The senescence-accelerated mouse prone 8 as a model for oxidative stress and impaired DNA repair in the male germ line

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Abstract

The discovery of a truncated base excision repair pathway in human spermatozoa mediated by OGG1 has raised questions regarding the effect of mutations in critical DNA repair genes on the integrity of the paternal genome. The senescence-accelerated mouse prone 8 (SAMP8) is a mouse model containing a suite of naturally occurring mutations resulting in an accelerated senescence phenotype largely mediated by oxidative stress, which is further enhanced by a mutation in the *Ogg1* gene, greatly reducing the ability of the enzyme to excise 8-hydroxy,2'-deoxyguanosine (8OHdG) adducts. An analysis of the reproductive phenotype of the SAMP8 males revealed a high level of DNA damage in caudal epididymal spermatozoa as measured by the alkaline Comet assay. Furthermore, these lesions were confirmed to be oxidative in nature, as demonstrated by significant increases in 8OHdG adduct formation in the SAMP8 testicular tissue ($P < 0.05$) as well as in mature spermatozoa ($P < 0.001$) relative to a control strain (SAMR1). Despite this high level of oxidative DNA damage in spermatozoa, reactive oxygen species generation was not elevated and motility of spermatozoa was found to be similar to that for the control strain with the exception of progressive motility, which exhibited a slight but significant decline with advancing age ($P < 0.05$). When challenged with Fenton reagents (H_2O_2 and Fe^{2+}), the SAMP8 spermatozoa demonstrated a highly increased susceptibility to formation of 8OHdG adducts compared with the controls ($P < 0.001$). These data highlight the role of oxidative stress and OGG1-dependent base excision repair mechanisms in defining the genetic integrity of mammalian spermatozoa.

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Introduction

The senescence-accelerated mouse prone 8 (SAMP8) is a naturally occurring mouse model containing a suite of mutations, the net effects of which are a significant reduction in life span and a phenotype displaying accelerated senescence (Takeda *et al.* 1997a, 1997b). The senescence-accelerated mouse resistant 1 (SAMR1) serves as a control, in that it is a related mouse strain that does not show accelerated ageing, but has a background that is similar to that of the SAMP8 strain. Research into the molecular basis of accelerated ageing in the latter has focused on the base excision repair pathway, particularly on the first enzyme in this pathway, OGG1, responsible for the removal of the highly mutagenic oxidative guanine adduct, 8-hydroxy,2'-deoxyguanosine (8OHdG). Of all the SAMP strains, the P8 variant has particularly low levels of OGG1 activity, reduced to 10–40% of the activity observed in the SAMR1 strain (Choi *et al.* 1999). All SAM mice have an arginine to a histidine substitution at codon 336, but the accelerated ageing SAMP strains have an additional mutation at codon 304. This mutation is thought to be responsible for the loss of OGG1 activity in SAMP8 mice, impairing catalytic activity and generating thermolability (Choi *et al.* 1999).

Oxidative stress has been shown to be a major factor in the aetiology of male infertility. Such stress is not only involved in the origins of defective sperm function (Aitken & Clarkson 1987, Aitken & Curry 2011), but also a major mediator of DNA damage in the male germ line (De Iuliis *et al.* 2009, Thomson *et al.* 2011), resulting in poor fertilisation rates, an increased risk of spontaneous abortion and morbidity in the offspring (Aitken & Curry 2011). Because the molecular structure of sperm chromatin approaches the physical limits of compaction, spermatozoa have a very limited capacity for DNA repair (Ward & Coffey 1991). The recent discovery of a highly truncated but functional base excision repair pathway in human spermatozoa containing only OGG1 (Smith *et al.* 2013) suggests that the abasic site generated subsequent to 8OHdG excision is ultimately repaired to completion by the oocyte prior to the first round of embryonic cell division. If OGG1 is the only element of the base excision repair pathway possessed by spermatozoa, it should be possible to gauge the significance of this particular system in delivering DNA repair in the germ line by studying the phenotype of animals in which this enzyme has been impaired. The SAMP8 mutants, therefore, offer an ideal opportunity to test the hypothesis

that DNA damage in the male germ line is largely oxidative in origin and that base excision by OGG1 is the major mechanism by which oxidative DNA damage in spermatozoa is regulated.

Materials and methods

Reagents

All reagents were purchased from Sigma–Aldrich, unless otherwise stated. Warmed, fresh Biggers, Whitten and Whittingham (BWW) medium (Biggers *et al.* 1971), containing 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25 mM NaHCO_3 , 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 20 mM HEPES buffer supplemented with 1 mg/ml polyvinyl alcohol (PVA), 5 units/ml penicillin and 5 mg/ml streptomycin, was used for all the experiments, and the osmolarity was maintained between 290 and 310 mOsm/kg (Biggers *et al.* 1971). All fluorescent probes were purchased from Molecular Probes, Inc. (Eugene, OR, USA).

Animal husbandry and mating experiments

The SAMR1/TaHsd and SAMP8/TaHsd strains originally of AKR/J background were purchased from Harlan Laboratories (Bicester, UK), and the colony was maintained at the Australian BioResources animal facility in Moss Vale, Sydney, Australia. Breeding pairs were a minimum of 3 months of age and a maximum of 8 months of age and were retired after a maximum of six litters. The number of pups per litter was recorded. Once housed in the University of Newcastle animal facility, mice were maintained in conditions of controlled temperature (22 °C) and a 12 h light:12 h darkness cycle with food and water *ad libitum*. From this point on, all animal care and procedures were approved according to the University of Newcastle animal ethics committee.

Caudal sperm extraction by perfusion

Adult male mice were asphyxiated via CO_2 inhalation, and the epididymides and testes were removed immediately, blotted free of blood and placed in pre-warmed water-saturated mineral oil. Pure suspensions of spermatozoa were obtained from the caudal region of the epididymis by perfusion, and the isolated spermatozoa were allowed to disperse for 10 min at 37 °C in 1 ml BWW/PVA, followed by centrifugation at 400 g for 3 min, and the pellet was resuspended in 1 ml BWW/PVA. All experiments were performed immediately while maintaining the spermatozoa at 37 °C.

Computer-aided sperm assessment

The movement characteristics of the spermatozoa were examined with a Hamilton Thorne IVOS Motility Analyzer, version 10.5K, at an incubation temperature of 37 °C. Each sample was loaded on to 20 µm-deep MicroCell slides (Microm, Thame, UK), and the analysis was performed on five random fields from each sample. At least 200 cells were

analysed. The settings for mouse spermatozoa were as follows: negative-phase-contrast optics, recording rate 60 frames/s, minimum contrast 50, minimum cell size four pixels; threshold values for progressively motile spermatozoa were average path velocity (VAP) > 50 µm/s and straightness (STR) > 80%; for slow cells, VAP cut-off was 7.4 µm/s and straight line velocity (VSL) cut-off 6.6 µm/s.

Dihydroethidium

For the assay, dihydroethidium (DHE) and the vitality stain SYTOX Green (SyG) were diluted in BWW/PVA and added to 5×10^5 spermatozoa in a final volume of 50 µl, comprising 45 µl of a purified caudal sperm suspension and 5 µl of a DHE/SyG mixture, equating to final concentrations of 2.0 and 0.05 µM respectively. The cells were then incubated in the dark at 37 °C for 15 min and aliquoted on to a warmed microscope slide, and the resulting red and green fluorescence was analysed immediately using an Axio Imager A1 fluorescence microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA) under fluorescence optics (470 nm excitation). At least 200 cells were counted and scored as live/negative, live/positive or dead.

MitoSOX Red

In order to measure the extent of superoxide generation by the mitochondria, 5×10^5 cells were stained with MitoSOX Red (MSR) (2 µM) and the vitality stain SyG (0.05 µM) for 15 min at 37 °C, shielded from light. Following incubation, 5 µl of cells were placed on a warmed microscope slide, and the resulting red and green fluorescence was analysed immediately using an Axio Imager A1 fluorescence microscope (Carl Zeiss Microimaging, Inc.) under fluorescence optics (470 nm excitation). At least 200 cells were counted and scored as live/negative, live/positive or non-viable.

JC1

In order to assess mitochondrial membrane potential, 45 µl of a sperm suspension (containing 5×10^5 cells) were co-incubated with 5 µl JC1 to give a final concentration of 20 µM. After incubation with JC1 for 15 min at 37 °C, the spermatozoa were placed on a pre-warmed microscope slide and immediately scored as having high mitochondrial membrane potential (orange/yellow) or low mitochondrial membrane potential (green) using an Axioplan 2 microscope (Carl Zeiss Microimaging, Inc.).

Chemiluminescence

For luminol–peroxidase-dependent chemiluminescence, 1×10^6 spermatozoa in 400 µl BWW were supplemented with 4 µl luminol (25 mM) and 8 µl HRP (11.52 U/ml). The samples were then run for 60 min at 37 °C in a Berthold AutoLumat luminometer LB-953 (Berthold, Bad Wildbad, Germany). Media blanks were run for every treatment in order to ensure that the signals recorded were not due to the spontaneous activation of the probe. The values obtained in

these media-only control incubations were subtracted from those obtained in the presence of spermatozoa.

Alkaline comet

Briefly, slides coated with agarose containing spermatozoa were immersed in a lysing solution of 2.5 M NaCl, 100 mM Na₂EDTA and 10 mM Trizma, pH 10, supplemented with 10 mM dithiothreitol (DTT) and 1% Triton X-100 for 1 h at 37 °C. These slides were subsequently placed in a horizontal gel electrophoresis tank (Millipore, Bedford, MA, USA) with an electrophoresis buffer (1 mM Na₂EDTA and 300 mM NaOH, pH 13) and cooled to 4 °C for 20 min to allow the DNA to unravel. Electrophoresis was run at 25 V for 5 min at 350–400 mA. Following electrophoresis, the slides were neutralised in a buffer containing 400 mM Trizma in ddH₂O, pH 7.5, for 5 min and stained with 20 µg/ml ethidium bromide and viewed immediately by fluorescence microscopy, and the images were captured using an Olympus DP70 camera (Olympus America, Center Valley, PA, USA). At least 100 cells were analysed per slide using Comet Assay IV Version 4.2 (Perceptive Instruments, Steeple Bumstead, UK), recording tail intensity and tail moment, excluding cells that did not lyse completely. All the experiments were run in duplicate.

Intracellular 8OHdG

Intracellular 8OHdG was assessed using the OxyDNA Assay kit (Calbiochem, San Diego, CA, USA) in combination with the LIVE/DEAD fixable dead stain. For this assay, 1.5×10^6 spermatozoa were treated with H₂O₂ supplemented with Fe²⁺ in a molar ratio of 2:1 in BWW/PVA for 1 h at 37 °C. The LIVE/DEAD stain was then added to the sperm suspensions (final concentration 1:10 000), after which the cells were washed twice with BWW/PVA at 500 g for 3 min and resuspended in 2 mM DTT and incubated at 37 °C for 45 min. Following treatment with DTT, the spermatozoa were washed twice with BWW at 500 g for 3 min and fixed in 2% paraformaldehyde at 4 °C for 15 min. The fixative was removed with two cycles of centrifugation and resuspension, and the washed cells were stored in 0.1 M glycine in PBS for up to 1 week at 4 °C. Ultimately, the stored spermatozoa were spun down at 500 g for 3 min, and the pellet was resuspended in a permeabilisation solution (10 mg sodium citrate and 10 µl Triton X-100 in 10 ml PBS). The cells were permeabilised for 5 min at room temperature, washed twice with a wash solution (Calbiochem) and then blocked in 3% BSA for 1 h at 37 °C. The cells were rinsed twice in the wash solution before the addition of a FITC-tagged fluorescent binding protein with a high affinity towards 8OHdG. To purify the fluorescent binding protein, a 1:50 dilution was made in the wash solution and added to a tube containing ~1 mg of activated charcoal. The charcoal-fluorescent binding protein solution was vortexed for 30 s and spun down at 13 000 g, and the supernatant was removed and repeatedly centrifuged until the charcoal was completely removed from the solution. A 50 µl sample of the FITC-tagged probe was added to the cell pellet and incubated at room temperature for 1 h and then washed once with PBS prior to the flow cytometric analysis.

Flow cytometry

8OHdG fluorescence was measured on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using 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. After gating out non-sperm events, 10 000 sperm events were recorded.

CMA3

In order to assess the efficiency of spermiogenesis, CMA3, a marker of protamination, was employed. Briefly, 5×10^5 caudal spermatozoa were fixed with 2% paraformaldehyde for 15 min at 4 °C, washed for 5 min and stored at 4 °C in 0.1 M glycine for a maximum of 1 week at 4 °C. An aliquot of the sperm suspension was settled on to a poly-L-lysine coverslip in a humidified chamber for at least 2 h at 4 °C. The spermatozoa were then permeabilised in 0.2% Triton X-100 in PBS for 15 min at 37 °C and washed once with McIlvaine's buffer. Coverslips were then stained with 25 µl CMA3 solution (0.25 mg/ml in McIlvaine's buffer) at room temperature for 20 min protected from light, washed twice with McIlvaine's buffer, and mounted with Mowiol and sealed. At least 100 cells were examined by fluorescence microscopy and scored as positive or negative.

Immunohistochemistry

Mouse testes were fixed with Bouin's solution, embedded in paraffin and sectioned to prepare slides. The sections were de-paraffinised and rehydrated, and antigens were retrieved by heating in a microwave at 1000 W for 15 min in sodium citrate (10 mM). The sections were blocked in 10% goat serum in 3% BSA for 1 h at 37 °C in a humidified chamber and then incubated with anti-γ-H2AX antibody (1:500, Abcam Cambridge, UK) overnight at 4 °C. The slides were washed thrice for 5 min with PBS followed by incubation with the appropriate Alexa-Fluor 594-conjugated secondary antibody for 1 h at room temperature in the dark. The sections were washed with PBS and mounted with the ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR, USA) as a DNA counterstain, sealed and viewed under an Axio Imager A1 fluorescent microscope (Carl Zeiss Microimaging, Inc.), and images were captured using an Olympus DP70 camera (Olympus America).

8OHdG detection in the testes

Mouse testes were homogenised and the DNA was extracted. For this purpose, ~100 mg of tissue were resuspended in 840 µl STE buffer (500 mM NaCl, 100 mM Tris-HCl, pH 8.0, and 10 mM EDTA), and then 50 µl 20% SDS (1% final), 10 µl 2-mercaptoethanol (1% final) and 100 µl 20 mg/ml Proteinase K (2 mg/ml final) were added and incubated at 55 °C for 6 h. DNA was extracted and purified using standard phenol/chloroform procedures. The purified DNA was resuspended in water and adjusted to 200 µg/ml. The DNA solution was then

Table 1 Impact of the SAMP8 phenotype on fertility.

	SAMR1	SAMP8
No. of litters	154	99
No. of pups born	895	656
Mean pups/litter ($\pm$ s.d.)	5.81 $\pm$ 2.59	6.63 $\pm$ 2.23*

* $P < 0.05$ for difference in mean pups/litter between SAMR1 and SAMP8 strains.

heated to 97 °C for 2 min and quickly put on ice for 5 min to facilitate denaturing. A 50 μ l sample of DNA was added to 12 μ l ZnCl₂ (10 mM), 9 μ l MgCl₂ (200 mM), 12 μ l sodium acetate (200 mM) and 5 μ l (1 U) nuclease P1 with 32 μ l water. This mixture was incubated overnight at 50 °C. The nucleotide preparation was then purified by solid-phase extraction. C18 reversed-phase cartridges were utilised, and the nucleotides were washed with 25 mM ammonium acetate and then eluted with 25 mM ammonium acetate containing 20% acetonitrile. The recovered nucleotides were then lyophilised. The powder was reconstituted in 50 μ l H₂O and analysed by LC–MS/MS (Q-ToF, Bruker, Bremen, Germany). Nucleotides were first identified by MS/MS and then the retention time was identified. The relative level of 8OHdG was defined as the peak area under the extracted ion chromatogram for 8OHdG divided by the peak area under the extracted ion chromatogram for dGMP (internal standard).

Statistical analysis

All experiments were replicated at least thrice on independent samples, and the results were analysed by one-way and two-way ANOVA using the SuperANOVA program (Abacus Concepts, Inc., Berkeley, CA, USA) on a MacIntosh G4 Powerbook computer; *post hoc* comparison of group means

was made by Fisher's protected least significant difference. Paired comparisons were made using a paired *t*-test using the StatView program (Abacus Concepts Inc.). Differences with a *P* value < 0.05 were considered significant.

Results

Evaluation of fertility and reproductive histology

In order to investigate the overall reproductive health of the SAMP8 strain, the number of pups/litter was recorded. There was no observable decrease in the number of pups per litter sired by the SAMP8 males (6.63 $\pm$ 2.23) compared with the SAMR1 (5.81 $\pm$ 2.59) males when mated with 3–8-month-old SAMP8 or SAMR1 females respectively. Rather, there was a significant increase in the number of pups/litter sired by SAMP8 mice (Table 1; $P < 0.05$). An assessment of the gross overall histology of the testes also failed to reveal any consistent significant aberrations in spermatogenesis when the SAMP8 and SAMR1 strains were compared (Fig. 1A). The assessment of sperm morphology, however, revealed that the SAMP8 spermatozoa failed to completely discharge their residual cytoplasm during epididymal transit, as indicated by the retention of cytoplasmic droplets at the base of the midpiece in a majority of spermatozoa, which were not present in age-matched SAMR1 spermatozoa (Fig. 1B, arrows).

Sperm function

With the aim of exploring the possibility of an accelerated ageing phenotype consistent with oxidative

Figure 1 Impact of the senescence-accelerated mouse prone 8 (SAMP8) phenotype on spermatogenesis. (A) Haematoxylin and eosin staining of the testes revealed no obvious aberrations in the reproductive organs of the senescence-accelerated mouse resistant 1 and SAMP8 males. Scale bars = 100 μ m. (B) Mature spermatozoa obtained from the caudal epididymides revealed the presence of cytoplasmic droplets in the terminally differentiated gametes (arrows). Scale bars = 10 μ m.

Figure 2 Sperm motility and mitochondrial membrane potential in the senescence-accelerated mouse prone 8 (SAMP8; black bars) and senescence-accelerated mouse resistant 1 (SAMR1; white bars) mice. (A) Computer-aided sperm assessment revealed that total motility in 4- and 8-month-old SAMP8 males was similar to that in age-matched SAMR1 males. (B) Progressive motility declined with age in the SAMP8 spermatozoa, being statistically significant at 8 months of age. (C) No statistically significant effect on mitochondrial membrane potential measured by JC1 was observed in the SAMP8 spermatozoa at 4 and 8 months of age. Motility and JC1 analyses were performed on at least three independent mice. Bars represent means $\pm$ S.E.M. * $P < 0.05$.

stress, computer-aided sperm assessment (CASA) was used to assess the motility of spermatozoa recovered from the caudal epididymides of 4- and 8-month-old males. As shown in Fig. 2A, total motility was maintained in the SAMP8 strain during the ageing process, being similar to that for the SAMR1 mice at each time point assessed. However, the percentage of spermatozoa displaying forward progressive movement was significantly lower for spermatozoa from 8-month-old SAMP8 mice compared with that of spermatozoa from age-matched SAMR1 controls (Fig. 2B; $P < 0.05$). This change was not accompanied by any detectable alternation of mitochondrial function, assessed using JC1, a cationic dye that indicates mitochondrial membrane potential (Fig. 2C). In order to determine whether the reduction in progressive sperm motility might be associated with increased free radical generation, DHE and MSR were employed to assess superoxide generation by the spermatozoa as a function of age. As shown in Fig. 3, there was no detectable difference in the levels of superoxide generated by the SAMR1 and SAMP8 spermatozoa, as detected with either probe (Fig. 3A and B). This lack of any strain-dependent differences in reactive oxygen species (ROS) generation was also confirmed by chemiluminescence utilising a luminol-peroxidase detection system. The latter did demonstrate an increase in ROS generation with time, but little difference was detected between the SAMR1 and SAMP8 spermatozoa (Fig. 3C).

Increased levels of oxidative DNA damage in the SAMP8 spermatozoa

The assessment of DNA integrity in the spermatozoa of the SAMR1 and SAMP8 males, as reported by the alkaline Comet assay, revealed a highly significant increase in the number of alkali-labile sites (abasic sites and unstable base adducts including oxidised bases) and strand breaks in the SAMP8 spermatozoa at 4 and 8 months of age (Fig. 4A; $P < 0.001$). The importance of oxidative stress in the generation of DNA

damage in spermatozoa was supported by parallel increases observed in the percentage of SAMP8 spermatozoa containing 8OHdG DNA adducts at 4 and 8 months of age (Fig. 4B; $P < 0.01$). Moreover, this increase in oxidative DNA damage was associated with a decrease in sperm chromatin protamination as reflected by highly significant increases in CMA3 fluorescence (Fig. 4C; $P < 0.001$).

Increased susceptibility to H_2O_2 -induced 8OHdG adduct formation in the SAMP8 spermatozoa

The hypothesis that SAMP8 mice are compromised in their ability to cope with oxidative DNA damage at the level of the mature gamete was supported by *in vitro* experiments involving the measurement of 8OHdG in spermatozoa exposed to increasing doses of H_2O_2 and Fe^{2+} at a ratio designed to promote Fenton chemistry. The results demonstrated an increased susceptibility of the SAMP8 spermatozoa to form oxidative DNA adducts at both doses of Fenton reagents (100 and 250 μ M) when compared with the SAMR1 controls (Fig. 5; $P < 0.001$). In light of this result, it would appear that normal mature spermatozoa have a functional base excision repair system capable of repairing oxidative damage in sperm DNA, which is noticeably defective in the SAMP8 mice.

Increased levels of 8OHdG and unresolved γ H2AX foci in the testes

To further explore the effect of the *Ogg1* mutation on DNA integrity in the male germ line, 8OHdG DNA adduct concentrations were quantified using LC-MS/MS from DNA extracted from whole-testis homogenates. This analysis revealed a significant increase in the concentration of 8OHdG adducts in the SAMP8 testis when compared with the SAMR1 testis (Fig. 6A; $P < 0.05$). Interestingly, immunohistochemistry also revealed an apparent rise in the number of unresolved γ H2AX foci in the SAMP8 sections at the late spermatid

Figure 3 Free radical generation in the senescence-accelerated mouse prone 8 (SAMP8; black bars) and senescence-accelerated mouse resistant 1 (SAMR1; white bars) spermatozoa. (A) The percentage of live spermatozoa generating superoxide anions from their mitochondria as detected by MitoSOX Red was similar for the SAMR1 and SAMP8 spermatozoa at 4 and 8 months of age. (B) There was also no detectable increase in the percentage of spermatozoa generating superoxide anions as detected by dihydroethidium. (C) Chemiluminescence utilising luminol–peroxidase as a reporter of ROS generation revealed a time-dependent increase in reactive species generation over 60 min; however, this increase was identical in the SAMR1 and SAMP8 spermatozoa. Experiments were performed on at least three independent mice. Bars represent means $\pm$ S.E.M.

stage compared with the SAMR1 sections (Fig. 6B). The formation of double-strand breaks is an essential process occurring at several places along the mitotic and meiotic pathways leading to full spermatogenesis (Blanco-Rodríguez 2009); however, residual double-strand breaks have the potential to negatively affect the offspring.

Discussion

It is well established that the excessive or uncontrolled production of ROS is a major mediator of DNA damage in human spermatozoa, which, in turn, is associated with poor reproductive outcomes and morbidity in the offspring (Aitken & Curry 2011). Until recently, sperm

chromatin has been considered to be metabolically silent by virtue of the high degree of chromatin compaction and complete lack of nuclear gene transcription, attributes that are unique to this cell type. The assumption that then followed from this consideration was that the mature male gamete was incapable of effecting DNA repair. However, it has recently been discovered that human spermatozoa possess a capacity for base excision repair of oxidised guanine residues via the action of OGG1 (Smith *et al.* 2013). Following exposure to H_2O_2 , spermatozoa are capable of excising oxidised base adducts from the DNA, which then appear in the extracellular space in a time- and dose-dependent manner (Smith *et al.* 2013). Moreover, this activity has been found to be sensitive to inhibition by cadmium (II), a well-characterised inhibitor of OGG1 activity (Smith *et al.* 2013). However, surprisingly, the base excision repair pathway in spermatozoa has been found to be restricted to the ability of OGG1 to excise 8OHdG adducts and form abasic sites. The next enzyme in the base excision repair pathway, apurinic endonuclease 1, responsible for the creation of 3'-OH groups necessary for lesion recognition and repair by polymerases, was found to be absent (Smith *et al.* 2013). The absence of 3'-OH groups immediately after an oxidative attack on spermatozoa had previously been indicated by the lack of a short-term response to such stress in the TUNEL assay, which is dependent on the availability of free 3'-OH DNA ends to enzymatically label the strand breaks (Ramos & Wetzels 2001, Smith *et al.* 2013). The essential scaffolding protein X-ray cross-complementing protein 1, common to a number of DNA repair pathways, has also been found to be absent in human spermatozoa, suggesting that the truncated base excision repair pathway present in these cells is confined to OGG1.

A complicated and largely uncharacterised suite of naturally occurring mutations plagues SAMP8 strains, culminating in the early onset of age-related morbidity related to oxidative stress and a markedly reduced life span (Bayram *et al.* 2012, Gan *et al.* 2012). This is significantly less than that observed in their accelerated senescence-resistant counterparts, denoted SAMR1, which served as the control strain for this study. A mutation uncovered in a previous investigation by Choi *et al.* (1999) in all SAM strains was an arginine to histidine substitution at codon 336 in the *Ogg1* gene. However, an additional mutation at codon 304 exclusive to the SAMP8 strain reduced the catalytic activity of this enzyme *in vitro* to 10–40% of that of the SAMR1 controls.

An analysis of the SAMP8 spermatozoa in relation to this impaired base excision repair pathway revealed a high level of DNA damage as measured by the Comet assay. Further characterisation of this damage revealed that it was largely oxidative in nature (Fig. 4B). Consistent with a mutation in *Ogg1*, the SAMP8

Figure 4 DNA integrity in caudal epididymal spermatozoa from the senescence-accelerated mouse prone 8 (SAMP8; black bars) and senescence-accelerated mouse resistant 1 (SAMR1; white bars) mice. (A) Comet analysis revealed highly significant increases in DNA damage in the SAMP8 spermatozoa at both 4 and 8 months compared with the SAMR1 controls. (B) The presence of 8-hydroxy-2'-deoxyguanosine adducts was also significantly elevated in the SAMP8 spermatozoa compared with the controls. (C) CMA3 staining similarly revealed significant defects in the efficiency of protamination at 4 and 8 months of age in the SAMP8 spermatozoa compared with their SAMR1 counterparts. Experiments were performed on at least three independent mice. Bars represent means $\pm$ S.E.M. ** $P < 0.01$, and *** $P < 0.001$.

spermatozoa also demonstrated an increased tendency to accumulate 8OHdG adducts in the presence of increasing concentrations of H_2O_2 and Fe^{2+} (Fig. 5). To further demonstrate that the increased DNA damage observed in the SAMP8 spermatozoa was a consequence of defective DNA repair rather than due to an increase in free radical generation, we utilised probes previously employed for the measurement of free radical generation in human spermatozoa (Aitken *et al.* 2013). In these analyses, no obvious signs of diminished mitochondrial membrane potential or excessive superoxide generation from the cytosol or mitochondria were evident (Figs 2C, 3A, B and C). In both SAMR1 and SAMP8 strains, a time-dependent increase in ROS generation was observed, signalling the onset of sperm capacitation (Ecroyd *et al.* 2003); however, no difference between the strains was evident in terms of the levels of ROS generation detected (Fig. 3C).

A slight but significant decrease in progressive motility was observed in spermatozoa from 8-month-old SAMP8 animals, and such a change is typical of spermatozoa experiencing oxidative stress (Gomez *et al.* 1998). In the absence of a change in superoxide anion formation, oxidative stress would be expected to arise as a consequence of a compromised antioxidant defence capability. In this context, the significant reductions in reduced glutathione (Nogués *et al.* 2006) and superoxide dismutase (Kurokawa *et al.* 2001) levels observed in the SAMP8 mice relative to the control SAMR1 strain would be expected to generate a state of oxidative stress throughout the body. The mechanisms underpinning these changes are not fully resolved but may involve a self-propagating cycle of oxidative damage, whereby the carbonyl modifications associated with free radical

attacks on proteins lead to the inactivation of antioxidant enzymes (Nabeshi *et al.* 2006), creating yet more oxidative stress and leading to a further deterioration of the antioxidant protection system.

The increase in 8OHdG adduct formation in whole testis DNA in the SAMP8 strain (Fig. 6A) is consistent with the observations of Choi *et al.* (1999), who observed an accumulation of 8OHdG in SAMP8 tissues and also elegantly demonstrated a decrease in the ability of OGG1 extracted from the SAMP8 testes to cleave 8OHdG in a controlled base excision experiment.

Figure 5 Increased susceptibility of the senescence-accelerated mouse prone 8 (SAMP8) spermatozoa to H_2O_2 -induced 8-hydroxy-2'-deoxyguanosine (8OHdG) adduct formation. Exposure of caudal spermatozoa to 0–250 μM H_2O_2 and Fe^{2+} at a 2:1 M ratio for 1 h revealed a highly significant increase in the vulnerability of the SAMP8 spermatozoa to 8OHdG adduct formation compared with the senescence-accelerated mouse resistant 1 (SAMR1) spermatozoa. Experiments were performed on at least three independent mice. Bars represent means $\pm$ S.E.M. *** $P < 0.001$.

Figure 6 Increased amounts of unrepaired 8-hydroxy, 2'-deoxyguanosine (8OHdG) adducts and unresolved γ H2AX foci in the senescence-accelerated mouse prone 8 (SAMP8) testes. (A) Mass spectrometric analysis of 8OHdG adducts in DNA extracted from whole testes revealed a significant increase in the ratio of 8OHdG adducts normalised to dGMP in the SAMP8 mice relative to the senescence-accelerated mouse resistant 1 (SAMR1) controls. Experiments were performed on at least three independent mice. Bars represent means $\pm$ S.E.M. * $P < 0.05$. (B) Immunohistochemistry using an antibody raised against γ H2AX revealed an apparent increase in the presence of this marker of double-strand breaks in the SAMP8 testis sections in the later stages of spermatogenesis. Experiments were performed in triplicate. Representative images are shown. Scale bars = 200 μ m.

Furthermore, in the SAMP8 seminiferous tubules, the presence of unresolved γ H2AX foci in spermatozoa was markedly increased compared with the SAMR1 strain, again emphasising the particularly high level of DNA strand breakage in these OGG1-compromised cells (Fig. 6B). Such breaks are known to occur in late spermatids as a means of relieving the torsional stress associated with sperm chromatin compaction; however, these lesions are subsequently labelled with γ H2AX and repaired in a topoisomerase-dependent process. This observation highlights the limited ability of post-meiotic haploid germ cells to address the increase in DNA strand breakage secondary to 8OHdG accumulation in SAMP8 mice and the resulting persistence of strand breaks from spermiogenesis onwards.

The finding that these oxidatively stressed spermatozoa exhibit poor chromatin compaction is in keeping with the results obtained in another oxidative stress model, the *Gpx5* knockout mouse (Chabory *et al.* 2009). Indeed, this inverse relationship between DNA compaction and DNA fragmentation has been observed on many occasions, including in the infertile population where CMA3 fluorescence is positively correlated with the presence of 8OHdG adducts and reactivity in the TUNEL assay (De Iuliis *et al.* 2009). The presence of CMA3 staining is positively associated with abnormal nuclear remodelling, including the presence of large nuclear vacuoles (Franco *et al.* 2012), and negatively correlated with pregnancy rates in assisted conception cycles (Sakkas *et al.* 1998, Nasr-Esfahani *et al.* 2001, Esterhuizen *et al.* 2002). CMA3 is believed to indicate the extent to which sperm chromatin is protaminated (Bizzaro *et al.* 1998). The positive relationship between DNA base oxidation and CMA3 fluorescence may reflect the powerful protective role normally played by cysteine-rich protamines, by virtue of the latter's ability to cross-link and stabilise the chromatin through the creation of intermolecular and intramolecular disulphide bridges (Balhorn 1982). Furthermore, the residual free thiols that are not involved in chromatin stabilisation

can function as sacrificial antioxidants or chelators of metals involved in the promotion of free radical reactions (Kasprzak 2002). If protamine thiols are alkylated by electrophilic lipid aldehydes generated as a consequence of oxidative stress, then they will not be able to protect the spermatozoa by stabilising the DNA or by acting as sacrificial antioxidants and the cells will be vulnerable to oxidative attack and 8OHdG adduct formation. Intriguingly, the protamines of metatherian mammals lack cysteines and, as a consequence, their chromatin is particularly vulnerable to oxidative DNA damage compared with eutherian spermatozoa (Bennetts & Aitken 2005). Within the Eutheria, the resistance of spermatozoa to oxidative attack increases with the number of cysteine residues present in the major sperm protamines (Enciso *et al.* 2011).

The discovery of functional OGG1 in human spermatozoa and the results obtained in this study indicating that mutations in the *OGG1* gene can affect the genetic integrity of the male germ line raise interesting questions regarding the impact of polymorphisms in the *OGG1* gene in humans and the occurrence of paternally derived *de novo* mutations. The Ser326Cys mutation occurs frequently in the human population and is reportedly associated with the development of cancer (reviewed by Karahalil *et al.* (2012)). Previous studies have identified a kinetic effect of this *OGG1* polymorphism, reducing the rate of 8OHdG excision by three- to fourfold (Zielinska *et al.* 2011, Kershaw & Hodges 2012). However, the potential effects of Ser326Cys *OGG1* germline mutations on human fertility and the transmission of potentially mutagenic 8OHdG base adducts to the offspring are yet to be determined.

It is remarkable that despite the high levels of oxidative DNA damage observed in the male germ line of the SAMP8 mice, fertility was not compromised. Significantly, however, the offspring produced by these mice are plagued with morbidity (reviewed in Takeda *et al.* (1997a, 1997b)), highlighting the significance of paternally derived 8OHdG adducts in transmitting highly

mutagenic DNA lesions on to the offspring. There are important implications of these results for the safety of assisted reproductive technologies involving the use of oxidatively damaged spermatozoa to achieve conceptions *in vitro* that would have been impossible *in vivo*. This study also highlights the need to study the ability of the oocyte to complete the base excision repair pathway initiated by OGG1 in the male germ line and to determine whether the ability of the oocyte to complete this task might be influenced by factors such as maternal age or exposure to environmental toxicants.

Declaration of interest

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

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