of penicillamine enabled a partial recovery of ARSA surface expression and zona pellucida adherence in H₂O₂-treated cells. These data suggest that the ability of low levels of oxidative stress to disrupt sperm function is mediated by the production of lipid aldehydes as a consequence of lipid peroxidation and their adduction to the molecular chaperone HSPA2 that is responsible for co-ordinating the assembly of functional zona pellucida-receptor complexes during sperm capacitation.

LIMITATIONS, REASONS FOR CAUTION: While these results extend only to one particular zona pellucida-receptor complex, we postulate that oxidative stress may more broadly impact upon sperm surface architecture. In this light, further study is required to assess the impact of oxidative stress on additional HSPA2-laden protein complexes.

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Introduction

The generation of reactive oxygen species (ROS) by mammalian spermatozoa is crucial for the activation of several physiological processes (Aitken et al., 1998; de Lamberd and Lamothe, 2009; Aitken and Curry, 2011). Not least of these is capacitation, a complex and tightly regulated series of transformations that spermatozoa must undergo in the female reproductive tract in order to interact with an oocyte and carry out fertilization (de Lamberd and Gagnon, 1993). While sperm capacitation is characterized by its key outcomes (hyperactivated motility, sperm–zona pellucida recognition and an acquired ability to undergo acrosomal exocytosis), it is also coupled with a suite of important cellular events that make it possible to monitor this process in vitro. These events include an influx of calcium and bicarbonate (Gadella and Harrison, 2000; Florman et al., 2008), increases in membrane fluidity (Davis et al., 1979), a redistribution of membrane rafts (Boerke et al., 2008; Nixon et al., 2009), rises in both intracellular pH (Vrendenburgh-Wilberg and Parrish, 1995; Aitken et al., 1998) and cAMP (Visconti et al., 1995a; Tardif et al., 2004) and the activation of numerous signalling cascades that underpin key protein phosphorylation events (Visconti et al., 1995b; O’Flaherty et al., 2003, 2005). Importantly, the stimulation and regulation of a number of these events by both exogenous and endogenous ROS is now well recognized in a number of species, including the human (de Lamberd and Gagnon, 1995; Aitken et al., 2003; O’Flaherty et al., 2006), mouse (Herrero et al., 2003) and the bovine (O’Flaherty et al., 2003).

Despite their fundamental importance for sperm capacitation, levels of ROS that exceed physiological relevance can stimulate a cascade of events leading to oxidative DNA damage and eventually an apoptotic-like death (Aitken, 2011). Additionally, peroxidative damage to the sperm plasma membrane caused by oxidative stress can result in an irrepairable loss of sperm function and long-term infertility (Aitken and Clarkson, 1998). Moreover, oxidative stress to spermatozoa is a particular risk in the in vitro systems that are used for assisted conception, as removal of seminal plasma during sperm preparation leaves these cells particularly susceptible to oxidative attack (Aitken and Clarkson, 1998).

Although sperm capacitation events have been intently studied over the past decade, an understanding of some of the key molecular mechanisms that underpin this process is still lacking (Aitken and Nixon, 2013). A notable example is that despite decades of work towards the identification of a single sperm surface receptor that mediates adhesion and binding to the zona pellucida, murine knockouts of putative zona pellucida receptors have generally failed to elicit complete infertility (as reviewed by Reid et al., 2011). However, these seemingly confounding studies have given rise to a novel concept that molecular chaperones may be involved in the capacitation-dependent assembly of zona-receptor complexes on the sperm surface during capacitation (Asquith et al., 2004; as reviewed by Bromfield and Nixon, 2013). In support of this proposal, recent work in our laboratory has identified a number of high molecular weight, multimeric protein complexes on the surface of human spermatozoa that show affinity for homologous zonae pellucidae (Redgrove et al., 2011, 2012). The most dominant of these complexes has been extensively characterized and comprises three key proteins that form a stable interaction in human spermatozoa, namely sperm adhesion molecule 1 (SPAM1, previously PH20), which is a hyaluronidase implicated in both cumulus cell matrix dispersal and sperm–zona pellucida binding (Lathrop et al., 1990; Kimura et al., 2009), Arylsulphatase A (ARSA), which has previously been implicated in sperm–egg adhesion and binding (Carmona et al., 2002; Tantibhedhyangkul et al., 2002) and a molecular chaperone of the heat shock protein 70 family, heat shock protein A2 (HSPA2), which has been the subject of studies both in our laboratory and independently, as a key marker of both sperm maturity and zona pellucida binding competence (Ergur et al., 2002; Huszar et al., 2007; Redgrove et al., 2012). Our collective evidence suggests that HSPA2 coordinates a capacitation-associated rearrangement of this complex (Redgrove et al., 2012). Thus, the hyaluronidase SPAM1 is present on the surface of non-capacitated sperm to aid in their penetration of the hyaluronic acid-rich matrix of cumulus cells surrounding the oocyte. Thereafter, capacitated sperm present ARSA on their surface to assist in their initial tethering to the zona pellucida via its interaction with the sulphated regions of glycans adorning the surface of the zona pellucida (Fig. 1).

Failure to bind to the zona pellucida is a commonly encountered attribute of defective spermatozoa within the subpopulation of patients exhibiting male infertility (Bastiaan et al., 2002; Liu et al., 2004). In light of the above findings, we postulate that the clinical disruption of sperm–zona pellucida binding may be facilitated by defects in the HSPA2-mediated assembly of zona-receptor complexes on the sperm surface during capacitation. In some cases, we have found that this involves an actual loss of the HSPA2 chaperone from the sperm proteome (Redgrove et al., 2012, 2013). However, we also propose that the same phenotype would result if the functionality of HSPA2 were somehow compromised. Since oxidative stress is a common feature of male infertility, we undertook the present study to determine whether this form of stress can inhibit the ability of human spermatozoa to bind to the zona pellucida and, if so, whether this loss of functionality is associated with a failure to assemble and present HSPA2-mediated zona-receptor complexes on the sperm surface.
Materials and Methods

Reagents

Unless specified, chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of research grade. The following conjugated antibodies were purchased to characterize proteins of interest: anti-phosphotyrosine [PT66-FITC (fluorescein isothiocyanate)] anti-phosphotyrosine [PT66-HRP (horse-radish peroxidase)]. Albumin, ammonium persulphate and 3-(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) were obtained from Research Organics (Cleveland, OH, USA); D-glucose, sodium hydrogen carbonate, sodium chloride, potassium chloride, calcium chloride, potassium orthophosphate and magnesium sulphate were all of analytical reagent grade, purchased from Merck (BDH Merck, Kilsyth, VIC, Australia). Tris was from ICN Biochemicals (Castle Hill, NSW, Australia), and Percoll from GE Healthcare (Rydalmere, NSW, Australia). Nitrocellulose was from GE Healthcare (Buckinghamshire, UK) while highly pure Coomassie Brilliant Blue G250 was obtained from Serva (Heidelberg, Germany). SYTOX green cell vitality stain was purchased from Invitrogen (Carlsbad, CA, USA). Mowiol 4-88 was from Calbiochem (La Jolla, CA, USA), and paraformaldehyde was supplied by ProSciTech (Thuringowa, Australia). A rabbit polyclonal antibody to HSPA2 was purchased from Sigma-Aldrich (Cat # HPA000798) along with a rabbit monoclonal antibody to ARSA (Cat # HPA005554). A mouse monoclonal antibody to SPAM1 was purchased from Abnova (Cat # H00006677-A01, Taipei City, Taiwan) and a mouse, monoclonal antibody to CD59 was purchased from Abcam (Cat # ab9183). Anti-4HNE rabbit polyclonal antibody (Cat # HNE11-S) was purchased from Jomar diagnostics (Stepney, SA, Australia). Appropriate HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma-Aldrich.

Ethical approval

The experiments described in this study were conducted using pooled human semen samples obtained with informed written consent from a panel of 15 healthy normozoospermic donors (the majority of proven fertility) in accordance with the University of Newcastle’s Human Ethics Committee guidelines (Approval No. H-2013-0319).

Preparation of human spermatozoa

Enrichment of human spermatozoa was achieved using 45 and 90% discontinuous Percoll gradients as described previously (Nixon et al., 2011). High-quality spermatozoa were recovered from the base of the 90% Percoll fraction and resuspended in a bicarbonate-free non-capacitating (NC) form of Biggers, Whitten and Whittingham medium (NC BWW; Biggers et al., 1979) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM CaCl2.2H2O, 1.2 mM KH2PO4, 1.2 mM MgSO4.7H2O, 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 (PVA, osmolarity of 300 mOsm/kg). The cells were then pelleted by centrifugation at 500 \( \times g \) for a further 15 min and resuspended at a concentration of 10 \( \times 10^6 \) cells/ml. All experiments were performed on samples pooled from two or more donors per replicate with a minimum of three replicates examined.

Induction of oxidative stress in human spermatozoa

Oxidative stress was induced in populations of non-capacitated human spermatozoa through treatments with either 4-hydroxynonenal (4HNE) (Sigma) or hydrogen peroxide (H2O2) (Sigma) at concentrations of 50, 100 and 150 \( \mu \)M. Both 4HNE and H2O2 were chosen to induce oxidative stress based on their efficacy established in previous studies (Aitken et al., 2011). Cells at a concentration of 10 \( \times 10^6 \) cells/ml were resuspended in 4HNE or H2O2 and then incubated for 1 h at 37°C. Treated spermatozoa were then washed once in NC BWW. Following treatment, cell motility assessments were conducted at 37°C with an HTM-IVOS CASA system (Hamilton-Thorne Biosciences, version 12.3) using chamber slides of 20 \( \mu \)m depth (Leja, GN Nieuw-Vennep, The Netherlands). HTM-IVOS settings were adjusted as follows for human spermatozoa: negative phase...
contrast optics, recording rate 60 frames/s, minimum contrast 80, minimum cell size 3 pixels, low size gate 1.0, high size gate 2.9, low intensity gate 0.6, high intensity gate 1.4, non-motile head size 6, non-motile head intensity 160, progressive VAP (average path velocity) threshold value, 25 μm/s, slow cells VAP cut-off, 5 μm/s, slow cells VSL (straight line velocity) cut-off, 11 μm/s and threshold STR (straightness) >80%. Progressive cells were those exhibiting a VAP of >25 μm/s and a STR of >80%. Approximately eight fields were assessed in order to generate data on an average of 207 (range 168–277) spermatozoa per sample. The following sperm motility parameters were analysed: total motility (%); forward progressive motility (%); amplitude of lateral head displacement (ALH; μm); track speed (VCL; μm/s). Additionally, the percentage of hyperactivated spermatozoa (of 200 cells) was assessed by examining the spermatozoa using phase contrast optics at room temperature. Cell vitality was assessed using an eosin vitality stain as previously described (Aitken et al., 2012a).

**Capacitation of human spermatozoa**

To induce capacitation in vitro, human spermatozoa were resuspended in a capacitating (CAP) form of BWW composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM CaCl2-2H2O, 1.2 mM K2HPO4, 1.2 mM MgSO4.7H2O, 25 mM NaHCO3, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 mg/ml streptomycin, 20 mM HEPES buffer and 1 mg/ml PVA (osmolarity of 300 mOsm/kg) and supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic adenosine monophosphate (dbcAMP). Cells were incubated in this medium for 3 h at 37°C under an atmosphere of 5% CO2: 95% air with cells resuspended at a concentration of 10 × 106 cells/ml. Non-capacitated cells were incubated for the same period of time (without exposure to CO2) in BWW prepared without NaHCO3 (NC BWW). Following incubation, cell motility, hyperactivation and vitality were assessed, as described above. Populations of spermatozoa were then used for the assays outlined below.

**Assessment of membrane fluidity and membrane raft localization**

Merocyanine 540 (Sigma) was used to assess membrane fluidity of human spermatozoa. Following capacitation, aliquots of human spermatozoa from each treatment were diluted to 1 × 106 cells/ml and incubated in SYTOX green vitality stain at 37°C for 10 min. This preparation was washed once in BWW and then incubated in 2.7 μM merocyanine 540 at 37°C for 10 min. Preparations were then washed once in BWW and 200 cells from each treatment were scored on a Zeiss fluorescence microscope at excitation wavelengths 590 nm (merocyanine 540) and 470 nm (SYTOX green) (Carl Zeiss, Thornwood, NY, USA). Merocyanine positive sperm were identified through bright red fluorescence over the entire head and the absence of SYTOX green staining.

The localization of membrane raft marker, GM1 ganglioside, was visualized in human spermatozoa by staining with Alexa Fluor 555-labelled B subunit of cholera toxin (CTB) as previously described (Nixon et al., 2011). For each treatment, 200 cells were classified into two fluorescent patterns (head and tail or head only labelling).

**Analysis of protein tyrosine phosphorylation**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis/western blotting Following treatment, human spermatozoa were pelleted via centrifugation and resuspended for protein extraction as previously described (Reid et al., 2012). Protein extracts were then boiled in the presence of NuPAGE LDS sample buffer (Invitrogen) containing 8% β-Mercaptoethanol, subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 4–12% Bis–Tris gels (Invitrogen) and then electrotransferred to nitrocellulose membranes using conventional western blotting techniques (Towbin et al., 1979). To detect proteins of interest, membranes were blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST, pH 7.4) then probed with HRP-conjugated anti-phosphotyrosine antibody (a-PT66) diluted 1:10 000 in TBST supplemented with 1% BSA under constant rotation for 1 h at room temperature. Membranes were washed in TBST (3 × 10 min) and labelled proteins were visualized using an enhanced chemiluminescence detection kit (ECL plus, Amershams Bioscience) according to the manufacturer’s instructions.

**Immunocytochemistry**

Following capacitation, spermatozoa were fixed in 4% paraformaldehyde, washed 3 × with 0.05 M glycine in phosphate-buffered saline (PBS) and then applied to poly-L-lysine-coated glass cover slips. Cells were permeabilised with 0.2% Triton X-100, then placed in a humid chamber and blocked in 3% BSA/PBS for 1 h. Coverslips were then washed in PBS and incubated in FITC-conjugated a-PT66 antibody diluted 1:100 with 1% BSA/PBS for 1 h at 37°C. Following this, coverslips were washed (3 × 5 min) in PBS before mounting in 10% mowiol 4–88 (Calbiochem) with 3% glycerol in 0.2 M Tris (pH 8.5) and 2.5% 1,4-diazabicyclo-(2.2.2)-octane (DABCO). Samples were examined with a Zeiss LSM 10 laser scanning confocal microscope (Carl Zeiss Pty, Sydney, Australia), and human spermatozoa were classified as phosphotyrosine (PT66) positive when displaying uniform fluorescence across the full length of the sperm flagella, as previously described (Urner and Sakkas, 2003).

**Zona pellucida binding assessment**

Human zonae pellucidae were obtained from IVF Australia clinics (Human Ethics Approval No. H-2013-0134) and stored in a high salt storage medium consisting of 1.5 M MgCl2, 0.1% dextran, 0.01 mM HEPES buffer and 0.1% PVA at 4°C. Prior to use, zona-intact oocytes were washed 3 × in PBS and four oocytes per treatment were placed in droplets of BWW under water-saturated mineral oil at 37°C in an atmosphere of 5% CO2: 95% air and allowed to equilibrate for 30 min. The spermatozoa were diluted to a concentration of 1 × 106 cells/ml and 20 μl of sperm suspension was added to each droplet of oocytes. Gametes were co-incubated under the same conditions for a further 30 min. Oocytes were then washed 3 × by serial aspiration through droplets of BWW to remove any unbound sperm. After washing in BWW, oocytes were mounted on glass slides under coverslips suspended at each corner by 80% paraffin wax and 20% Vaseline gel and the number of motile sperm bound to each zona pellucida was assessed using phase contrast microscopy (Zeiss Axioscan 2).

**Agonist-induced acrosome reaction**

To induce acrosomal exocytosis, human spermatozoa were incubated for 30 min with 0.5% v/v dimethylsulfoxide (DMSO) vehicle control or 2.5 μM calcium ionophore (A23187). Sperm were then incubated in pre-warmed hypo-osmotic swelling media (HOS; 0.07% w/v sodium citrate; 1.3% w/v fructose) for a further 30 min at 37°C. Sperm preparations were washed in PBS, placed on poly-L-lysine-coated slides and allowed to air dry. The cells were then permeabilized in ice-cold methanol and subjected to immunocytochemistry with TRIIT-labelled peanut agglutinin lectin (PNA; I:200), as previously described (Redgrove et al., 2013). The acrosomal status of human spermatozoa was assessed with a Zeiss LSM 10 laser scanning confocal microscope. Acrosome reacted cells were identified by the appearance of a curled tail and either the complete absence of PNA staining over the acrosomal region or the restriction of this labelling to the equatorial segment of the sperm head (Rathi et al., 2001).

**Surface labelling of human spermatozoa**

Non-capacitated, 4HNE-treated and H2O2-treated sperm suspensions were incubated with primary antibody (Anti-SPAM1, Anti-ARSA or
Anti-CD59; diluted 1:100) for 1 h. The cells were subsequently washed 2 × with BWW and incubated with FITC-conjugated secondary antibody (diluted 1:500) for a further 30 min. Following three washes with BWW, the cells were incubated with 20 mg/ml of propidium iodide (PI) for 1 min and assessed for surface fluorescence using a Zeiss fluorescence microscope (t = 0 min). Following capacitation of untreated, 4HNE-treated and H2O2-treated spermatozoa, fresh sperm suspensions were incubated with primary antibody for 1 h, washed in BWW (×2) and incubated in corresponding FITC-conjugated secondary antibodies for 30 min. After three washes, these cells were also incubated in PI and immediately assessed for surface fluorescence (t = 180 min). Antibodies against CD59 were used as a positive control for assessment of protein expression on the surface of human spermatozoa, as previously described (Redgrove et al., 2012), and the proportion of sperm expressing ARSA and SPAM1 on their surface was recorded for 200 cells from each treatment.

**Blue Native PAGE**

Both H2O2-treated and capacitated spermatozoa were prepared for blue native PAGE (BN-PAGE) as previously described (Redgrove et al., 2013). Briefly, cell pellets were resuspended in native lysis buffer consisting of 1% n-dodecyl b o -maltoside, 0.5% Coomassie Blue G250 and a cocktail of protease inhibitors (Roche, Mannheim, Germany) and incubated at 4 °C on an orbital rocker for 30 min. Samples were centrifuged for 20 min at 14,000 × g and then dialysed against Blue Native cathode buffer (purchased from Invitrogen, Carlsbad, CA, USA) overnight at 4 °C. Dialysed native protein lysates were loaded onto blue native polyacrylamide gels (NativePAGE Novex 4–16%, Bis–Tris gels; Invitrogen) and resolved using a NativePAGE cathode and anode buffer (Redgrove et al., 2011) and run at 4 °C at 100 V for the duration of the stacking gel layer and at 200 V for the resolving gel layer for ~3 h. Following retrieval from the cassettes, gels were either stained with Coomassie G250 or prepared for western blotting with an antibody to HSPA2.

**Proximity ligation assay**

Duolink in situ proximity ligation assays (PLAs) were conducted in accordance with the manufacturers’ instructions on fixed human spermatozoa adhered to poly-l-lysine-coated coverslips (OLINK Biosciences, Uppsala, Sweden). Samples were blocked in Duolink blocking solution and then incubated with primary antibodies (anti-SPAM1, anti-ARSA, anti-HSPA2 and anti-tubulin) overnight at 4 °C. Oligonucleotide-conjugated secondary antibodies (PLA probes) were then applied for 1 h at 37 °C and ligation of the PLA probes was performed, and the signal amplified according to the manufacturer’s instructions. The fluorescent signal generated when molecules are in close association (<40 nm) was visualized using fluorescence microscopy and could be quantified by recording the proportion of 100 spermatozoa displaying a collection of red fluorescent dots over the sperm head. Specificity of this reaction was ensured by performing proximity ligation with antibodies to the target antigens combined with anti-tubulin antibodies with which they should not interact.

**Immunoprecipitation**

Capacitated and 4HNE-treated spermatozoa were prepared as above. Cell lysis was performed on populations of ~ 100 × 10^6 cells from each treatment at 4 °C for 2 h in lysis buffer consisting of 10 mM CHAPS, 10 mM HEPES, 137 mM NaCl and 10% glycerol with the addition of protease inhibitors (Roche). Lysis was completed with a centrifugation step performed at 14,000 × g at 4 °C for 20 min. The cell lysates were then added to 50 μl aliquots of washed protein G Dynabeads and incubated under rotation to preclar at 4 °C for 1 h. Anti-HSPA2 antibody at a concentration of 10 μg in 200 μl of PBS was conjugated to fresh aliquots of washed (supernatant removed) Dynabeads by incubation for 2 h at 4 °C under rotation. Following antibody binding the cross-linking reagent, 3,3′-dithiodi(4-sulfosuccinimidylpropionate) (DTSSP), was added at a final concentration of 2 mM and cross-linking was performed at room temperature for 30 min after which 20 mM TRIS was added to each tube for an additional 15 min at room temperature to quench the reaction. Beads were washed (3 ×) in 200 μl of lysis buffer and the wash supernatants were kept and stored at ~20 °C.

Immunoprecipitation was then performed by adding 1 ml precleared lysate to HSPA2 antibody-bound beads and incubating under rotation overnight at 4 °C. After incubation, supernatant was transferred to a clean tube and washed (3 ×) in 200 μl of PBS. Finally, beads were resuspended in 100 μl of PBS and transferred to a fresh tube to avoid co-elution of proteins bound to the tube.

Target antigen was eluted from the beads by boiling in the presence of SDS-loading buffer containing 8% β-mercaptoethanol. The same elution was performed on precleared beads and these solutions were loaded onto a NuSep 4−20% Tris–glycine gel for analysis via SDS–PAGE. In addition, bead-only and antibody-only controls were prepared by loading 10 μl of protein-G bead slurry and 5 μl of anti-HSPA2 in the presence of SDS-loading buffer into appropriate gel lanes. The third wash of the antibody-bound beads for each treatment was also loaded onto the gel after boiling in the presence of SDS-loading buffer. A duplicate gel was also prepared for immunoblotting and both were resolved at 150 V for ~1 h. Electro-transfer of proteins was performed as previously described (Redgrove et al., 2013). To detect proteins of interest, membranes were blocked in 3% w/v BSA in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST, pH 7.4) then probed with anti-HSPA2 antibody diluted 1:1000 in TBST supplemented with 1% BSA under constant rotation overnight at 4 °C. Membranes were washed in TBST (3 × 10 min) and then incubated in anti-rabbit HRP-conjugated secondary antibody (diluted 1:10000) for 1 h at room temperature. Following three washes in TBST, proteins were visualized using an enhanced chemiluminescence detection kit (ECL plus, Amersham Bioscience) according to the manufacturer’s instructions. Following this, membranes were stripped for 30 min at room temperature in ReProbe solution (G Biosciences, St. Louis, MO, USA), blocked in 5% w/v skim milk in TBST and probed with anti-4HNE antibody diluted 1:1000 in TBST supplemented with 3% skim milk overnight at 4 °C. After three washes, membranes were incubated in anti-rabbit HRP-conjugated secondary antibody (1:1000) for 1 h at room temperature before being visualized as above.

**Penicillamine treatment**

To investigate the effect of aldehydes on sperm function, the nucleophile D-penicillamine was used to covalently bind aldehydes, thereby limiting their bioavailability, as described previously (Aitken et al., 2012). Briefly, human spermatozoa were prepared via Percoll centrifugation and then exposed to 50 μM H2O2 for 1 h at 37 °C (as above). Following this treatment, cells were washed once in BWW and then capacitated for 3 h in CAP BWW supplemented with either 0.5 or 1 mM D-penicillamine (Sigma). Following capacitation, spermatozoa were washed in BWW and either processed for western blotting with an antibody to HSPA2 or prepared for western blotting with an antibody to HSPA2.

**Statistical analysis**

All experiments were replicated at least three times with independent samples and data are expressed as mean values ± SE. Statistical analysis was performed using a two-tailed, unpaired Student’s t-test using Microsoft Excel (Version 14.0.0; Redmond, Washington, DC, USA). Differences were considered significant for *P* < 0.05.

**Results**

Sperm motility and viability after treatment with 4HNE and H2O2

To establish the concentrations of 4HNE and H2O2 to be used for this series of experiments, a dose-dependent study was conducted to
evaluate both sperm motility and viability following treatment with each agent. Concentrations of 50, 100 and 150 μM were trialled based on previous studies (Aitken et al., 2011, 2012a).

As expected, dose-dependent decreases in both viability and motility were recorded after treatment with all concentrations of 4HNE or H₂O₂ (Fig. 2). Despite this, at the lowest concentration of each agent (50 μM), motility remained at 65 and 62% for 4HNE and H₂O₂, respectively. For the purpose of this study, only low levels of oxidative stress were desirable to evaluate subtle differences in sperm capacitation and zona pellucida binding ability without explicitly compromising the ability of these cells to function. For this reason, concentrations of 50 μM of each agent were chosen for subsequent analyses. These concentrations of 4HNE and H₂O₂ are well within the range attained under conditions of oxidative stress, which in the case of 4HNE can reach 5 mM (Uchida, 2003) while in the case of H₂O₂, a concentration of 50 μM is within the range found in many biological fluids (Halliwell et al., 2000).

For the purpose of the next series of experiments, spermatozoa that were treated with 50 μM 4HNE or 50 μM H₂O₂ were then washed free of these agents and capacitated for 3 h in BWW supplemented with 3 mM pentoxifylline and 5 mM dbcAMP.

Assessment of capacitation status after treatment with 4HNE and H₂O₂

During the initial phase of capacitation in the female reproductive tract, mammalian spermatozoa experience a rapid loss of membrane sterols in response to increased levels of bicarbonate and calcium in the extracellular environment (Flesch et al., 2001). This loss of membrane sterols promotes a degree of membrane fluidization through a ‘scrambling’ of the lipid components of the plasma membrane (Boerke et al., 2008). In vitro, this capacitation-driven membrane fluidization can be evaluated using a fluorescent probe, Merocyanine 540, which is incorporated into spermatozoa that have a degree of membrane destabilization (Flesch et al., 2001). This probe was therefore used in our study to quantify the number of sperm that were able to undergo this capacitation-dependent process after treatment with 50 μM 4HNE or H₂O₂ (Fig. 3A).

Unexpectedly, the percentage of sperm that were positive for Merocyanine 540 after treatment with either ROS generating agent was statistically similar (P ≥ 0.05) to the percentage of spermatozoa exhibiting a positive merocyanine response under standard capacitating conditions. Interestingly, the percentage of spermatozoa that displayed evidence of membrane fluidization was greater having undergone pretreatment with 4HNE (87.5%) or H₂O₂ (74.5%) than the capacitated control (CAP; 54%). Notably, all treatments showed a statistically significant (P ≤ 0.05) increase in the proportion of spermatozoa displaying a positive merocyanine response compared with the non-capacitating control devoid of bicarbonate (NC; 18%).

An additional correlate of the capacitation process that has been documented in human spermatozoa (Nixon et al., 2011) is the redistribution of membrane rafts from a uniform distribution throughout both the sperm head and flagella in non-capacitated cells to a discernible aggregation within the apical region of the sperm head, following capacitation (Fig. 3B). This capacitation-dependent shift in raft distribution is thought to be permitted by the fluidization of the plasma membrane and can be monitored using fluorescently labelled cholera toxin B (CTB) that has affinity for the G₄₃₁ ganglioside structural components.

**Figure 2.** Effect of oxidative stress on human sperm motility and viability. Non-capacitated human spermatozoa were treated with either 50, 100 or 150 μM 4HNE or H₂O₂ for 1 h and then capacitated in BWW supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic AMP. A dose-dependent decrease in motility was observed using a HTM-IVOS CASA system and a dose-dependent reduction in sperm viability was observed through use of an Eosin vitality stain. Statistical analyses were performed using a Student’s t-test, *P < 0.05; **P < 0.01.
of membrane rafts (Nixon et al., 2010, 2011). Using this marker, the number of human spermatozoa displaying the CTB labelling pattern typical of CAP cells was recorded after incubation under NC or CAP conditions and after treatment with 4HNE or H2O2. Consistent with the results observed for membrane fluidization, in an environment of enhanced oxidative stress a similar percentage of human spermatozoa displayed the restricted apical CTB labelling pattern after treatment with 4HNE (71%) and H2O2 (71%) compared with that observed under CAP conditions (66.5%). In contrast, only 13% of sperm incubated under NC conditions displayed any evidence of membrane raft redistribution to the sperm head.

To further explore these findings, the ability of human spermatozoa to undergo protein tyrosine phosphorylation after exposure to oxidative stress was evaluated. Through immunocytochemistry, only 8% of cells incubated under NC conditions displayed typical PT66 positive labelling of the whole flagella. This was in contrast to CAP spermatozoa that presented evidence of complete flagellar protein tyrosine phosphorylation in 74% of the population. When these cells were treated with either 4HNE or H2O2 prior to capacitation, no significant difference in the percentage of cells displaying complete PT66 labelling was observed compared with the capacitated control with 75 and 65% of cells from these treatments, respectively, displaying labelling patterns typical of a capacitated cell (Fig. 4A). These results were consistent with western blotting analyses (Fig. 4B), which revealed a similar profile and overall level of phosphotyrosine expression following treatment with either 4HNE or H2O2 (Fig. 4B).

Collectively, this evaluation of membrane fluidization, raft reorganization and capacitation-induced protein tyrosine phosphorylation suggests that human spermatozoa treated with low levels of 4HNE and H2O2 remain capable of undergoing the changes necessary to achieve the early stages of capacitation, in vitro. However, one of the most dynamic properties acquired by capacitating sperm is the ability to recognize and bind to the zona pellucida (Dun et al., 2010). To explore the impact of low levels of oxidative stress on the functional end-points of sperm capacitation, the remaining experiments of this study evaluated

**Figure 3** Assessment of human sperm membrane fluidization and membrane raft localization in response to oxidative stress. To assess the capacitation state of human spermatozoa pretreated with 4HNE or H2O2 prior to capacitation, merocyanine 540 was selected to monitor membrane destabilization and fluidization and fluorescently labelled CTB was used to localize the G_{M1} gangliosides of membrane rafts. Spermatozoa treated with 4HNE or H2O2 were washed free of these agents and then capacitated for 3 h in BWW supplemented with 3 mM pentoxifylline and 5 mM dibutyryl cyclic AMP. Live cells were then either stained with Merocyanine 540/SYTOX green or incubated in Alexa Fluor 555-labelled CTB and fixed with 4% paraformaldehyde. Non-capacitated (NC), capacitated (CAP), 4HNE-treated (4HNE) and H2O2-treated (H2O2) cells were assessed for membrane fluidity by the recording the proportion of 200 viable cells displaying a Merocyanine positive signal across three biological replicates (red fluorescence over the sperm head), images were taken on a fluorescence microscope using a ×40 objective (A) and spermatozoa were assessed for raft localization by scoring 200 cells across three biological replicates displaying the peri-acrosomal labelling pattern typical of a capacitated cell. Images were taken on a fluorescence microscope using a ×100 objective (B). Statistical analyses were performed using a Student’s t-test, *P < 0.05; **P < 0.01.
the ability of 4HNE- and H$_2$O$_2$-treated human spermatozoa to engage in interactions with the zona pellucida.

Evaluation of the zona pellucida binding ability of human sperm after treatment with 4HNE and H$_2$O$_2$

To examine the impact of 4HNE and H$_2$O$_2$ on the zona pellucida binding ability of human spermatozoa, a binding assay incorporating human ova was used (Fig. 5A). As expected, a significantly greater number of CAP human sperm were capable of interacting with the zona pellucida when compared with those incubated under NC conditions ($P < 0.001$) with an average of 33 sperm bound/oocyte after capacitation and an average of only two sperm bound/oocyte after a 3 h incubation in media devoid of bicarbonate (7% of CAP control). Despite having comparable levels of motility and number of hyperactivated cells within the samples (Supplementary data, Table SI), human spermatozoa treated with 4HNE and H$_2$O$_2$ prior to capacitation were unable to bind tightly to the zona pellucida with an average of only seven spermatozoa bound/oocyte for 4HNE-treated cells (22% of CAP control) and two spermatozoa bound/oocyte following H$_2$O$_2$ treatment (7% of CAP control). These numbers were significantly lower than those achieved under the same capacitating conditions without prior exposure to 4HNE and H$_2$O$_2$ ($P < 0.01$). Treatment with H$_2$O$_2$, in particular, reduced the binding ability to a level statistically similar to that achieved under NC conditions ($P > 0.05$). While not the focus of these studies, treatment with 4HNE and H$_2$O$_2$ also had a significant impact on the ability of human spermatozoa to undergo an agonist-induced acrosome reaction, with a 2-fold decrease in the number of acrosome reacted spermatozoa detected in populations exposed to such insults prior to capacitation (Supplementary data, Fig. S1).

The impact of oxidative stress on the co-ordinated surface expression of SPAM1 and ARSA during capacitation

Given the important role of ARSA in mediating contact between the sperm surface and the sulphated residues of zona pellucida glycans, a reduction in expression of this receptor on the surface of capacitated human sperm might be expected to have deleterious consequences on the binding ability of these cells during gamete co-incubation. This study revealed that ~78% of NC cells (untreated, $t = 0$) were surface labelled with anti-SPAM1 compared with only ~17% with anti-ARSA (Fig. 5B). However, incubation in CAP media led to a significant decrease in the surface expression of SPAM1 such that only 34% of the viable sperm population was positively labelled. In contrast, ARSA surface expression showed a dramatic increase after capacitation, resulting in ~62% of the viable population being labelled after 180 min, a result that reflects flow cytometry data published by Redgrove et al. (2013). However, the capacitation-dependent increase in ARSA surface expression was significantly compromised by pretreatment of the cells with reagents capable of inducing oxidative stress. In this context, only 21 and ~10% of live spermatozoa exhibited surface expression of ARSA following 4HNE and H$_2$O$_2$ treatments, respectively (Fig. 5B). Importantly, CD59 was detected on the surface of >80% of spermatozoa across all

![Figure 4](https://academic.oup.com/humrep/article-abstract/30/11/2597/2384764/5B)
treatments indicating that the induction of oxidative stress had a non-specific effect on the integrity of the plasma membrane and its capacity to express surface markers. Similarly, HSPA2 was found on the surface of 7% of spermatozoa regardless of treatment. While SPAM1 surface expression was significantly reduced in CAP cells (as shown by Redgrove et al., 2012), its surface expression did not appear affected by 4HNE and \( \text{H}_2\text{O}_2 \). To ensure that the reduction in ARSA surface expression observed in response to oxidative stress did not reflect a complete loss of this protein from the cell, an aliquot of spermatozoa from each treatment was permeabilized prior to ARSA labelling. This permeabilization step resulted in distinct ARSA fluorescence over the sperm head in >85% of cells, regardless of treatment (Supplementary data, Fig. S2C).

![Figure 5](https://academic.oup.com/humrep/article-abstract/30/11/2597/2384764)

**Figure 5** Assessment of zona pellucida binding competence of human spermatozoa after treatment with 4HNE and \( \text{H}_2\text{O}_2 \). Non-capacitated sperm were treated with either 50 \( \mu \text{M} \) 4HNE or 50 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), capacitated and then (A) co-incubated with human zona-intact oocytes (images were taken on a fluorescence microscope using a \( \times40 \) objective) or (B) incubated in either anti-ARSA or anti-SPAM1 antibodies to examine the surface expression of SPAM1 and ARSA over a 180-min period of capacitation. The number of zona pellucida-bound sperm was recorded and presented as a percentage of the capacitated control. (B) Untreated, 4HNE-treated and \( \text{H}_2\text{O}_2 \)-treated spermatozoa were incubated in capacitating media and aliquots were sampled and assessed at 0 (non-capacitated) and 180 min (capacitated) time points. The presence of SPAM1, ARSA and HSPA2 on the surface of live spermatozoa was assessed using appropriate primary antibodies, followed by an Alexa Fluor-conjugated secondary antibody and PI as a counterstain to assess cell viability. Positive control incubations were labelled with anti-CD59. The percentage of live sperm expressing surface fluorescence in each population was evaluated using a fluorescence microscope, scoring 200 cells across three biological replicates. Statistical analyses were performed using a Student’s t-test. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \).
Evaluation of protein complex assembly/function after oxidative stress

To further explore the relationship between oxidative stress and the assembly of the SPAM1/ARSA zona pellucida-receptor complex, BN-PAGE was performed with populations of spermatozoa treated with 4HNE or H2O2. Immunoblot experiments with anti-HSPA2 antibodies revealed that these agents did not greatly affect the profile of HSPA2-laden complexes present in spermatozoa (Fig. 6A and B) and the ~200 kDa complex containing SPAM1, ARSA and HSPA2 (Redgrove et al., 2013) resolved at the correct molecular weight in all samples. Additionally, probing these BN-PAGE blots with anti-ARSA antibodies confirmed that ARSA was not lost from the complex through treatment with 4HNE and H2O2 (Supplementary data, Fig. S2A). These data suggest that 4HNE or H2O2 does not perturb the interactions that underpin the formation of sperm protein complexes. However, to determine if subtle changes in protein interactions occur within this complex after a period of oxidative stress, a proximity ligation technique was employed.

Previous studies of this complex using both proximity ligation and co-localization have revealed the close apposition SPAM1 and ARSA as well as the regulatory chaperone HSPA2 in the anterior region of the spermatozoa. (Fig. 6A) Detection of 200 kDa HSPA2/SPAM1/ARSA complex in native lysates of human spermatozoa after treatment with 4HNE and H2O2. Aliquots of non-capacitated and capacitated spermatozoa, and capacitated spermatozoa pretreated with 4HNE or H2O2 were subjected to native protein lysis and the resulting protein complexes were visualized by Coomassie staining or prepared for western blotting with anti-HSPA2. Coomassie staining revealed the presence of a 200 kDa protein complex previously described by Redgrove et al. (2012) in native lysates of both treated and untreated samples. HSPA2 was shown to resolve in this 200 kDa complex through probing of corresponding western blots with anti-HSPA2 antibodies. Importantly, the presence of HSPA2 within this complex did not appear affected by treatment with 4HNE or H2O2. (B) This was verified through band densitometry analysis comparing the density of anti-HSPA2 labelled bands across three replicate blots to the band density of the blue native PAGE 200 kDa gel bands. (C) Populations of non-capacitated, capacitated, 4HNE-treated and H2O2-treated human spermatozoa were fixed in paraformaldehyde and allowed to settle onto poly-L-lysine-coated slides. These samples were then blocked in Duolink blocking solution, followed by incubation with primary antibodies (anti-SPAM1 and anti-ARSA; anti-SPAM1 and anti-tubulin; anti-ARSA and anti-tubulin) and oligonucleotide-conjugated secondary antibodies (proximity ligation assay (PLA) probes). The PLA probes were then ligated and the signal was amplified according to the manufacturer’s instructions (OLINK Biosciences). The fluorescent signal generated when molecules are in close association (<40 nm) was visualized using fluorescence microscopy, using either a ×100 or ×20 objective.

Figure 6 Effect of oxidative stress on the interaction between SPAM1 and ARSA in human spermatozoa. (A) Detection of 200 kDa HSPA2/SPAM1/ARSA complex in native lysates of human spermatozoa after treatment with 4HNE and H2O2. Aliquots of non-capacitated and capacitated spermatozoa, and capacitated spermatozoa pretreated with 4HNE or H2O2 were subjected to native protein lysis and the resulting protein complexes were visualized by Coomassie staining or prepared for western blotting with anti-HSPA2. Coomassie staining revealed the presence of a 200 kDa protein complex previously described by Redgrove et al. (2012) in native lysates of both treated and untreated samples. HSPA2 was shown to resolve in this 200 kDa complex through probing of corresponding western blots with anti-HSPA2 antibodies. Importantly, the presence of HSPA2 within this complex did not appear affected by treatment with 4HNE or H2O2. (B) This was verified through band densitometry analysis comparing the density of anti-HSPA2 labelled bands across three replicate blots to the band density of the blue native PAGE 200 kDa gel bands. (C) Populations of non-capacitated, capacitated, 4HNE-treated and H2O2-treated human spermatozoa were fixed in paraformaldehyde and allowed to settle onto poly-L-lysine-coated slides. These samples were then blocked in Duolink blocking solution, followed by incubation with primary antibodies (anti-SPAM1 and anti-ARSA; anti-SPAM1 and anti-tubulin; anti-ARSA and anti-tubulin) and oligonucleotide-conjugated secondary antibodies (proximity ligation assay (PLA) probes). The PLA probes were then ligated and the signal was amplified according to the manufacturer’s instructions (OLINK Biosciences). The fluorescent signal generated when molecules are in close association (<40 nm) was visualized using fluorescence microscopy, using either a ×100 or ×20 objective.
of the sperm head after in vitro capacitation (Redgrove et al., 2013). To further evaluate the effect of oxidative stress on interactions between ARSA and SPAM1, the Duolink PLA was performed on non-capacitated, capacitated, 4HNE-treated and H2O2-treated cells. As shown in Fig. 6C, the punctuate fluorescent spots that indicate a close interaction between two proteins (<40 nm apart) were no longer detectable over the anterior head region of spermatozoa treated with either 4HNE or H2O2. This suggests that SPAM1 and ARSA were not able to form a stable interaction in a majority of 4HNE- or H2O2-treated human spermatozoa, with only 8 and 12% of cells showing evidence of PLA fluorescence, respectively (data not shown). This was in contrast to both NC and CAP sperm where 65 and 76% of cells, respectively, showed evidence of an interaction between SPAM1 and ARSA, as indicated by the red fluorescence within the sperm head (Fig. 6C). Importantly, the specificity of this reaction was confirmed through dual labelling with a combination of the anti-SPAM1 and anti-ARSA antibodies with that of an unrelated antibody (anti-tubulin). These results suggested that rather than a complete deregulation of this zona pellucida complex assembly, the reduction of ARSA expression on the surface of capacitated human spermatozoa may be due to a loss of the tight coordination/association between members of this complex during capacitation in cells experiencing the effects of oxidative stress.

### Investigation of HSPA2 as a target for modification by 4HNE after exposure to oxidative stress

As our model suggests that the chaperone activity of HSPA2 is required for the co-ordinated presentation of ARSA on the sperm surface, we followed up this series of experiments investigating potential impacts of oxidative stress on HSPA2.

In order to investigate the potential adduction of HSPA2 by 4HNE in capacitated and oxidative stressed human spermatozoa, an immunoprecipitation strategy was adopted. Human sperm lysates extracted from both H2O2-treated and capacitated cells were immunoprecipitated with anti-HSPA2 antibodies and eluted proteins were sequentially probed with anti-HSPA2 (to confirm the specificity of the IP; Fig. 7A) and anti-4HNE (Fig. 7B) to identify potential protein adducts. As shown in Fig. 7A and B, the HSPA2 protein was effectively isolated as a predominant band at ~70 kDa in the eluates from both CAP- and H2O2-treated cells. An additional band of ~55 kDa was also detected in these samples; however, this may correspond to the heavy IgG chain given that a band of similar size was detected in the antibody-only control. The specificity of this immunoprecipitation was confirmed through the use of antibody-only and bead-only controls, as well as a ‘precleared’ control. The experiment was replicated three times using pooled semen samples and representative blots are depicted.

![Image](https://example.com/image.png)

**Figure 7** Examination of HSPA2/4HNE interaction in human spermatozoa. Lysates of capacitated and H2O2-treated, capacitated human spermatozoa were incubated with protein G Dynabeads conjugated with anti-HSPA2 antibodies. The beads were washed, and then bound proteins were eluted and resolved on SDS–PAGE gels before being transferred to nitrocellulose membranes. (A) Membranes were probed with anti-HSPA2 antibodies to confirm the efficacy of immunoprecipitation before being stripped and then reprobed with 4HNE antibodies (B). Controls included an antibody-only control (Ab only) in which antibody-conjugated beads were incubated in the absence of cell lysate. In addition, a whole sperm lysate was included to confirm the identity of the co-precipitated proteins as well as a preclear eluate control. The experiment was replicated three times using pooled semen samples and representative blots are depicted.

Anti-4HNE were also probed for the presence of ARSA. While bands were detected in the elution lanes at the appropriate size (~55 kDa), ARSA appeared to be constitutively modified by 4HNE with no change detected between CAP sperm lysates and lysates from H2O2-treated spermatozoa (Supplementary data, Fig. S2B).

Given these results it would be tempting to speculate that 4HNE adduction to the HSPA2 chaperone may give rise to the loss of sperm–zona pellucida recognition observed in this study. If this was the case, we reasoned that it would be possible to prevent the effects of this lipid aldehyde by introducing nucleophilic scavengers to limit its bioavailability. Penicillamine has previously been shown to significantly reduce the cellular expression of 4HNE owing to its ability to covalently bind the aldehyde (Aitken et al., 2012a). In view of this protective effect, the remaining experiments of this study focused on analysing whether penicillamine could ameliorate the negative outcomes of 4HNE adduction to HSPA2, ARSA surface expression and zona pellucida binding in oxidatively stressed human spermatozoa.

### The effect of penicillamine treatment on zona pellucida binding, ARSA surface expression and 4HNE adduction of HSPA2

To evaluate the effect of penicillamine on ARSA expression on the sperm surface, live human spermatozoa were labelled with anti-ARSA and examined through fluorescence microscopy. These analyses revealed a
significant increase in the number of labelled spermatozoa after addition of both 0.5 and 1 mM penicillamine when compared with H2O2-treated sperm. Indeed, incubation with 0.5 and 1 mM penicillamine resulted in a 2- and 3-fold increase in the number of spermatozoa surface labelled with ARSA compared with that of the H2O2-treated sperm population ($P = 0.05; P = 0.01$), respectively (Fig. 8A).

To assess whether these effects were related to a relief of 4HNE adduction to HSPA2, immunoprecipitation was performed using
anti-HSPA2. Through immunoblotting analysis with anti-HSPA2, 70 kDa bands were observed in both H2O2-treated and H2O2/penicillamine-treated eluates (Fig. 8B). However, when these eluted proteins were probed with anti-4HNE antibodies, a distinct loss of 4HNE adducts was revealed in the penicillamine-treated sperm eluate.

Given these results, we sought to test the ability of penicillamine to limit the impact of H2O2 on sperm function by examining the ability of these cells to interact with homologous zona pellucidae. Surprisingly, while H2O2-treated cells showed a marked reduction in zona pellucida binding ability compared with the CAP sperm control (6% of CAP control; Fig. 8C and D), the co-incubation of capacitating sperm with penicillamine enabled a near complete recovery of zona pellucida binding ability (97% of capacitated control; Fig. 8D and E). This result was statistically significant from both the non-capacitated control and H2O2-treated cells, while the capacitated control and penicillamine-treated cells were statistically similar. Importantly, these results were not mediated by differences in sperm total motility or hyperactivated motility, as shown in Supplementary data, Table SI.

Discussion

ROS are well recognized as key regulators of mammalian sperm capacitation, yet a fine balance exists between a beneficial presence of ROS to promote cholesterol efflux (Boerke et al., 2013), cAMP production (Zhang and Zheng, 1996; Ickowicz et al., 2012), protein tyrosine phosphorylation (Aitken et al., 1998; Leclerc et al., 1997) and the acrosome reaction (de Lamirande et al., 1998) and the detrimental presence of excess ROS that can lead otherwise viable cells down an intrinsic apoptotic-like pathway (Aitken et al., 2012). The present study suggests that levels of oxidative stress that might be encountered in vivo (Uchida, 2003; Aitken et al., 2012) have relatively little effect on events associated with early capacitation, such as membrane fluidity, raft redistribution and protein tyrosine phosphorylation. However, such low levels of stress can, nevertheless, have detrimental downstream effects on sperm-zona pellucida interaction. Moreover, this study has suggested that the inhibition of zona pellucida binding under these circumstances is associated with the impaired expression of the zona pellucida receptor, ARSA, on the sperm surface.

The disrupted presentation of ARSA on the sperm surface is, in turn, thought to be associated with the dysregulation of a sperm–zona pellucida-receptor complex that becomes expressed during capacitation comprising the regulatory chaperone HSPA2 in close association with SPAM1/ARSA (Redgrove et al., 2012, 2013). Following exposure to oxidative stress, this complex does not disappear; however, its molecular constituents no longer exist in sufficient proximity to one another to give a positive signal when interrogated in a PLA. This loss of functional association is in turn thought to be due to the post-translational modification of HSPA2 as a consequence of addition by the lipid aldehyde, 4HNE.

4HNE is the major cytotoxic aldehyde generated by the attack of free radicals on ω-6 polyunsaturated fatty acids during lipid peroxidation and accumulates more readily in biomembranes than in free solution due to its lipophilic nature (Esterbauer et al., 1982; Esterbauer et al., 1986; Uchida, 2003). The molecule itself exhibits a wide range of biological functions such as the inhibition of protein and DNA synthesis, inactivation of enzymes and stimulation of phospholipase C (Uchida, 2003; Carini et al., 2004). However, in addition to these functions, the electrophilic nature of 4HNE causes it to form stable covalent adducts with the nucleophilic functional groups of cysteine, lysine and histidine residues of numerous proteins to form both Michael and Schiff base adducts, thereby introducing carbonyl groups into proteins and altering their function (Uchida and Stadtman, 1992; Butterfield, 2002; Perluigi et al., 2012).

Although there are several potential explanations for the loss of ARSA surface expression following oxidative stress, the increase in HSPA2-bound 4HNE adducts supports a mechanism involving non-enzymatic post-translational protein modification by 4HNE. Certainly, the use of the antioxidant penicillamine to counteract the deleterious effects of H2O2 on sperm function suggests that this damage is mediated by electrophilic aldehyde(s). Penicillamine has been shown to significantly reduce the expression of 4HNE in human spermatozoa by intercepting 4HNE as it is produced, through covalent interaction, while simultaneously chelating transition metals, such as iron and copper, that catalyse the cascades of lipid peroxidation that are responsible for the formation of these aldehydes (Aitken et al., 2012). Our immunoprecipitation analyses suggest that the use of penicillamine during human sperm capacitation was able to protect/relieve HSPA2 from 4HNE adduction and subsequently a majority of H2O2-treated cells were able to present ARSA on their surface and participate in zona pellucida recognition. In light of this, we propose that oxidative stress induces lipid peroxidation and the subsequent generation of 4HNE (and likely other reactive aldehydes) targets HSPA2, causing modifications to the protein and consequently its molecular chaperone activity. Such modifications then appear to destroy the ability of this chaperone to orchestrate the reciprocal surface expression of ARSA and SPAM1 (Redgrove et al., 2012, 2013).

Although investigating potential sites of 4HNE addition and the manner in which these modifications may compromise the structure and/or function of HSPA2 is beyond the scope of this study, in a number of independent studies 4HNE has been shown to interfere with the activities of various signalling kinases, such as mitogen-activated protein kinases (MAPKs; Sampey et al., 2007) and protein kinase C (Harry et al., 2012), and to regulate or dysregulate cell processes such as apoptosis, proliferation and differentiation (Leonarduzzi et al., 2004). It is likely that 4HNE addition could modify the ATPase activity of HSPA2 and thus prevent it from fulfilling its role in protein trafficking or refolding events, as has been previously documented for HSP72 in the rat (Carbone et al., 2004, 2005). In these studies, 4HNE modification of cytosolic HSP72 led to an inhibition of protein refolding function through covalent modification of Cys267 in the ATP binding site of the molecule, purportedly through a thiol-specific mechanism of inactivation (Carbone et al., 2004). Moreover, in a rat model of alcohol-induced oxidative stress (Carbone et al., 2005) it has been demonstrated that 4HNE deregulates the activity of an alternative heat shock protein, HSP90, through a similar mode of thiol modification.

Taken together, these data suggest that heat shock proteins are key targets for 4HNE addition reactions and that their chaperoning activity is particularly susceptible to such an insult. While similar studies need to be carried out with human HSPA2 to determine whether equivalent mechanisms may contribute to its dysregulation, it is tempting to conclude that 4HNE addition directly leads to a loss of HSPA2 chaperone activity and a subsequent perturbation of zona pellucida protein complex assembly.
Within the patient population levels of oxidative stress are, of course, variable (Aitken et al., 2010) and the suite of proteins that are usually targeted for adduction by cytotoxic aldehydes will vary as a consequence. Although this study did not seek to identify additional proteins or protein complexes that interact with 4HNE, this may be a useful approach to determine whether proteins involved in the initiation of sperm plasma membrane remodelling events may also be compromised by reactive aldehyde modification in the same manner as HSPA2. As 4HNE has been implicated in the inactivation of several key kinases, the modification of PKA or PKC activity by reactive aldehydes may also have downstream effects. Specifically, this may affect the phosphorylation status of key proteins involved in the activation of chaperone proteins, such as HSPA2, responsible for the remarkable membrane modification and protein reshuffling events that are crucial for the success of sperm– zona pellucida interaction (Lefievre et al., 2004).

In conclusion, this series of experiments has established a clear link between oxidative stress, impaired function of the SPAM1/ARSA/HSPA2 zona pellucida-receptor complex in human spermatozoa and a severely reduced ability to recognize and adhere to the zona pellucida. This suggests that the coordinated functions of both SPAM1 and ARSA

**Figure 9** Model of impaired zona pellucida interaction due to oxidative stress during sperm capacitation. Incubation of sperm in the presence of the reactive aldehyde 4HNE or H2O2 results in the production of mitochondrial and cytoplasmic ROS. This often leads to a loss of mitochondrial membrane potential, a loss of motility, lipid peroxidation and the production of further reactive aldehydes such as 4HNE. While this commonly leads to DNA damage, it can also lead to the modification of proteins through protein adduction that can alter their functions. The heat shock protein 70 family of chaperones are key targets for 4HNE adduction (Aitken et al., 2012a) and hence we propose in this model that adduction of HSPA2 by 4HNE may result in a loss of chaperone activity and a consequential inability to coordinate the presentation of zona pellucida receptors on the sperm surface in preparation for sperm– zona pellucida adhesion.
may play a vital role in the initial tethering of human spermatozoa to the zona pellucida. Moreover, we propose that the dysregulation of SPAM1 and ARSA complex assembly and/or presentation may be linked to a reduction in the chaperone function of HSPA2 owing to its modification by the cytotoxic lipid peroxidation product 4HNE (Fig. 9). Given that diminished HSPA2 expression in the sperm of infertile men has been causally linked to a loss of zona pellucida binding ability (Huszar et al., 2006; Redgrove et al., 2012), the implications of this work may extend to an increase in understanding of male factor infertility, particularly in patients that present with this particular defect. Certainly, this study suggests that the chaperone HSPA2 is sensitive to the effects of oxidative stress, highlighting an important direction for further study into a causative link between non-enzymatic post-translational modifications and a loss of the functional presence of HSPA2 in cases of male infertility.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles

E.G.B. contributed to study design, conducted the experiments and generated the manuscript. R.J.A. contributed to study conception and design, data interpretation and manuscript editing. A.L.A. provided technical assistance. E.A.M. contributed to study design and data interpretation and B.N. contributed to study design, data interpretation, manuscript preparation and editing.

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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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