

The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1

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Summary

DNA repair has long been considered impossible in human spermatozoa due to the high level of DNA compaction observed in these cells. However, detailed examination of the base excision repair pathway in human spermatozoa has revealed the presence of an enzyme critical to this pathway, 8-oxoguanine DNA glycosylase 1 (OGG1). This glycosylase was associated with the sperm nucleus and mitochondria and could actively excise 8-hydroxy-2'-deoxyguanosine (8OHdG), releasing this adduct into the extracellular space. This activity was significantly reduced in the presence of cadmium (II), a recognized inhibitor of OGG1, in a time- and dose-dependent manner ($P < 0.001$). Remarkably, spermatozoa do not possess the downstream components of the base excision repair pathway, apurinic endonuclease 1 (APE1) and X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1). The absence of these proteins was particularly significant, as APE1 is required to create a 3'-hydroxyl (3'-OH) terminus at the apurinic site created by OGG1, which would be recognized by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. As a result, TUNEL was unable to detect oxidatively induced DNA damage in spermatozoa following exposure to hydrogen peroxide. In the same cells, intracellular and extracellular 8OHdG could be clearly detected in a manner that was highly correlated with the outcome of the sperm chromatin structure assay (SCSA). However, incubation of these cells for 48 hours revealed a time-dependent increase in TUNEL positivity, suggesting the perimortem activation of a nuclease. These results emphasize the limited capacity of mature spermatozoa to mount a DNA repair response to oxidative stress, and highlight the importance of such mechanisms in the oocyte in order to protect the embryo from paternally mediated genetic damage.

Key words: Human spermatozoa, OGG1, DNA damage, Oxidative stress

Introduction

A high level of DNA damage in the male germ line is a hallmark of human infertility. The clinical consequences of such a loss of genetic integrity can be found in the associations that have been observed between DNA damage in human spermatozoa and a wide range of adverse reproductive outcomes including poor rates of fertilization, impaired embryonic development, an increased incidence of miscarriage and significant morbidity in the offspring, including cancer (Aitken et al., 2009; Zini et al., 2008). Although the detailed aetiology of this damage is still unresolved, it has been established that such losses of DNA integrity are highly correlated with the efficiency of DNA compaction during the terminal stages of spermiogenesis and evidence of oxidative stress (De Iuliis et al., 2009). Of the four DNA bases, guanine is the most susceptible to oxidation by virtue of its low oxidation potential (Klungland and Bjelland, 2007). As a result, significant correlations have been observed between the levels of 8-hydroxy-2'-deoxyguanosine (8OHdG) expression by human spermatozoa and DNA fragmentation as recorded by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, as well as the expression of fertility *in vivo* (De Iuliis et al., 2009; Thomson et al., 2011). 8OHdG is considered to be a highly mutagenic DNA lesion by virtue of its propensity to

form a stable base pair with adenine, resulting in G:C→T:A transversion mutations following DNA replication, if the lesion escapes repair (Wood et al., 1992). It is therefore essential that 8OHdG lesions are removed from DNA-damaged spermatozoa lest these lesions subvert the normality of gene expression during embryonic development.

Unfortunately very little is known about DNA repair pathways in differentiating human spermatozoa other than that they are unlikely to exist. During spermiogenesis, sperm chromatin is extensively remodelled, with positively charged protamines replacing the histones as the spermatozoon's genetic material is packaged into toroid-like structures spanning ~50 kb linked by matrix attachment regions (Balhorn et al., 2000; Ward, 1993). This chromatin packaging arrangement approaches the physical limits of molecular compaction and is theoretically designed to protect the paternal genome from damage (Sawyer et al., 2003). However such high levels of compaction also place limits on the capacity of sperm chromatin to execute DNA repair. Mammalian cells are equipped with a highly coordinated base excision repair pathway for the removal of 8OHdG. 8-Oxoguanine DNA glycosylase 1 (OGG1) is a bifunctional *N*-glycosylase/DNA lyase involved in the excision of 8OHdG, the action of which results in an apurinic site (AP site) as well as a nick in the

phosphodiester backbone yielding a 3', α , β -unsaturated aldehyde and a 5'-deoxyribosephosphate. Apurinic endonuclease 1 (APE1) then cleaves the AP site to form a 3'-OH group adjacent to the 5'-deoxyribosephosphate in preparation for the insertion of a new guanine nucleotide by polymerase β . The phosphodiesterase activity of ligase III completes the base excision repair pathway by sealing the nick in the backbone (Mol et al., 2000). In the study presented herein, we have examined the integrity of this pathway in human spermatozoa and demonstrated, for the first time, the existence of a highly truncated OGG1 mediated base excision repair pathway in these cells, responsible for the creation of abasic sites.

Results

Human spermatozoa release 8OHdG into the surrounding medium upon exposure to H₂O₂ in a dose-dependent manner

The base excision repair pathway initially requires excision of the base adduct by OGG1, therefore we initiated this study by determining whether exposure of human spermatozoa to oxidative stress resulted in the release of 8OHdG into the extracellular space. Incubating human spermatozoa in the presence of H₂O₂ for 2 hours resulted in a dose-dependent increase in the percentage of cells containing 8OHdG ($P < 0.001$; Fig. 1A). Significantly, 8OHdG was also detected in the supernatant following incubation with H₂O₂ in a parallel dose-dependent manner ($P < 0.01$; Fig. 1B). The sperm chromatin structure assay (SCSA), which assesses the ability of sperm chromatin to resist acid denaturation, detected a concomitant increase in DNA damage in response to H₂O₂ (Fig. 1C). However oxidative stress was not accompanied by an increase in the percentage of cells containing 3'-OH termini on the phosphodiester backbone, as revealed by a lack of TUNEL

positivity within the 2-hour time frame of this study (Fig. 1D). Thus, human spermatozoa appear capable of responding to oxidative stress by eliminating 8OHdG, suggesting a capacity for base excision repair within the highly condensed chromatin structure typical of spermatozoa. However, the second step in this pathway, involving the generation of 3'-OH groups via the intervention of APE1 appeared to be missing (Fig. 1D).

OGG1 is present in human spermatozoa

To confirm the mechanism by which 8OHdG is released from spermatozoa, the presence of the principal enzyme involved in the removal of 8OHdG via the base excision repair pathway, OGG1, was examined using an antibody raised against this enzyme. In untreated spermatozoa OGG1 was detected in the midpiece, which houses the mitochondrial genome (Fig. 2A). However upon treatment with H₂O₂, OGG1 could be detected in the nucleus, as well as the midpiece (Fig. 2B). Since spermatozoa are transcriptionally and translationally silent, the sudden appearance of OGG1 in the sperm nucleus could not have been due to the synthesis of new enzyme in response to the oxidative stress induced in the presence of H₂O₂. Furthermore, the condensed chromatin structure in combination with the extremely compartmentalized architecture typical of spermatozoa would have prevented an intracellular translocation of OGG1 from the midpiece to the nucleus. We therefore hypothesized that this change in location was the result of improved penetration of OGG1 antibodies into the sperm nucleus as a result of chromatin-relaxing strand breaks associated with H₂O₂ exposure. In order to test this hypothesis strand breaks were artificially created within the sperm nucleus with DNase 1; following such treatment OGG1 was readily detected in the sperm nucleus (Fig. 2C). Western blot analysis also confirmed the presence of OGG1 in human spermatozoa with a band detected at the anticipated molecular mass of 38 kDa (Fig. 2D). In order to further confirm the presence of OGG1, this antibody was employed to isolate OGG1 from whole cell lysates via immunoprecipitation (Fig. 2E). Western blot confirmed that the immunoprecipitation had successfully isolated a 38 kDa protein recognised by the OGG1 antibody and this band was excised from the corresponding polyacrylamide gel, and thoroughly scrutinised by mass spectrometry. Peptides unique to OGG1 were obtained from the 38 kDa band covering 72.2% of the total sequence, including the peptide at the C terminus which confers nuclear localisation (Fig. 2F) (Nishioka et al., 1999). Finally, PCR analysis employing primers specific for OGG1 mRNA revealed clear signals for OGG1 variants 1a (the predominant somatic form) and 2a in human testes cDNA but no signal from sperm mRNA (Fig. 2G). This apparent lack of OGG1 message was postulated to be a consequence of the low levels of residual mRNA present in these cells as a consequence of their virtual lack of cytoplasm. We therefore used a nested PCR strategy, which involves further amplification of the original PCR product in order to compensate for low levels of mRNA, and succeeded in detecting clear bands for OGG1 variants 1a and 2a in high-quality, functional spermatozoa in 4 out of 5 donors (Fig. 2E). There was no detectable mRNA for transcript variants, OGG1b and OGG1c or OGG2b-OGG2e in the male germ line using both conventional and nested PCR strategies. Although the significance of residual mRNA transcripts in mature spermatozoa is unclear, it is evident from the foregoing that this enzyme is not only present in human spermatozoa but also functionally capable of exerting its glycosylase activity.

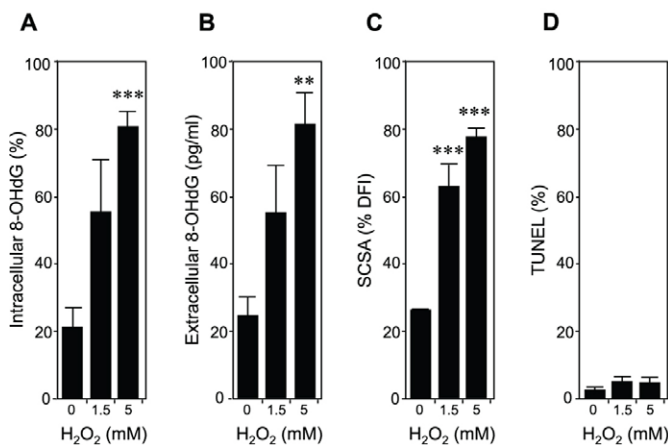


Fig. 1. Human spermatozoa release 8OHdG into the surrounding medium upon exposure to H₂O₂ in a dose-dependent manner. Human spermatozoa were treated with increasing doses of H₂O₂ for 2 hours. H₂O₂ treatment resulted in (A) a dose-dependent increase in intracellular 8OHdG and (B) a simultaneous increase in the concentration of free 8OHdG detected in the supernatant. (C) The treatment of spermatozoa with increasing concentrations of H₂O₂ decreased the ability of the chromatin to withstand acid denaturation measured by the SCSA. (D) No increase in the percentage of spermatozoa exhibiting DNA strand breaks with 3'-OH groups was detected with TUNEL. Analyses based on three independent semen samples. Error bars represent \pm s.e.m. ** $P < 0.01$; *** $P < 0.001$.

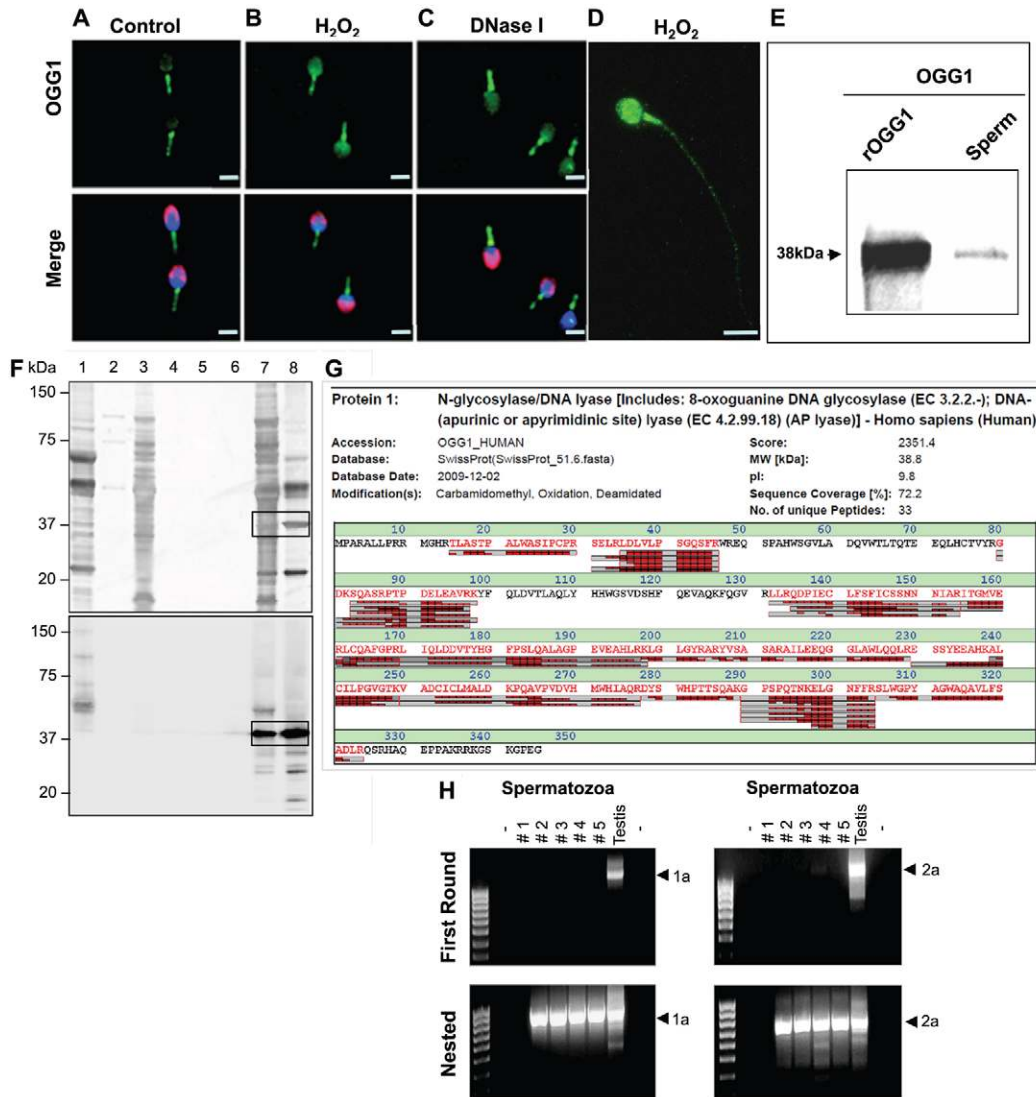


Fig. 2. OGG1 is present in human spermatozoa. (A–C) Upper panels: (A) Immunocytochemical localisation of OGG1 predominantly to the midpiece of human spermatozoa when no treatment was applied. (B) Immunocytochemical localisation of OGG1 to the midpiece and the nucleus of human spermatozoa when incubated in the presence of 2 mM H_2O_2 overnight. (C) Immunocytochemical localisation of OGG1 to the midpiece and nucleus of human spermatozoa when the chromatin was fragmented with DNase I. (A–C) Lower panels: the acrosome was counterstained with PNA (pink) and the nucleus counterstained with DAPI (blue). (D) Higher-magnification image of OGG1 localization in H_2O_2 -treated spermatozoa showing clear localization to the midpiece and nucleus. (E) Demonstration by western blot analysis that OGG1 is present in human spermatozoa and exhibits a molecular mass of ~38 kDa, matching recombinant OGG1 (rOGG1). (F) Silver stained polyacrylamide gel showing proteins precipitated using an antibody raised against OGG1. Lanes are as follows: 1, antibody only; 2, beads and lysate only; 3, sperm lysate post immunoprecipitation; 4–6, PBS washes; 7, sperm proteins eluted from the antibody-conjugated beads; 8, rOGG1 eluted from the antibody-conjugated beads. The box indicates the bands that were excised from the sperm and rOGG1 lanes for mass spectrometry analysis, which corresponded to the OGG1 band, as demonstrated by western blot analysis using an antibody raised against OGG1 (lower panel, box) (G) Mass spectrometry analysis of the 38 kDa band identified OGG1a in human spermatozoa. (H) Nested PCR analysis revealed the presence of OGG1a and OGG2a transcript variants in high-density spermatozoa, but not OGG1b,c or OGG2b-e. Experiments were performed in triplicate and representative images are shown. Scale bars: 5 μ m.

The downstream base excision repair enzymes APE1 and XRCC1 are not present in human spermatozoa

APE1, the enzyme responsible for creating 3'-OH termini adjacent to the AP sites generated by OGG1, was not detected in human spermatozoa using immunocytochemistry (Fig. 3A) or western blot analysis (Fig. 3B). Furthermore relieving the degree of chromatin compaction using H_2O_2 and DNase I as used previously for the detection of OGG1, failed to reveal a positive signal. Similarly, X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1), a scaffolding enzyme

involved in the stabilization of the base excision repair pathway was not detected in human spermatozoa using immunocytochemistry (Fig. 3C) or western blot (Fig. 3D) and again chromatin relaxation strategies failed to reveal a positive signal. These data therefore indicated that, unlike somatic cells, spermatozoa only possess an ability to remove the 8OHdG base adduct but lack the machinery required to repair oxidative DNA lesions beyond this first step. The lack of APE1 suggests why oxidative stress can create 8OHdG lesions and a concomitant signal in the SCSA assay but failed to generate a response in the

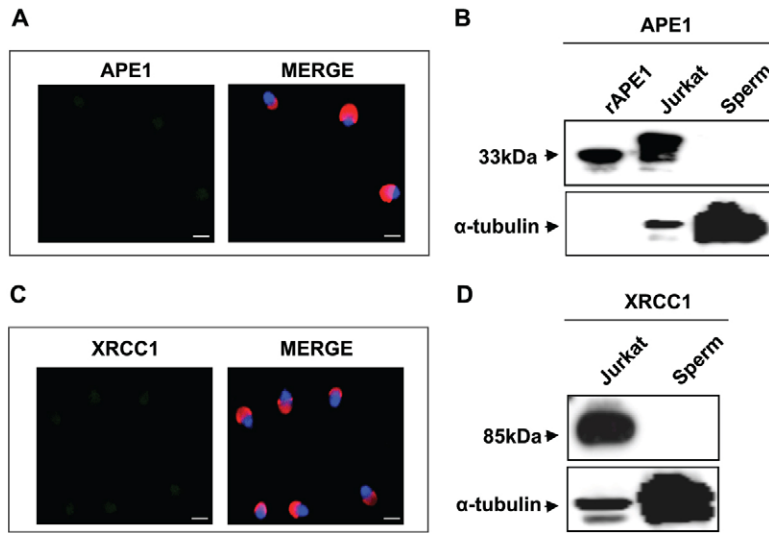


Fig. 3. The downstream base excision repair enzymes APE1 and XRCC1 are not present in human spermatozoa.

(A) Immunocytochemical analysis of human spermatozoa with antibodies raised against APE1 revealed that this base excision repair enzyme was not found in spermatozoa, regardless of the degree of chromatin fragmentation achieved by H_2O_2 and DNase 1 treatment. The acrosome was counterstained with PNA (pink) and the nuclear DNA counterstained with DAPI (blue). (B) Western blot analysis confirmed that APE1 was not expressed in human spermatozoa (lane 3); however it was detected at the correct molecular mass of 33 kDa in the recombinant APE1 positive control sample (lane 1) and a Jurkat whole cell lysate (lane 2). (C) Immunocytochemical analysis of human spermatozoa with antibodies raised against XRCC1 revealed that this scaffolding enzyme, which plays a role in the base excision repair pathway, was also not found in spermatozoa. (D) Western blot analysis confirmed that XRCC1 was not expressed in human spermatozoa (lane 2); however, it was detected at the correct molecular mass of 85 kDa in Jurkat whole cell lysate (lane 1). The abundant presence of sperm proteins was confirmed using an antibody raised against α -tubulin (B,D). Experiments were performed in triplicate and representative images are shown. Scale bars: 5 μ m.

TUNEL assay, which depends upon the presence of available 3'-OH termini (Fig. 1D).

Cadmium inhibits the repair of 8OHdG

To further explore the functionality of OGG1 in spermatozoa, these cells were exposed to cadmium (II) chloride ($CdCl_2$), a well-recognized inhibitor of OGG1 (Zharkov and Rosenquist, 2002). When $CdCl_2$ alone was added to human spermatozoa there was no observable change in the intracellular or extracellular levels of 8OHdG over a 2-hour incubation period (Fig. 4A,B). However, when DNA oxidation was induced with H_2O_2 , co-incubating spermatozoa with increasing doses of $CdCl_2$ resulted in a highly elevated level of intracellular 8OHdG ($P < 0.001$; Fig. 4A), accompanied by a corresponding decrease in the concentration of 8OHdG expelled to the exterior of the cell ($P < 0.001$; Fig. 4B), suggesting inhibition of OGG1-mediated base excision. Cadmium, unlike Fenton-type metals, cannot produce reactive oxygen species directly, so it was unlikely that the increased intracellular accumulation of 8OHdG observed in the presence of this cation was due to oxidative stress created by a direct interaction between H_2O_2 and $CdCl_2$. To ensure that this was the case, oxidative lesions were induced by incubating spermatozoa in 2 mM H_2O_2 for 2 hours. The spermatozoa were then washed to remove any residual H_2O_2 and subsequently incubated for 24 hours in the presence or absence of 100 μ M $CdCl_2$ (Fig. 4C). $CdCl_2$ effectively reduced the concentration of 8OHdG released into the ambient medium to control levels following H_2O_2 treatment ($P < 0.05$), providing further evidence that $CdCl_2$ has the ability to inhibit 8OHdG excision in human spermatozoa and demonstrating that the increased intracellular 8OHdG levels were not due to an interaction between $CdCl_2$ and H_2O_2 . These findings were confirmed in an independent data set using a single dose of $CdCl_2$ (100 μ M) to suppress the OGG1 response to a range of H_2O_2 concentrations, employing an incubation time of 2 hours (Fig. 4D,E). These data revealed a clear dose-dependent increase in the percentage of cells expressing high levels of intracellular 8OHdG adducts on exposure to H_2O_2 , which was significantly increased by the presence of $CdCl_2$ ($P < 0.001$; Fig. 4D). In contrast, the same

dose of $CdCl_2$ significantly reduced the amount of 8OHdG released into the extracellular space ($P < 0.001$; Fig. 4E) in keeping with the anticipated inhibition of OGG1. If the incubation time was extended to 16 hours, the amount of 8OHdG accumulating in the extracellular space increased five fold, however in the presence of $CdCl_2$ this increase was significantly curtailed ($P < 0.001$; Fig. 4F). SCSA analysis at this 16 hour time point revealed a significant suppressive effect of cadmium (II) on the levels of DNA fragmentation observed in this assay ($P < 0.001$; Fig. 4G), in keeping with the notion that OGG1 activity creates the abasic sites detected by this assay.

OGG1 and the measurement of DNA damage in human spermatozoa using SCSA and TUNEL assays

To determine the impact of OGG1 activity on the major assays used clinically to assess DNA damage in human spermatozoa, these cells were incubated in the presence of increasing concentrations of H_2O_2 (0.25–5 mM) for 2 hours and the levels of DNA damage recorded with SCSA, TUNEL and 8OHdG assays. The results of this study demonstrated that SCSA was an exquisitely sensitive marker for oxidative DNA damage, generating an R^2 value of 0.87 ($P < 0.001$) when intracellular 8OHdG and SCSA [% DNA fragmentation index (DFI)] were compared (Fig. 5A). Consistent with the aforementioned findings, the levels of 8OHdG released into the extracellular space also correlated strongly with SCSA (% DFI) ($R^2 = 0.47$; $P < 0.001$; Fig. 5B). By contrast, no change in total TUNEL activity was observed within 2 hours (Fig. 5C), despite the high levels of oxidative DNA damage observed in these cells and the presence of destabilized chromatin capable of generating significant SCSA signals. Thus, in confirmation of the data presented in Fig. 1, the response of human spermatozoa to oxidative stress did not result in the generation 3'-OH ends that could be detected with a TUNEL assay.

Notwithstanding these findings, it is also evident that *in vivo* spontaneous TUNEL signals are observed in human spermatozoa which correlate well with the presence of intracellular 8OHdG (De Iuliis et al., 2009). In somatic cells, TUNEL detects the 3'-OH termini of DNA strand breaks created via the activation and

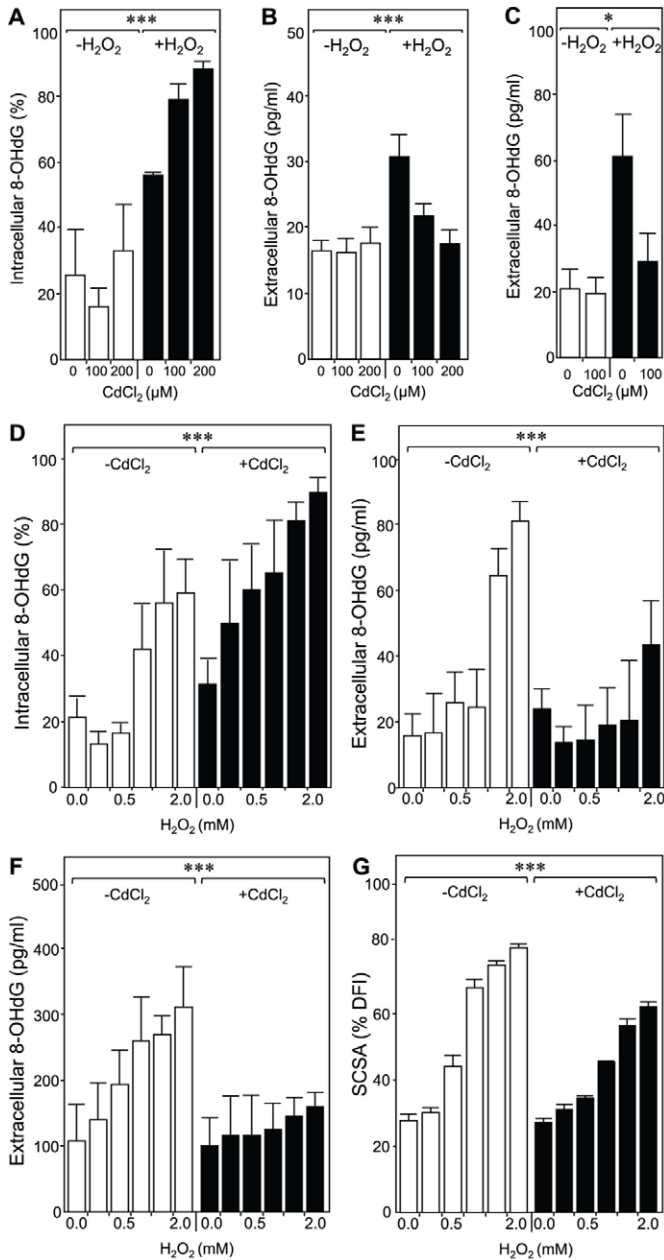


Fig. 4. Cadmium inhibits the repair of 8OHdG in spermatozoa. (A) A CdCl₂ dose response in the presence or absence of 2 mM H₂O₂ for 2 hours resulted in a dose-dependent increase in intracellular 8OHdG. (B) There was a corresponding dose-dependent decrease in the concentration of free 8OHdG detected in the extracellular space. (C) Following 2 hours of treatment with H₂O₂, the presence of 8OHdG in the extracellular space was reduced in the presence of CdCl₂ in the ensuing 14 hours. (D) Two hours of treatment with increasing doses of H₂O₂ in the presence of CdCl₂ resulted in an increased percentage of spermatozoa positive for intracellular 8OHdG. (E) In the presence of CdCl₂ a significant decrease in extracellular 8OHdG was detected following 2 hours of incubation. (F) A significant decrease in the concentration of 8OHdG detected in the supernatant following H₂O₂ treatment in the presence of CdCl₂ over 16 hours. (G) Reduced ability of SCSA to detect DNA damage in the presence of CdCl₂. Analyses based on three independent semen samples. Error bars represent \pm s.e.m. * P <0.05; *** P <0.001.

release of nucleases that destroy the nuclear genome in the final stages of apoptosis. In spermatozoa the physical separation of nuclear DNA from the midpiece, which houses most of the residual cytoplasm and all of the mitochondria, impedes the nuclear translocation of these activated nucleases during apoptosis. Thus nucleases such as caspase activated DNase (CAD), apoptosis inducing factor (AIF) or endonuclease G (EndoG) all remain resolutely locked in the midpiece following the induction of apoptosis and never penetrate the nuclear compartment (Koppers et al., 2011).

The fact that a majority of spontaneously TUNEL-positive spermatozoa in the human ejaculate have lost their vitality (Mitchell et al., 2011) suggested that the generation of TUNEL-reactive 3'-OH termini in sperm DNA is a perimortem change that takes place some time after the initial oxidative insult. To test this hypothesis, we conducted a time-dependent study to determine whether exposure to H₂O₂ could generate TUNEL signals after prolonged incubation. As anticipated, after 2 hours of exposure there was no evidence of TUNEL positivity and thus no correlation with intracellular 8OHdG formation (Fig. 5C) or SCSA (Fig. 5D). After 24 hours however, 3'-OH DNA termini had started to appear and a moderate correlation between TUNEL and SCSA % DFI appeared ($R^2=0.58$; $P<0.05$; Fig. 5E) and by 48 hours the correlation between SCSA % DFI and TUNEL was highly significant ($R^2=0.85$; $P<0.001$; Fig. 5F). Interestingly, the correlations between TUNEL and viability followed the same trend; after 2 hours a slight decrease in viability was observed but there was no detectable TUNEL signal (Fig. 5G), however by 24 hours vitality had decreased significantly and the population of TUNEL-positive cells exhibited a corresponding increase ($R^2=0.73$; $P<0.001$; Fig. 5H). After 48 hour exposure, viability and TUNEL were very highly correlated ($R^2=0.91$; $P<0.0001$; Fig. 5I) indicating that the 3'-OH groups detected by TUNEL are a peri- or post-mortem change in human spermatozoa.

Discussion

By virtue of their highly specialised structure and function, spermatozoa are characterized by a limited level of antioxidant protection, rendering these cells particularly vulnerable to oxidative stress (Aitken and Curry, 2011). Furthermore these cells are replete with substrates for free radical attack including the polyunsaturated fatty acids that dominate their lipid profile (Jones et al., 1979; Koppers et al., 2010) and the DNA in the sperm nucleus and mitochondria (Aitken et al., 1998). The nuclear DNA is partially protected from damage by virtue of its highly condensed state (Sawyer et al., 2001) however this strategy is evidently of limited efficacy because human spermatozoa are characterized by high levels of DNA fragmentation (Aitken and De Iulius, 2010; Irvine et al., 2000). Moreover, this DNA damage appears to be clinically significant, having negative impacts on fertilization, the quality of the resulting pre-implantation embryo, the incidence of miscarriage in the ensuing pregnancy, and the health and wellbeing of the offspring (Aitken, 1999; Zini, 2011). These observations raise important questions about the competence of the terminally differentiated gamete to execute DNA repair. Very little is known about this process in spermatozoa other than the fact that it is severely compromised. In haploid germ cells, homologous recombination cannot occur because of the absence of a sister chromatid. However, spermatids (the stage of germ cell differentiation prior to the formation of spermatozoa) are

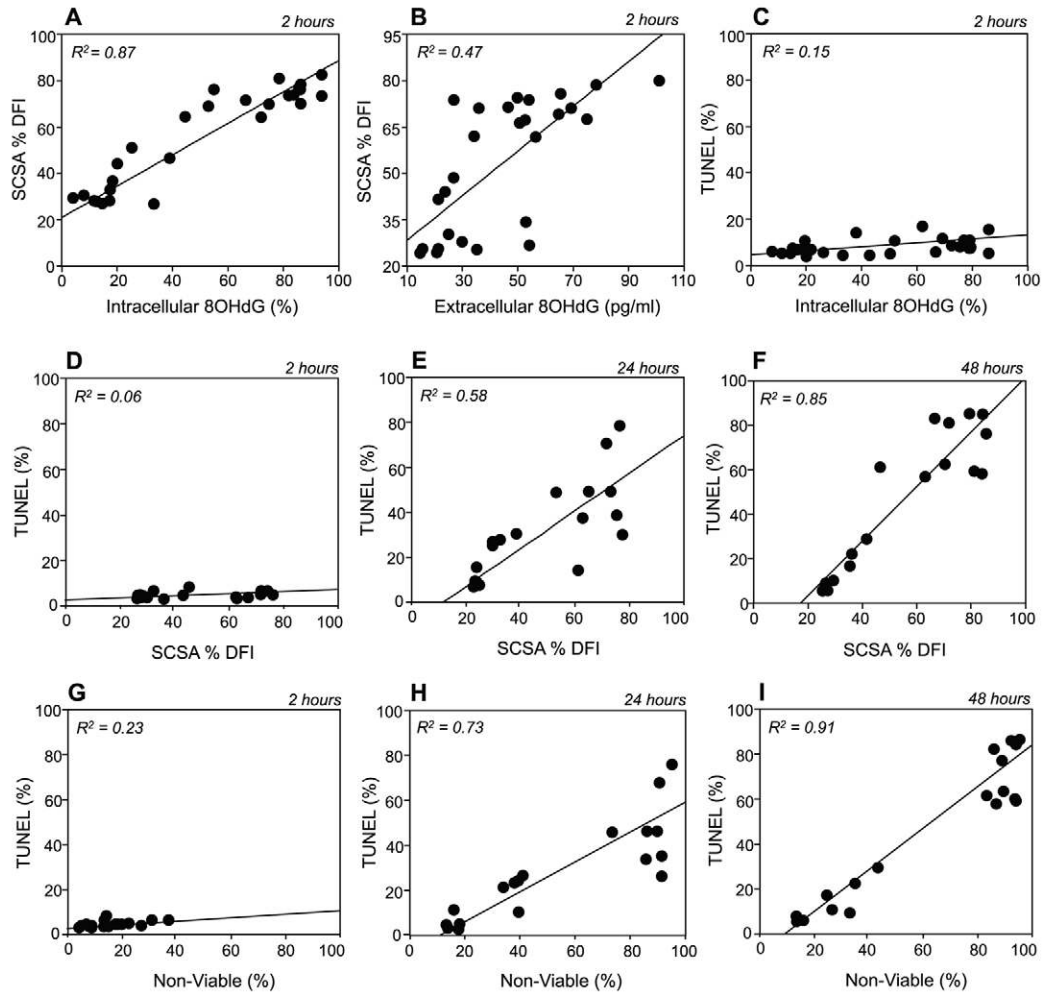


Fig. 5. OGG1 and the measurement of DNA damage in human spermatozoa with SCSA and TUNEL assays. Following 2 hours of incubation in 0–4 mM H_2O_2 there was (A) a highly significant correlation between SCSA %DFI and intracellular 8OHdG ($R^2=0.87$) and (B) correlation between SCSA %DFI and the concentration of free 8OHdG in the supernatant ($R^2=0.47$). However, there was no correlation between (C) intracellular 8OHdG and TUNEL ($R^2=0.04$) or (D) SCSA %DFI and TUNEL ($R^2=0.06$). (E) After 24 hours of incubation with increasing doses of H_2O_2 , SCSA %DFI and TUNEL were moderately correlated ($R^2=0.58$) and (F) after 48 hours of incubation in increasing concentrations of H_2O_2 , SCSA %DFI and TUNEL were very strongly correlated ($R^2=0.85$). (G) There was no correlation between TUNEL and cell death after 2 hours of incubation in increasing doses of H_2O_2 ($R^2=0.23$). (H) However, at 24 hours there was a high correlation between TUNEL and cell death ($R^2=0.73$), and (I) an almost perfect correlation between TUNEL and cell death after 48 hours indicating that TUNEL-detectable 3'-OH group formation was occurring perimortem. Analyses based on three independent semen samples for the data comprising each panel.

known to possess some capacity to repair double strand breaks via the creation of γ H2AX foci and recruitment of elements from the non-homologous end-joining pathway (NHEJ) including Parp1 and XRCC1 (Ahmed et al., 2010; Leduc et al., 2008a). Because the NHEJ pathway is an error prone process, it has been suggested that some of the DNA damage carried by defective human spermatozoa is a result of aberrant repair during the early stages of spermiogenesis (Leduc et al., 2008b).

In the mature gamete, the presence of a chromatin structure dominated by protamines, together with the high level of compaction, places severe limitations on the DNA repair competence of these cells. There are some data to suggest that fully differentiated spermatozoa can not only create γ H2AX foci in response to oxidative stress created by H_2O_2 or adriamycin but also recruit repair proteins to the damaged chromatin in the form of RAD50 and 53BP1 (Li et al., 2006; Li et al., 2008). These studies suggest a remarkable degree of molecular movement

within a highly condensed chromatin structure that has previously been regarded as relatively inert, and in a cell that is virtually transcriptionally and translationally silent. Independent verification of these data is now eagerly anticipated. In the meantime, the only DNA repair strategy that appears to be available to spermatozoa is a severely truncated base excision repair pathway. This study has identified, for the first time, the presence of OGG1 in human spermatozoa at the mRNA and protein levels. This essential base excision repair enzyme was localized to the midpiece, which contains the mitochondrial genome, and was also buried deep in the chromatin where its presence could only be detected once this structure had been relaxed by DNA strand breaks induced by H_2O_2 or DNase 1. Under conditions of oxidative stress, we revealed that this base excision repair enzyme is functional in actively cleaving out oxidative DNA base adducts that then appear in the extracellular space. The specific OGG1 isoform in the male germ line, at the

mRNA level and the protein level is OGG1a, the predominant active isoform localised to the nucleus of somatic cells. Mature spermatozoa also contained the mRNA for the mitochondrial-specific active isoform, OGG2a.

Following the induction of oxidative stress, OGG1 appears to work in isolation to remove 8OHdG, creating an AP site. We could not detect APE1, which would be needed to create 3'-OH termini on the phosphodiester backbone in order for polymerases to insert an unmodified nucleotide and ligase III to seal the nick on the 3' and 5' ends of the backbone, with XRCC1 playing a scaffolding role (Hoeijmakers, 2001). The absence of APE1 and XRCC1 sets spermatozoa apart from most other cells types and is presumably a reflection of the highly compacted, inert nature of sperm chromatin. Rather than complete the base excision repair pathway, we predict that the AP site created by OGG1 in DNA damaged spermatozoa is normally repaired to completion following fertilization, at a time that precedes the S-phase of the first mitotic division in the one-cell embryo (Shimura et al., 2002). Indeed we further propose that it is errors created by the oocyte during this early round of DNA repair that mediates the impact of paternal factors associated with the induction of oxidative stress in the germ line, such as age and smoking, on the health and wellbeing of the offspring (Aitken, 1999).

Consistent with previous studies reporting an inhibitory role of cadmium (II) on the repair capabilities of the OGG1 enzyme (Zharkov and Rosenquist, 2002), the release of 8OHdG by human spermatozoa was significantly inhibited by cadmium (II) in a dose- and time-dependent manner, as indicated by significantly elevated levels of intracellular 8OHdG in concert with a significant decrease in the levels of 8OHdG detected in the extracellular space (Fig. 4). Alarming, cadmium (II) has a half life in the body of 15–20 years and is found in high concentrations in cigarette smoke (Satarug and Moore, 2004). A study by Shen et al. (Shen et al., 1997) comparing the spermatozoa of smoking and non-smoking men reported a significant increase in 8OHdG lesions in the spermatozoa of smokers. There is a strong body of evidence linking paternal cigarette smoking with cancer in the offspring (Lee et al., 2009) and it seems likely that the increased levels of 8OHdG in spermatozoa from smokers are due to inefficient repair by OGG1. If these highly mutagenic lesions overwhelm the repair capacity of the oocyte, G→T transversions may occur, increasing the mutational load carried by the embryo and the progeny's subsequent susceptibility to cancer.

The finding that OGG1 is a key player in the truncated base excision repair pathway in human spermatozoa also has implications for methods used to detect DNA damage for diagnostic purposes. Specifically, when this highly specialised cell type experiences oxidative stress the only defence it possesses is the ability to cleave out the oxidized base adduct and create an abasic site. The SCSA assay detects such sites, however the lack of APE1 to generate the 3'-OH termini essential for the TUNEL assay, means that the latter is incapable of detecting the early stages of oxidative DNA damage. Only after the cells have been incubated for 24–48 hours at 37°C does the TUNEL signal start to appear, generating results that are highly correlated with the outcome of the SCSA assay. The late onset of TUNEL positivity explains why a majority of spermatozoa that are positive with this test are no longer viable (Mitchell et al., 2011). Whether the progressive appearance of TUNEL reactivity reflects the release of an endogenous nuclease as the structural

integrity of the spermatozoa becomes compromised perimortem has not yet been determined. Whatever mechanism is involved in the increase in TUNEL positivity, the fact that TUNEL-positive spermatozoa are moribund or dead means that under normal physiological circumstances, such cells are incapable of fertilization and therefore pose little threat to the offspring. Rather, this study highlights the importance of developing a highly sensitive, reproducible assay for measuring 8OHdG in viable cells that are capable of fertilization (Aitken et al., 2010). The inherent susceptibility of human spermatozoa to the formation of oxidative base adducts coupled with the truncated nature of the base excision repair pathway in these cells also places emphasis on the fidelity of the DNA repair pathways in the oocyte in order to reduce the risk of paternally mediated defects in the progeny. Unfortunately the nature of the DNA repair pathways in the female germ line and the way in which they might be impacted by age or lifestyle factors such as smoking, are currently unknown.

Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. Fresh Biggers, Whitten and Whittingham (BWW) medium was used for all experiments, supplemented with 1 mg/ml polyvinyl alcohol, 5 units/ml penicillin and 5 mg/ml streptomycin, and the osmolarity was kept between 290 and 310 mOsm/kg (Biggers et al., 1971).

Semen samples

The use of semen samples for research purposes was approved by an institutional human ethics committee and the State Minister for Health. A cohort of unselected, normozoospermic donors, mainly university students of unknown fertility status, supplied semen samples for this study. Semen samples were produced by masturbation into a sterile container and delivered to the laboratory within 1 hour of ejaculation.

Sperm preparation

Fractionation of semen samples was achieved using a 44% and 88% discontinuous Percoll centrifugation gradient (GE Healthcare, Castle Hill, Australia) (Nixon et al., 2005). Following centrifugation for 30 minutes at 500 g, the seminal plasma fraction and the low density layer were removed, and the high-density fraction predominantly containing spermatozoa with a high percentage of normal morphology, motility and vitality, was washed for a further 15 minutes in 5 ml BWW/PVA. The resulting pellets were diluted to a concentration of 50×10^6 /ml in BWW/PVA.

Treatment of spermatozoa

5×10^6 spermatozoa were centrifuged at 500 g for 5 minutes and the pellet resuspended in 0–5 mM H_2O_2 in BWW in a final volume of 100 μ l. Treatments involving $CdCl_2$ (ICN Pharmaceuticals Inc., Aurora, OH) were resuspended in 90 μ l of H_2O_2 -containing medium then to the cell suspension 10 μ l of 1 mM $CdCl_2$ (prepared in the corresponding concentration of H_2O_2) to give a final concentration of 100 μ M was added, or 10 μ l of 2 mM $CdCl_2$ to give a final concentration of 200 μ M. Sperm were incubated at 37°C for 2–48 hours. Following incubation, samples were centrifuged at 500 g for 5 minutes and the supernatant transferred to a fresh tube and stored at $-80^\circ C$ until 8OHdG ELISA analysis. The cell pellet was resuspended in 500 μ l BWW and 100 μ l of the cell suspension was transferred to a fresh tube, snap frozen in liquid nitrogen and stored at $-80^\circ C$ until SCSA analysis. The remaining cell suspension was centrifuged at 500 g for 5 minutes and the cell pellet resuspended in LIVE/DEAD® Fixable Dead Cell Stain (far red) (Molecular Probes, Eugene, OR) in BWW for 30 minutes at 37°C. The spermatozoa were then washed once in BWW/PVA for 5 minutes at 500 g before being resuspended in 2 mM dithiothreitol for 30 minutes at 37°C to reduce the disulfide bonds contributing to protamine cross linking. Following this, spermatozoa were washed and fixed in 2% paraformaldehyde for 15 minutes at 4°C, washed with PBS and stored in 0.1 M glycine in PBS at 4°C for the TUNEL assay and intracellular 8OHdG assay.

Intracellular 8-hydroxy-2'-deoxyguanosine

Intracellular guanine oxidation was measured using OxyDNA Assay Kit, Fluorometric (Calbiochem, CA) which contains a FITC-conjugated binding protein with high affinity for 8OHdG. Spermatozoa prepared as above were analysed for 8OHdG formation within 1 week of preparation. 2×10^6 spermatozoa

were permeabilised in 0.1% Triton X-100 supplemented with 1 mg/ml sodium citrate in PBS for 5 minutes at room temperature, washed once in the wash solution supplied by the manufacturers and the cell pellet blocked in 50 μ l of 3% BSA (Research Organics) and incubated for 1 hour at 37°C. Following blocking, samples were centrifuged for 5 minutes at 500 *g* in wash solution. FITC-conjugated binding protein was diluted 1:50 in wash solution and further purified by adding 1 mg of activated charcoal to absorb any excess free FITC. This solution was vortexed for 30 seconds and centrifuged for 2 minutes at 10,000 *g* until all residual charcoal was removed. Spermatozoa were resuspended in 50 μ l of purified FITC-tagged fluorescent binding protein and incubated in the dark at 37°C for 1 hour, centrifuged at 500 *g* for 5 minutes, resuspended in 500 μ l PBS and transferred to FACS tubes for analysis by flow cytometry as described below.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

DNA fragmentation was measured using the TUNEL assay (Roche Diagnostics, Mannheim, Germany) as described previously (Mitchell et al., 2011) on 2×10^6 spermatozoa prepared above. The assay was performed within 1 week of sample preparation and the presence of free 3'-OH groups measured by flow cytometry.

Flow cytometry

TUNEL and intracellular 8OHdG fluorescence was measured on a FACSCalibur flow cytometer (BD) using an argon laser excitation at 488 nm coupled with emission measurements using 530/30 band pass (green) using the FL1 channel. Cell vitality was determined by the percentage of cells emitting far red fluorescence at 665 nm using the FL-3 detector. 10,000 sperm events were recorded after non-sperm events were gated out.

Extracellular 8-hydroxy-2'-deoxyguanosine

For the detection of DNA repair products in the extracellular space, an 8-hydroxy-2'-deoxyguanosine-specific ELISA kit (Abcam, Cambridge, UK) was used according to the manufacturer's instructions. Briefly 50 μ l of supernatant, prepared as described above, was thawed and carefully aliquoted into the base of the wells on the microtitre plate provided. BWW/PVA was used for the preparation of standards. 50 μ l of the 8-hydroxy-2'-deoxyguanosine AChE tracer and monoclonal antibody were added to the appropriate wells and the plate was incubated at 4°C for 18 hours. The wells were washed five times with wash buffer and 200 μ l Ellman's reagent added to each well and the plate incubated for 90 minutes on an orbital shaker at room temperature. The absorbance was read on a Fluostar Optima spectrophotometer (BMG LabTechnologies, Durham, NC) and the concentration of 8OHdG in the sperm-conditioned medium was calculated according to the manufacturer's instructions.

Sperm chromatin structure assay (SCSA)

The SCSA was performed as described by Evenson and Jost (Evenson and Jost, 2000). Briefly, 1×10^6 spermatozoa in 100 μ l of BWW/PVA were snap frozen in liquid nitrogen and stored at -80°C until further analysis. Immediately prior to assessment, the spermatozoa were thawed at 37°C for 1 minute and placed on ice. 100 μ l of the sperm suspension was added to a FACS tube with 200 μ l of acid detergent solution. After 30 seconds, spermatozoa were stained with 600 μ l of acridine orange staining solution. Using a FACScan™ Flow Cytometer (BD) debris was gated out and 5000 sperm events were acquired per sample. The ratio of single stranded (red) to double stranded (green) fluorescence (%DFI) was calculated using CellQuest™ software (BD).

Immunolocalization of OGG1, APE1 and XRCC1

The localization of the enzymes participating in the base excision repair pathway was detected using immunocytochemistry. 2×10^6 spermatozoa were treated with BWW or H₂O₂ (2 mM) for 16 hours at 37°C then washed in BWW for 5 minutes at 500 *g*. The chromatin was decondensed using 2 mM dithiothreitol for 30 minutes at 37°C, washed and fixed in 2% paraformaldehyde in PBS for 15 minutes at 4°C. Spermatozoa was washed and stored in 0.1 M glycine in PBS at 4°C. 50 μ l of spermatozoa were settled on poly-L-lysine coated coverslips at 4°C for at least 2 hours in a humidified chamber then permeabilised at room temperature for 15 minutes in 0.2% Triton X-100 in PBS. For DNase I treatment, cells were exposed to 2 mg/ml DNase I (Roche) in PBS supplemented with 10 mM MgSO₄ for 1 hour at 37°C, washed three times then blocked with 10% goat serum in 3% BSA/PBS for 1 hour at 37°C. The blocking solution was removed and the sperm coated coverslips incubated with primary antibodies against OGG1 (1:100, Novus Biologicals, Littleton, CO), XRCC1 (1:100, Abcam) and APE1 (1:100, Abcam) in 1% BSA at 4°C overnight in a humidified chamber. The primary antibody was washed off with PBS and 50 μ l of 1% BSA with 1:200 dilution of goat Alexa Fluor 488 (Invitrogen) secondary antibody against the species in which the primary antibody was raised, was added to the cells, which were then incubated for 1 hour at 37°C. The secondary antibody was washed off thoroughly and the spermatozoa incubated in 0.005 mg/ml of TRITC-tagged peanut agglutinin for 15 minutes at room temperature in order to label the

acrosome. The coverslips were washed and mounted with ProLong Gold Antifade Reagent With DAPI (Molecular Probes, Eugene, OR) as a DNA counter stain, and immunolocalization examined using a LSM510 laser scanning confocal microscope equipped with argon and helium/neon lasers (Carl Zeiss Pty, Sydney, Australia). A secondary-only control was used as a reference to determine background fluorescence.

Protein extraction for 1D SDS-PAGE/western blot

For 1D SDS-PAGE and western blot, spermatozoa were washed with PBS and the cell pellet extracted with sodium dodecyl sulfate (SDS) extraction buffer (0.5% SDS, 10% sucrose in 0.1875 M Tris pH 6.8) containing a protease inhibitor cocktail (Roche) at 100°C for 5 minutes, vortexed vigorously and the insoluble cellular debris removed by centrifugation at 10,000 *g* for 15 minutes at 4°C. Quantification of the isolated protein supernatant was achieved using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions. 10–20 μ g of sperm protein as well as 1 μ g of recombinant human OGG1 and APE1 (New England BioLabs, Ipswich, MA) or a Jurkat whole cell lysate (Novus Biologicals) were boiled in SDS/PAGE sample buffer (SDS extraction buffer as above supplemented with 2% 2-mercaptoethanol, bromophenol blue and 10% glycerol) for 5 minutes and resolved on Tris-glycine 4–20% polyacrylamide gels (NuSep, Sydney, Australia). The resolved proteins were then transferred onto nitrocellulose membranes under a constant current of 350 mA for 1 hour. The nitrocellulose membranes were blocked overnight in 5% skim milk powder in TBS (Tris-buffered saline: 100 mM Tris/HCl, pH 7.6, and 150 mM NaCl) (pH 7.4) supplemented with 0.1% Tween 20 (TBST). Membranes were rinsed in TBST and probed overnight with primary antibodies against OGG1 (Epitomics, Burlingame, CA) at a concentration of 1:7500, APE1 at 1:1000 (Abcam) and XRCC1 at 1:1000 (Abcam) in 1% skim milk powder in TBST. Membranes were then further probed for 1 hour with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody at room temperature. Following a further three washes in TBST, cross-reactive proteins were visualized using an ECL (enhanced chemiluminescence) kit (GE Healthcare) according to the manufacturer's instructions.

Immunoprecipitation of OGG1

SDS extracted whole cell lysate prepared as described above was dialysed using a Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, Rockford, IL) against $1 \times$ PBS at 4°C for 24 hours in order limit the effect of micelles and to remove residual SDS. Approximately 60 μ l (per treatment) of protein G magnetic beads (Millipore) were washed three times in PBS. This was followed by conjugation with 5 μ g anti-OGG1 antibody overnight at 4°C with constant mixing. Following conjugation, the antibody-bead complexes were washed two times and then covalently cross-linked by incubation in 15 mM bis(succinimidyl) penta(ethylene glycol) (Pierce) for 2 hours at 4°C. The cross-linking reaction was quenched using 1 M Tris and the conjugated beads were washed as above. A control sample of beads was also left un-conjugated and was incubated with PBS only. Each of the conjugated bead preparations were then incubated with 500 μ g of 0.5% w/v SDS sperm lysate (prepared as described above) that had been pre-cleared against un-conjugated beads to limit non-specific interactions. After an overnight incubation at 4°C with constant mixing, the beads were washed three times prior to boiling in SDS running buffer containing 8% 2-mercaptoethanol at 100°C for 5 minutes. Precipitated proteins were resolved on a 10% polyacrylamide gel and silver stained, or transferred to a nitrocellulose membrane and probed for OGG1 as described above.

Silver staining

Silver staining was achieved by fixing the polyacrylamide gel for 1 hour in 50% v/v methanol and 12% v/v acetic acid. The gels were further fixed two times for 15 minutes each in 10% v/v methanol and 12% v/v acetic acid. Gels were washed three times for 10 minutes in 10% v/v methanol then sensitised for 1 minute with 1.63 mM sodium thiosulfide, immediately washed three times in water before incubating with 12 mM silver nitrate for 6 minutes. The gels were washed a further three times in water prior to incubation with the developing buffer (0.28 M sodium bicarbonate, 0.02% v/v formaldehyde, 25 μ M sodium thiosulfide). Once bands had resolved, the gels were washed for 2 minutes in 1% v/v acetic acid.

Mass spectrometry identification of proteins of interest

Protein bands corresponding to the western blot location of OGG1 isolated by immunoprecipitation were carefully excised and separation of tryptic peptide mixtures was achieved by nanoscale reversed phase high-pressure liquid chromatography [nano-AQUITY system (Waters)] in combination with on-line electrospray ionization mass spectrometry. The mass spectrometric analysis was performed on an LTQ XL-linear ion trap system (Thermo Scientific).

Bioinformatics

Thermo RAW files were converted into MASCOT Generic Format using MASCOT Daemon 2.3.2 and imported into Bruker's ProteinScape platform

(Bruker Daltonics) for database searching. Searches were performed against the SwissProt database (version 57.15) using in house licensed MASCOT server (version 2.3.02, Matrix Science), with the species subset set to *Homo sapiens* and the number of allowed trypsin missed cleavages set to 2. Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine and phosphorylation of serine, threonine, and tyrosine were set as variable modifications. The parent ion tolerance was set to 1.4 Da with fragment ion set to 0.7 Da. Peptide thresholds were set requiring a False Positive Rate of less than 0.05% and a MASCOT score of greater than 40. Those spectra meeting these criteria were validated by manual inspection on a residue-by-residue basis to ensure accurate γ - and b -ion detection with overlapping sequence coverage.

RT-PCR of human OGG1 isoforms in spermatozoa

To determine the presence of residual OGG1 isoform transcripts in human spermatozoa, 5 μ g of total RNA was reverse transcribed with oligo(dT)₁₅ primer (Promega), and M-MLV Reverse Transcriptase (Promega, Madison, WI). RT-PCR was then performed to detect known transcript variants and isoforms of the OGG1 gene. The first set of primers was designed to detect the differing isoforms of transcript variant 1, namely isoforms 1a, 1b and 1c. (GenBank accession numbers NM_002542, NM_016819 and NM_016820 respectively). For the first round of PCR, the forward primer sequence was 5'-AACAGCACCGTGTGGCGAG-3'; the reverse primer sequence was 5'-TGAGCATGGCGGGATTGGCG-3'. These primers are predicted to generate bands of 1110 bp (base pairs), 1354 bp and 1127 bp for isoforms 1a, 1b and 1c respectively. The PCR reaction conditions were as follows: 1 cycle of 94°C for 5 minutes; 35 cycles of 95°C for 45 seconds, 69°C for 45 seconds, 72°C for 2 minutes; 1 cycle of 72°C for 10 minutes. For nested PCR, a 1:100 dilution of the first round product was used as the template and the conditions were the same as above. For nested PCR, the forward primer sequence was 5'-TGGAGGAGCAAAGTCTGCACA-3'; the reverse primer sequence was 5'-GATTGGCGCAGTCCGCACT-3'. These primers are predicted to generate bands of 839 bp, 1083 bp and 856 bp for isoforms 1a, 1b and 1c respectively.

The second set of primers was designed to detect the differing isoforms of transcript variant 2, namely isoform 2a, 2b, 2c, 2d, and 2e (GenBank accession numbers NM_016821, NM_016826, NM_016827, NM_016828 and NM_016829 respectively). For the first round of PCR, the forward primer sequence was 5'-GCTCTGAGCTGCGCCTGGAC-3'; the reverse primer sequence was 5'-AGGCTGGAGGAAGCTGGGG-3'. These primers are predicted to generate bands of 1000 bp, 799 bp, 617 bp, 1100 bp and 1053 bp for isoforms 2a, 2b, 2c, 2d, and 2e respectively. The PCR reaction conditions were as follows: 1 cycle of 94°C for 5 minutes; 35 cycles of 95°C for 45 seconds, 69°C for 45 seconds, 72°C for 2 minutes; 1 cycle of 72°C for 10 minutes. For nested PCR, a 1:100 dilution of the first round product was used as the template and the conditions were the same as above. For nested PCR, the forward primer sequence was 5'-AGCAGGCCACACCAGACGA-3'; the reverse primer sequence was 5'-TGCATTGCCAAGGAGGCCCG-3'. These primers are predicted to generate bands of 711 bp and 510 bp, 328 bp, 811 bp and 764 bp for isoforms 2a, 2b, 2c, 2d, and 2e respectively. The PCR products were run on 1.5% agarose gels and the DNA was purified from the gel using the Wizard Gel Clean-Up Kit (Promega). The DNA was sequenced at the Australian Genomic Research Facility (Sydney).

Statistics

All experiments were replicated at least three times on independent samples and the results analyzed by one- and two-way ANOVA using the SuperANOVA program (Abacus Concepts Inc., CA, USA) on a MacIntosh G4 Powerbook computer; post hoc comparison of group means was by Fisher's PLSD (Protected Least Significant Difference). Paired comparisons were conducted using a paired t -test using the Statview program (Abacus Concepts Inc., CA). Differences with a P value of <0.05% were regarded as significant.

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Author contributions

T.B.S. originally conceived the study, while T.B.S. and R.J.A. designed and interpreted the experiments. T.B.S., M.D.D., N.D.S., B.J.C. and H.S.C. participated in the different aspects of the analyses, and T.B.S. and R.J.A. collaborated in generating a draft of the manuscript, which was then edited and approved by the remaining authors.

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