Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation

Ryan Moazamian1,2,3, Ashley Polhemus1,2,3, Haley Connaughton1,2, Barbara Fraser1,2, Sara Whiting1,2, Parviz Gharagozloo3, and Robert John Aitken1,2,3,*

1Priority Research Centre in Reproductive Science, Discipline of Biological Sciences, Faculty of Science and IT, University of Newcastle, University Drive, Callaghan, New South Wales 2308, Australia 2Hunter Medical Research Institute (HMRI), Newcastle, New South Wales 2310, Australia 3CellOxess LLC, 15 Roszel Street, Princeton, NJ 08540, USA

*Correspondence address. Tel: +61-2-4985-4466; Fax: +61-2-4921-5669; E-mail: john.aitken@newcastle.edu.au

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ABSTRACT: Oxidative stress is known to compromise human sperm function and to activate the intrinsic apoptotic cascade in these cells. One of the key features of oxidatively stressed spermatozoa is the induction of a lipid peroxidation process that results in the formation of aldehydes potentially capable of disrupting sperm function through the formation of adducts with DNA and key proteins. In this study, we have examined the impact of a range of small molecular mass aldehydes generated as a consequence of lipid peroxidation on human sperm function and also compared the two most commonly formed compounds, 4-hydroxynonenal (4HNE) and malondialdehyde (MDA), for their relative ability to reflect a state of oxidative stress in these cells. Dramatic differences in the bioactivity of individual aldehydes were observed, that generally correlated with the second order rate constants describing their interaction with the model nucleophile, glutathione. Our results demonstrate that acrolein and 4HNE were the most reactive lipid aldehydes, inhibiting sperm motility while augmenting reactive oxygen species production, lipid peroxidation, oxidative DNA damage and caspase activation, in a dose-dependent manner (P < 0.001). In contrast, a variety of saturated aldehydes and the well-known marker of oxidative stress, MDA, were without effect on this cell type. While MDA was not cytotoxic per se, its generation did reflect the induction of oxidative stress in vivo and in vitro in a manner that was highly correlated with the bioactive lipid aldehyde, 4HNE. Despite such overall correlations, individual patient samples were observed in which either MDA or 4HNE predominated. Given the relative cytotoxicity of 4HNE, we propose that this aldehyde should be the preferred criterion for diagnosing oxidative stress in the male germ line.

Key words: human spermatozoa / lipid peroxidation / lipid aldehydes / oxidative stress

Introduction

Reactive oxygen species (ROS) are major byproducts of sperm metabolism that play critical roles in the physiological regulation of sperm capacitation and the pathological induction of DNA damage and impaired fertilizing potential (Aitken et al., 1995, 1998b; Aitken and Curry, 2011). From a physiological perspective, ROS have been implicated in the tyrosine phosphorylation events associated with the attainment of a capacitated state, as a consequence of their ability to inhibit tyrosine phosphatase activity and induce cAMP generation (Aitken, 1997; Leclerc et al., 1997; Aitken et al., 1998a, b; Lewis and Aitken, 2001; O’Flaherty et al., 2006; Awda and Buhr, 2010; Aitken and Curry, 2011; Donà et al., 2011). In addition, ROS are thought to play key roles in inducing cholesterol oxidation during capacitation, facilitating sterol efflux from the sperm plasma membrane and enhancing plasma membrane fluidity (Aitken 2011; Brouwers et al., 2011).

The continued generation of ROS by capacitating spermatozoa eventually leads to a state of cell senescence characterized by high levels of oxidative damage to the spermatozoa and is accompanied by accelerated mitochondrial ROS generation, as these cells enter a truncated version of the intrinsic apoptotic cascade (Koppers et al., 2011). One consequence of the high levels of ROS generation observed in stressed populations of mammalian spermatozoa is the induction of lipid peroxidation cascades that culminate in the formation of electrophilic lipid aldehydes such as 4-hydroxynonenal (4HNE), malondialdehyde (MDA) and acrolein (ACR) (Jones et al., 1979; Aitken et al., 1989, 1993a, b; Rao et al., 1989). These electrophiles increase the oxidative stress experienced by spermatozoa through their capacity to bind covalently to the
nucleophilic centers of vulnerable proteins, such as succinic acid dehydrogenase, in the mitochondrial electron transport chain (ETC) (Aitken et al., 2012, 2013). The addition of these proteins leads to the disruption of mitochondrial electron transport, resulting in an efflux of electrons that subsequently become associated with the universal electron acceptor, oxygen, generating superoxide anion (Aitken et al., 2013).

As a result of this self-perpetuating redox cycle, any factor that initiates oxidative stress in spermatozoa (including low levels of antioxidant protection or exposure to ROS as a consequence of cigarette smoking, obesity, infection or inadvertent exposure to UV radiation or transition metals) will, via the generation of lipid aldehydes, lead to the sustained generation of mitochondrial ROS and the induction of high levels of oxidative damage in these cells (de Lamirande and Lamothe, 2009; Aitken et al., 2013, 2014a, b; Amaral et al., 2013). Spermatozoa are particularly vulnerable to lipid peroxidation because they are richly endowed with polyunsaturated fatty acids, which are highly susceptible to free radical attack (Aitken et al., 2006, 2007; Wathes et al., 2007; Koppers et al., 2010). The lipid peroxidation pathway initiated under such circumstances is extremely complex, generating a wide range of lipid aldehydes. Studies of lipid peroxidation in spermatozoa have variously used MDA or 4HNE as biomarkers for this process. However, no systematic studies have been conducted to determine which lipid aldehyde species are the most damaging to human spermatozoa or which are the most sensitive indicators of oxidative stress in these cells from a diagnostic perspective. This study set out to address these limitations in our knowledge.

Materials and Methods

Reagents and solutions

All chemicals and reagents were obtained from Sigma-Aldrich (St Louise, MO, USA) unless otherwise stated, and all fluorescent probes were purchased from Invitrogen (Carlsbad, CA, USA). Disodium hydrogen phosphate was purchased from Merck (Darmstadt, Germany). The sperm incubation medium used throughout this study was BWW (Biggers et al., 1971) containing 1 mg/ml polyvinyl alcohol, 2.1 mg/ml sodium bicarbonate and 1 mg/ml glucose. Osmolarity was adjusted to achieve a value of 290–310 mOsm/kg. For certain experiments sodium bicarbonate was omitted from the medium and the osmolarity was adjusted to the above range with sodium chloride. For certain experiments sodium bicarbonate was omitted from the medium and the osmolarity was adjusted to the above range with sodium chloride. The major aldehydes used in these studies included MDA, ACR (Fluka, Buchs, Switzerland) and 4HNE (Cayman Chemicals, Ann Arbor, MI), although additional aldehydes (2,4-heptadienal, 2-octenal, valeraldehyde, hexanal; Sigma-Aldrich) were used in the structure activity relationship (SAR) analysis. Log P values for these aldehydes were accessed from SciFinder (Chemical Abstracts Service: Columbus, OH, 2006) and calculated using ACD/Labs software, version 8.14; ACD/Labs 1994–2006.

Preparation of human spermatozoa

Human semen samples were obtained from donors with their written, informed consent under the auspices of Institutional and State Government ethical approval. Most samples were from males of unknown fertility status and were members of our university donor panel. In addition, where indicated, these samples were supplemented with 30 unselected, consecutive samples obtained from a local assisted conception program, to determine the diagnostic significance of different aldehydes in a clinical population. All samples were donated following 48 h abstinence and provided to laboratory staff within 1 h of donation. Liquefaction was allowed to occur over at least 30 min before the spermatozoa were fractionated on a discontinuous two-step Percoll gradient (80%/40%) as described (Aitken et al., 2003).

Flow cytometry measurements

Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) with a 488-nm argon ion laser. Emission measurements were made using 530/30 band pass (green/FL-1), 585/42 band pass (red/FL-2) and > 670 long pass (far red/FL-3) filters. Forward scatter and side scatter measurements were used to generate a scatter plot for gating sperm cells only, measuring 10 000 events per sample while excluding any excess contaminating cells. FACSDiva software (BD Biosciences) was used to collect and analyse all data, including gating for sperm populations. For measuring cytoplasmic ROS, mitochondrial ROS and cell viability, dihydroethidium (DHE), MitoSOX™ Red (MSR) and SYTOX Green were used, respectively, as described (Aitken et al., 2013) and the results were expressed as the percentage of live, positive cells. This focus on live cells was essential because commercial preparations of MitoSOX™ red and DHE are contaminated with small amounts of the parent ethidium compound which can breach the plasma membrane of dead cells and stain the nuclei, falsely suggesting the generation of ROS. For the MSR and DHE measurements, a positive control was included in each run by adding 50 μM arachidonic acid (Aitken et al., 2013).

BODIPY C11 was used as a measure of lipid peroxidation (Aitken et al., 2007) and the analysis was again confined to live cells, since the assay depends on the active oxidation of the probe, which can only occur in metabolically active, viable spermatozoa. In this case, LIVE/DEAD fixable dead cell stain (Life Technologies, Gaithersburg, MD, USA) was used to detect cell viability simultaneously, and the results of the flow cytometry assays were presented as the percentage of live cells generating a positive BODIPY C11 signal.

Fluorochrome-labeled inhibitor of caspase activity (FLICA; Immuno-Chemistry Technologies, Bloomington, MN, USA) was used to assess apoptosis (Koppers et al., 2011) while the OxyDNA Assay (Calbiochem, La Jolla, CA, USA) was deployed as a measure of oxidative DNA damage (Aitken et al., 2010). Anti-4HNE antibody (Jomar Diagnostics, Adelaide, SA, Australia) and anti-MDA antibody (Abcam, Cambridge, UK) were used to measure 4HNE/MDA levels within spermatozoa. For the 4HNE and MDA assays, spermatozoa were incubated for 30 min at 37°C with anti-4HNE (1:50) or anti-MDA (1:100), with dilutions made in BWW. The spermatozoa were then incubated for 10 min at 37°C in the secondary antibody, AlexaFluor 488 goat anti-rabbit IgG (1:200) and washed twice before being resuspended in 200 μl BWW and analysed by flow cytometry. For the FLICA, aldehyde and OxyDNA assays, results are expressed as the total percentage of positive cells because cells rapidly transition from live to dead as the levels of caspase activation, aldehyde generation and oxidative DNA damage increase.

Gel electrophoresis and western blotting

Protein extraction was performed with sodium dodecyl sulfate (SDS) extraction buffer (20% SDS, 10% sucrose in 0.1875 M Tris, pH 6.8) containing protease inhibitor (Roche Applied Science) at 100°C for 5 min. For conventional 1D western blotting, the primary antibodies used were anti-4HNE (1:500) and anti-MDA (1:1000), diluted in 1% skim milk and incubated at 4°C overnight. The secondary antibody used was HRP-labeled goat anti-rabbit (1:1000), diluted in 1% skim milk, while the loading control was a mouse anti-alpha tubulin antibody diluted 1:4000 in 1% BSA. All incubations were for 1 h at room temperature while respective blocking solutions (5% skim milk/BSA) were administered for 1 h at 4°C before all primary and secondary antibody incubations.
**Immunocytochemistry**

Sperm were fixed in 2% paraformaldehyde for 15 min at 4°C and settled on poly-L-lysine coated coverslips. Cells were blocked for 1 h with 3% BSA-PBS + 10% goat serum. Cells were then probed with a 1:50 dilution of anti-4HNE, anti-MDA and anti-ACR (Novus Biologicals, Littleton, CO, USA) in 1% BSA-PBS overnight at 4°C. All slides were stained with goat-anti-rabbit Alexa fluor 488 secondary antibody (1/400) and counterstained for DNA with 4′,6-diamidino-2-phenylindole (1:2000) for 10 min, after which they were mounted with MOWIOL solution and images were captured on an Olympus Fluoview FV10i Confocal microscope.

**Experimental determination of second order rate constants**

In order to determine the reactivity of selected aldehydes with the model nucleophile, glutathione (GSH), 150 μl of 5,5′-dithiobis-2-nitrobenzoic acid (2 M in phosphate buffer, pH 7.6) was added to each well of a 96-well plate. Equal volumes of the GSH (0.5 mM in phosphate buffer, pH 7.6) and aldehyde solutions (0.625–25 mM in phosphate buffer pH 7.6) were combined and mixed at t = 0. At t = 0, and at each subsequent time point, 3 × 150 μl of the reaction mixture was added to each of three wells of the 96-well plate. The absorbance was immediately read at 405 nm. The absorbance values were then used to calculate the second order reaction rate constant for each compound, as per Bohme et al. (2010), by using a curve fitting approach based on a least sum of squares. Each reaction was conducted three times at 24°C.

**Statistics**

All experiments were replicated at least three times on independent samples. Datasets were analysed by ANOVA using the JMP11 program (SAS Institute, Raleigh, NC, USA) on a DELL XPS computer with Tukey–Kramer honest significant difference post hoc comparison of group means. Differences with a P-value of < 0.05% were regarded as significant.

**Results**

**Lipid aldehydes and mitochondrial ROS production in human spermatozoa**

An analysis of the impact of lipid aldehydes (MDA, 4HNE and ACR) on sperm motility, vitality and mitochondrial free radical generation revealed dramatic differences between these electrophiles in terms of their levels of bioreactivity. MDA, frequently reported as a marker of peroxidative damage (Jones et al., 1979; Alvarez et al., 1987; Fraczek et al., 2014), surprisingly exhibited no detectable cytotoxicity at doses as high as 200 μM. Moreover, MDA did not significantly influence either motility or mitochondrial ROS generation even when exposure was extended to 24 h (Fig. 1A–D). In contrast, both 4HNE and ACR induced highly significant

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**Figure 1** Impact of MDA on human sperm function after 2 and 24 h exposure. (A) Motility; (B) vitality measured with the Far Red Live/Dead stain; (C) cellular ROS generation in live cells measured using flow cytometry, with DHE as the probe for ROS and SYTOX green as the vitality stain; (D) mitochondrial ROS generation by live cells measured using flow cytometry with MitoSOX red (MSR) as the probe for ROS and SYTOX green as the vitality stain. Pos = positive control generated by exposing the same populations of spermatozoa to arachidonic acid (50 μM). n = 3 independent samples, except DHE where n = 7.
(\(P < 0.001\)) dose-dependent decreases in sperm motility and viability at physiological concentrations (Figs 2A and B, 3A and B). This was particularly evident with ACR, which triggered a significant (\(P < 0.001\)) loss of motility at all doses above 25 \(\mu\)M following just 2 h exposure (Fig. 3A). With 4HNE, motility was only significantly reduced after a 2 h exposure with the highest doses of aldehyde assessed (Fig. 2A), 100 \(\mu\)M (\(P < 0.05\)) and 200 \(\mu\)M (\(P < 0.01\)). After 24 h exposure both 4HNE and ACR induced highly significant declines in sperm motility (\(P < 0.001\)) at all doses above 12.5 \(\mu\)M (Figs 2A and 3A), which is well within the physiological range (Uchida, 2003). This loss of motility was followed by a loss of viability, which only became evident after 24 h exposure to both 4HNE (Fig. 2B) and ACR (Fig. 3B) but not MDA (Fig. 1B).

This differential ability of lipid aldehydes to induce motility loss and cell death was reflected in the impact of these aldehydes on ROS generation at both the cellular level (measured with DHE) and from the mitochondria (measured with MSR). Thus, while MDA had no impact on cellular or mitochondrial ROS generation, even after 24 h exposure (Fig. 1C and D), both 4HNE (Fig. 2C and D) and ACR (Fig. 3C and D) generated highly significant increases in ROS which were detectable with both DHE and MSR. In the case of 4HNE, a statistically significant response was only detected after 2 h when DHE was used as the probe in the presence of 200 \(\mu\)M aldehyde (Fig. 2C and D). However, after 24 h a highly significant dose–response was observed with 4HNE (\(P < 0.001\)) at both cellular and mitochondrial levels. ACR was even more effective than 4HNE, stimulating significant responses with both DHE and MSR within 2 h that were further amplified after 24 h, such that at this time point, all doses of ACR generated a statistically significant response with both probes (Fig. 3C and D).

**Lipid peroxidation, apoptosis and oxidative DNA damage**

In order to determine whether the apparent differences between aldehydes in their ability to compromise sperm function and activate ROS generation were reflected in their capacity to stimulate lipid peroxidation, the fluorescent probe BODIPY C11 was used. Oxidation of the polyunsaturated butadienyl portion of this dye results in a shift of the fluorescence emission peak from \(\approx 590\) nm to \(\approx 510\) nm. When this probe was loaded into spermatozoa and they were then exposed to MDA, no change in the oxidation status of the dye was evident, even after 24 h (Fig. 4A). In contrast, both 4HNE and ACR induced lipid peroxidation, which in the case of the former was statistically significant after 2 h at the highest dose tested (200 \(\mu\)M) but was evident with both aldehydes after 24 h (Fig. 4B and C).

This general pattern was repeated when another barometer of oxidative stress in spermatozoa was assessed, the induction of oxidative DNA damage.
damage to generate 8-hydroxy-2′-deoxyguanosine (8OHdG) adducts (Valavanidis et al., 2009). In this case, there was little change in the 8OHdG levels following exposure to MDA (Fig. 5A), however, both ACR and 4HNE elicited a significant dose-dependent 8OHdG response after 24 h ($P < 0.001$; Fig. 5B and C).

Since oxidative stress has been associated with the induction of apoptosis in spermatozoa (Koppers et al., 2011), the relative ability of MDA, 4HNE and ACR to trigger an intrinsic apoptotic response in human spermatozoa was investigated using the FLICA assay for caspase activation as an end-point (Fig. 6A–C). In accordance with the lipid peroxidation data, a dose-dependent increase in caspase activation was observed with both 4HNE and ACR, which was evident within 2 h ($P < 0.001$) with 4HNE and with both ACR ($P < 0.001$) and 4HNE ($P < 0.001$) after 24 h (Fig. 6B and C). On the contrary, MDA treatment had no significant effect on caspase activation within spermatozoa (Fig. 6A).

### Causative relationship between aldehyde induced oxidative stress and motility loss

In order to determine whether the induction of oxidative stress with 4HNE and ACR was directly responsible for the loss of motility presented in Figs 2 and 3, the bioreactivity of these aldehydes was compared in the presence and absence of bicarbonate ion. The rationale behind this experiment is that ROS generation by mammalian spermatozoa is known to be dependent on the presence of extracellular bicarbonate in the medium (Aitken et al., 1998a; Ecroyd et al., 2003). Thus to elucidate whether the motility loss associated with ACR and 4HNE exposure was mediated by, or simply correlated with, ROS generation, spermatozoa were exposed to an immobilizing dose of aldehyde (200 $\mu$M) for 2 h in the presence and absence of bicarbonate (Fig. 7). Under these circumstances, the addition of 4HNE in the presence of bicarbonate induced a highly significant burst of mitochondrial ROS (Fig. 7A) and completely immobilized the spermatozoa (Fig. 7B). In contrast, in the absence of extracellular bicarbonate, no significant increase in mitochondrial ROS was observed (Fig. 7A) and yet the spermatozoa still lost their motility (Fig. 7B). The induction of ROS generation by aldehydes such as 4HNE is therefore not directly responsible for the loss of sperm motility but rather a reflection of their alkylating activity, as discussed below.

### SAR analysis of aldehydes

In light of the major differences between lipid aldehydes in their capacity to trigger motility loss and mitochondrial ROS generation, we extended the range of aldehydes assessed in this study in order to generate...
pertinent SAR data. For this purpose, we selected a range of aldehydes that are known to be generated in cells under conditions of oxidative stress (valeraldehyde, hexanal, 2,4-heptadienal and 2-octenal; Domínguez et al., 2013). Dose-dependent analyses of these compounds revealed that neither of the saturated aldehydes, valeraldehyde and hexanal, had any significant impact on sperm motility or mitochondrial ROS generation (Fig. 8A–D). In contrast, 2,4-heptadienal induced a significant ($P < 0.001$) increase in ROS generation at the highest dose tested (200 μM) after 2 h which was further accentuated after 24 h incubation, generating significant increases in ROS at all doses above 25 μM (Fig. 8F). This increase in ROS generation was accompanied by a dramatic loss of sperm motility after 24 h, which was also significant at all doses above 25 μM (Fig. 8E). Furthermore, 2-octenal generated an increase in mitochondrial ROS generation after 24 h incubation, which was statistically significant ($P = 0.001$) at the highest doses tested (100 and 200 μM), and associated with a loss of motility at 200 μM ($P < 0.05$;
range of values that generally mirrored their bioreactivity. Thus, the fastest reaction rate was observed with the most reactive aldehyde, ACR, resulting in a second order rate constant of $3063.9 \pm 144.2 \text{ l mol}^{-1} \text{ min}^{-1}$, with the variance around this measurement reflecting the speed of the reaction and the attendant difficulty in making accurate measurements (Table I). The next most reactive aldehyde, 4HNE, exhibited a second order rate constant ($125.3 \pm 3.3 \text{ l mol}^{-1} \text{ min}^{-1}$) which was a log order less than ACR. The next most reactive compounds, 2-octenal ($16.6 \pm 0.5 \text{ l mol}^{-1} \text{ min}^{-1}$) and 2,4-heptadienal ($7.0 \pm 0.7 \text{ l mol}^{-1} \text{ min}^{-1}$) gave second order rate constants that were a log order lower again, while the remaining aldehydes (MDA, valeraldehyde and hexanal) did not react with GSH under the conditions of our assay, in keeping with their lack of bioreactivity. The fact that 2,4-heptadienal had a slightly lower second order rate constant than 2-octenal and yet appeared to have a higher impact on ROS production, could not be explained by its log $P$-value (reflective of lipophilicity and hence a compound’s capacity to cross the plasma membrane into the interior of the cell) which was actually lower than that of 2-octenal (Table I).

## Protein adduction of lipid aldehydes

Given the apparent differences in chemoreactivity between aldehydes as exemplified by MDA and 4HNE, we next sought to examine whether such disparate reactivities were correlated with differences in the patterns of proteins alkylated by these compounds. A representative western blot is shown in Fig. 9. This analysis revealed a marked difference between these aldehydes in terms of their protein alkylation profiles. The donors featured in this analysis all revealed very similar patterns of spontaneous protein alkylation when probed with antibodies against specific aldehydes. With 4HNE, there were multiple protein adducts formed ranging from 180 to 34 kDa in size (Fig. 9). Several proteins were also alkylated with MDA; however, the pattern was different with a majority of the binding activity focused on a major protein with a molecular mass 75 kDa (Fig. 9).

These differences in alkylation pattern were associated with changes in the subcellular localization and intensity of spontaneous signals generated following immunocytochemical analysis of the major aldehyde species in these cells. Thus an anti-ACR antibody gave extremely bright signals in the sperm midpiece, where the mitochondria are housed and a more variable signal in the sperm acrosome (Fig. 10A and B). Probing spermatozoa with anti-4HNE gave a similar pattern of localization, suggesting that proteins vulnerable to alkylation by this aldehyde are located in the mitochondria and acrosome (Fig. 10C and D). Staining with an anti-MDA antibody generated a much weaker immunocytochemical signal although the sperm midpiece was again identified as a major site of aldehyde expression, frequently accompanied by staining of the post-acrosomal region of the sperm head (Fig. 10E and F).

## Relationship between MDA and 4HNE as markers of oxidative stress

Given the above differences between the two most commonly monitored lipid aldehydes (MDA and 4HNE) in terms of both the rate constants that characterize their interactions with proteins and their patterns of protein alkylation, it was of interest to know whether the diagnostic information generated by the analysis of these compounds was correlated. To achieve this end, spermatozoa from both the low- and high-density regions of discontinuous colloidal silicon gradients were analyzed for their ability to trigger apoptosis in human spermatozoa using the FLICA assay (ImmunoChemistry Technologies, Bloomington, MN, USA) to measure pan caspase activation. (A) Dose-dependent study with MDA; (B) dose-dependent study with 4HNE; (C) dose-dependent study with ACR. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 4$ independent samples for ACR and $n = 5$ independent samples for MDA and 4HNE.

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**Figure 6** Analysis of the ability of lipid aldehydes to trigger apoptosis in human spermatozoa using the FLICA assay (ImmunoChemistry Technologies, Bloomington, MN, USA) to measure pan caspase activation. (A) Dose-dependent study with MDA; (B) dose-dependent study with 4HNE; (C) dose-dependent study with ACR. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 4$ independent samples for ACR and $n = 5$ independent samples for MDA and 4HNE.
assessed by flow cytometry to determine the relative distribution of MDA and 4HNE positivity. This analysis involved 30 unselected patients from a local infertility clinic and revealed a close relationship between these readouts of peroxidative damage. Thus, both MDA and 4HNE were able to detect the known differences in oxidative stress between high- and low-density Percoll fractions (Aitken et al., 2007), successfully identifying the lower levels of peroxidative damage that characterize the high-density sperm populations (P, 0.001; Fig. 11A and B). Furthermore, across the entire dataset, a high degree of correlation was evident (r = 0.797) in the proportion of cells that were positive for 4HNE and MDA (Fig. 11C) although occasional deviations from this proportional relationship were observed (Fig. 11C, arrowed).

In order to confirm the close relationship between 4HNE and MDA in reflecting peroxidative damage to spermatozoa, these cells were challenged with increasing doses of H2O2 and the levels of 4HNE and MDA formed within a 3 h exposure period, were assessed (Fig. 11D). The results of this analysis revealed an initial slight decline in lipid aldehyde levels at low doses of H2O2 up to 0.5 mM; however, once the antioxidant protective mechanisms of these cells had been overwhelmed, the percentage of cells positive for 4HNE and MDA rose rapidly. Importantly, there was no significant difference between the levels of peroxidative damage recorded regardless of whether 4HNE or MDA was used as the end-point (Fig. 11D), indicating that while these aldehydes may be very different in terms of their cytotoxicity, they are generally similar in their ability to reflect levels of oxidative stress in the male germ line.

**Discussion**

Human spermatozoa, despite their tightly compacted chromatin, are highly susceptible to oxidative insult with the concomitant induction of lipid peroxidation and DNA damage (Aitken and Curry, 2011). Various environmental and pathophysiological factors initiate the oxidative stress cycle in spermatozoa featuring the breakdown of lipids, the formation of aldehydes, the stimulation of ROS formation by the mitochondria and the subsequent perpetuation of a pathway that ultimately leads to apoptosis (Koppers et al., 2011). The susceptibility of mammalian spermatozoa to lipid peroxidation has been appreciated since the pioneering studies of Thaddeus Mann et al. at the University of Cambridge in the 1970s (Jones et al., 1978, 1979), however, the biological activity of specific lipid aldehydes and their relative merits as indicators of oxidative stress in the male germ line have not been investigated.

Formation of small molecular mass alkenals such as MDA, 4HNE and ACR occur through the abstraction of hydrogen atoms from bis-allylic sites on polyunsaturated fatty acids (Aitken et al., 1993b, 2014b; Yin et al., 2011). The hydrogen abstraction results in the formation of a lipid radical, which then reacts with ground state oxygen to generate a peroxy radical which, to stabilize, abstracts hydrogen atoms from adjacent lipids to create the corresponding hydroperoxide, thereby propagating the lipid peroxidation cycle. Polyunsaturated fatty acids are highly susceptible to peroxy radical attack because the presence of double bonds lowers the bond-dissociation energy of the allylic hydrogen atom in the methylene bridge situated between two contiguous ethylenic double bonds. Such low-bond dissociation energies favor the hydrogen abstraction process and, as a result, polyunsaturated fatty acids, such as docosahexaenoic acid and arachidonic acid, are the main precursors for small molecular mass electrophilic lipid aldehydes (Yin et al., 2011).

Male infertility is a common clinical condition that affects at least 1 in 20 of the male population (McLachlan and de Kretser, 2001) and is known to involve excess free radical generation and the induction of lipid peroxidation (Aitken and Clarkson, 1987; Aitken et al., 1993a, b; Suleiman et al., 1996; Tavilani et al., 2005). Traditionally, MDA has been used as a diagnostic criterion for oxidative damage to spermatozoa (Jones et al., 1979; Alvarez et al., 1987; Aitken et al., 1989; Gomez et al., 1998; Tavilani et al., 2005). Experiment designed to demonstrate that the loss of motility with aldehydes such as 4HNE is not dependent on mitochondrial ROS generation. (A) 4HNE stimulates a significant increase in mitochondrial ROS generation, which is inhibited if bicarbonate is omitted from the incubation medium (Aitken et al., 1998a); (B) despite the suppression of mitochondrial ROS production, motility is still compromised with 4HNE, suggesting that this biological effect is not mediated by ROS but due to the direct adduction of proteins involved in motility expression. ***P < 0.001; n = 3 independent samples.
Although 4HNE has recently been used to monitor lipid peroxidation in these cells (Aitken et al., 2012), no studies have yet been performed to determine how these lipid aldehydes compare in terms of their biological potency and diagnostic relevance. This study set out to address this question.

Since MDA is a by-product of lipid peroxidation, it has long been assumed that this aldehyde is a good marker of lipid peroxidative damage. Our findings reveal that MDA is in fact an unreactive lipid aldehyde that has little impact on the biological behavior of spermatozoa with exposure levels that are within and even above the physiological range.

**Figure 8** A range of additional aldehydes that are typically generated during oxidative stress were also tested for their ability to suppress motility and activate mitochondrial ROS generation in live cells after 2 and 24 h exposure. (A and B) Valeraldehyde; (C and D) hexanal; (E and F) 2-4 heptadienal and (G and H) 2-octenal. *p < 0.05; **p < 0.01; ***p < 0.001; n = 3 independent samples.
In contrast to MDA, 4HNE primarily exists as an α,β-unsaturated aldehyde readily capable of Michael addition reactions. These structural differences between 4HNE and MDA were reflected in the western blot and immunocytochemical analyses, both of which indicated that 4HNE is more reactive than MDA. Thus, 4HNE bound to a wider variety of proteins compared with MDA in human spermatozoa (Fig. 9) and exhibited a more intense staining pattern with particular emphasis on the sperm midpiece, where the mitochondria are located. Previous studies have observed that 4HNE binds to succinic acid dehydrogenase in the mitochondria and have postulated that this action perturbs the flow of electrons in the mitochondrial ETC, resulting in electron leakage and superoxide anion production (Aitken et al., 2012). As a consequence of this ability of 4HNE to form adducts with mitochondrial proteins, oxidative stress becomes a self-propagating cycle in spermatozoa; lipid peroxidation results in the formation of lipid aldehydes that bind to proteins in the mitochondrial ETC, stimulating the generation of yet more mitochondrial ROS, peroxidation and lipid aldehyde formation. The cellular damage induced by this lipid peroxidation cascade may involve a direct attack by ROS, as in the induction of oxidative DNA damage (Fig. 5) or the direct alkylation of proteins important for sperm function. One clear example of the latter is motility loss, which can be induced by 4HNE even under conditions where mitochondrial ROS production is impaired (Fig. 7), suggesting that oxidative stress is not directly involved in the mediation of 4HNE-induced motility loss. Furthermore, a recent analysis of the ability of 4HNE to suppress sperm motility revealed that this inhibitory activity was not associated with any change in intracellular ATP and could not be reversed by axonemal exposure to ATP in a Triton X-demembranation-ATP reactivation motility model (Baker et al., 2015). Thus, 4HNE appears to render the motor apparatus of the sperm flagellum unresponsive to ATP via the collateral adduction of proteins associated with the axoneme. In this context, it should be remembered that alkylating agents have long been known to suppress sperm motility (Mann and Lutwak Mann, 1981) and that one of the sperm proteins recently found to be alkylated by 4HNE in a detailed proteomic screen, is dynein heavy chain, a major constituent of the sperm axoneme (Baker et al., 2015).

In general, the cytotoxicity of lipid aldehydes was found to be a function of their alkylating activity and correlated extremely well with the second order rate constants describing their interaction with the model nucleophile, GSH. The most reactive aldehyde in this context was the α,β-unsaturated aldehyde, ACR, which, as the strongest electrophile examined in this study, reacted extremely rapidly with GSH. ACR is a major toxic metabolite generated by the anticancer drug, cyclophosphamide and may also be formed intracellularly by enzymatic oxidation of polyamine metabolites and/or as a consequence of the peroxidation of polyunsaturated fatty acids (Alarcon, 1970; Hales, 1982; Esterbauer et al., 1991). The cytotoxicity of this compound has been well documented with other mammalian cell types (Krokan et al., 1985) although this is the first detailed report of ACR toxicity in spermatozoa. Given the high concentrations of polyamines in the male reproductive tract (Lefèvre et al., 2011), ACR toxicity may well be a physiological contributor to impaired sperm viability in vivo. In this context, the recent description of a sperm surface aldo-keto reductase (AKR1B7) on the surface of mammalian spermatozoa with a demonstrable capacity to detoxify ACR, may be highly germane (Jagoe et al., 2013).

The remaining aldehydes tested exhibited levels of bioactivity that correlated well with the second order rate constant data. The saturated

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**Table I** Second order rate constants and log P-values for compounds assessed in this study.

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Second order rate constant, 1 mol⁻¹ min⁻¹</th>
<th>log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrolein</td>
<td>3063.9 ± 144.2</td>
<td>0.263 ± 0.283</td>
</tr>
<tr>
<td>4-Hydroxynonenal</td>
<td>125.3 ± 3.3</td>
<td>1.897 ± 0.320</td>
</tr>
<tr>
<td>2-Octenal</td>
<td>16.6 ± 0.5</td>
<td>2.809 ± 0.282</td>
</tr>
<tr>
<td>2,4-Heptadienal</td>
<td>7.0 ± 0.7</td>
<td>1.891 ± 0.290</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>Unreactive</td>
<td>−0.987 ± 0.272</td>
</tr>
<tr>
<td>Valeraldehyde</td>
<td>Unreactive</td>
<td>1.423 ± 0.222</td>
</tr>
<tr>
<td>Hexanal</td>
<td>Unreactive</td>
<td>1.932 ± 0.223</td>
</tr>
</tbody>
</table>

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**Figure 9** Western blot analysis of the proteins spontaneously adducted in populations of human spermatozoa recovered from the high-density region of Percoll gradients using antibodies against 4HNE and MDA. The loading control was α-tubulin; n = 3 independent samples.

This does not mean that MDA is without cytotoxicity, as its cytotoxic effect has been detected in dose-dependent studies conducted with human fibroblasts (Michiels and Remacle, 1991). However, as far as spermatozoa are concerned, MDA appears to be relatively benign. The reason for this is that the tautomeric MDA molecule predominately stabilizes in the enolate form at physiological pH, thus becoming less reactive (Esterbauer et al., 1991). At acidic pH, however, MDA adopts an α,β-unsaturated aldehyde structure, becoming a powerful electrophile engaging in Michael addition reactions with polyunsaturated fatty acids. Within the acidic microenvironment of the sperm acrosome (a lysosome-like vesicle), it is possible that MDA exists mainly in this more reactive form but, at the neutral pH-values that pervade the rest of the cell, MDA does not interact effectively with spermatozoa, in contradiction to the common assumption (e.g. Hsieh et al., 2006).
aldehydes, hexanal and valeraldehyde were lacking in significant bioactivity under the conditions tested (Fig. 8) and lacked any detectable capacity to alkylate GSH. The remaining aldehydes, 2,4-heptadienal and 2-octenal, were capable of interacting with GSH in vitro and both compounds exhibited a limited capacity to impair motility and stimulate mitochondrial ROS generation by human spermatozoa. However, their relative bioactivities were out of step with their GSH reactivity; 2-octenal is the more reactive with GSH while 2,4-heptadienal had a more

Figure 10 Immunocytochemical analysis to determine the localization of proteins spontaneously adducted by lipid aldehydes in human spermatozoa incubated for 24 h at 37°C. (A and B) Stained with anti-ACR antibody; (C and D) stained with anti-4HNE antibody and (E and F) stained with anti-MDA antibody. Representative images shown. Scale bar = 20 μM.
profound impact on sperm mitochondrial ROS production (Fig. 8). Such results emphasize that there are additional attributes of these compounds, in addition to their electrophilicity, that define their ultimate cytotoxicity towards spermatozoa. Relative hydrophobicity is clearly an important property in this context since it will determine the ability of these compounds to cross the sperm plasma membrane and gain access to intracellular sites. However, examination of the log P-values for the compounds tested (Table I) in this study revealed that 2-octenal has a higher log P-value than 2,4-heptadienal and, as a result, should penetrate more readily into the intracellular space. Alternative explanations might be that 2-octenal is more readily metabolized by sperm associated aldehyde dehydrogenases; however, a detailed analysis of these enzymes in spermatozoa has not yet been conducted.

Notwithstanding the observed differences between individual lipid aldehydes in their cytotoxicity toward spermatozoa in vitro, in vivo these aldehydes will, of course, be generated as complex mixtures. Under these circumstances, it is of importance to know how well the generation of individual aldehydes is correlated under conditions of oxidative stress. In order to address this point, we took the two most commonly used end-points of lipid peroxidation, MDA and 4HNE and compared them in clinical samples. Our results indicate that even though these aldehydes have very different cytotoxicities, their production is highly coordinated. As a result, both these compounds were found to reflect the oxidative stress observed in subpopulations of poor-quality spermatozoa recovered from the low density region of Percoll gradients (Fig. 11A and B). Similarly, both aldehydes responded in a generally coordinated manner to an exogenous oxidative stress in the form of H2O2 (Fig. 11D). Finally, the spontaneous levels of MDA and 4HNE recorded in individual sperm populations were highly correlated (r = 0.797; P < 0.001; n = 60; Fig. 11C). Of course, as with any biological system, there are outliers on either side of the regression line with individual samples generating a relative excess of either MDA or 4HNE (Fig. 11C), so clearly these markers of lipid peroxidation are not completely interchangeable. Since only 4HNE is cytotoxic, it would appear to be the peroxidation criterion of choice when diagnosing the significance of oxidative stress in the etiology of defective sperm function and male infertility.

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Authors’ roles

R.M. and A.P. contributed to the study design, conducted the majority of the studies and generated the first draft of the manuscript. H.C. and S.W. assisted with the technical aspects of the study while B.F. performed the analysis of second order rate constants and contributed to data interpretation. R.J.A. conceived and designed the study, participated in data interpretation and edited the manuscript; P.G. contributed to the study design and edited the manuscript.
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Conflict of interest
None declared.

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