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www.publish.csiro.au/journals/rfd *Reproduction, Fertility and Development*, 2011, **23**, 633–637

Differential resistance of mammalian sperm chromatin to oxidative stress as assessed by a two-tailed comet assay

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Abstract. Protamines of eutherian species are cysteine-rich molecules that become cross-linked by disulfide bonds during epididymal transit, whereas the protamines of most marsupial species lack cysteine residuals. The present study made use of the differences in protamine structure between eutherian and metatherian mammal spermatozoa to examine the comparative resistance of sperm DNA to oxidative damage in three eutherian species (*Mus musculus*, *Homo sapiens*, *Sus domesticus*) and three metatherian species (*Vombatus ursinus*, *Phascolarctos cinereus*, *Macropus giganteus*). Sperm DNA fragmentation of samples exposed to increasing concentrations of hydrogen peroxide was assessed by means of the two-tailed comet assay. The sperm DNA of the marsupial species studied were significantly more sensitive to oxidative stress than the spermatozoa of eutherian species. Such susceptibility is consistent with the lack of disulfide cross-linking in marsupial sperm chromatin and suggests that the oxidation of thiols to disulfides for chromatin condensation during epididymal transit in eutherian mammals is likely to be important in order to provide stability and protect these cells from the genotoxic effects of adverse environments.

Introduction

It is well known that structural remodelling of sperm chromatin helps in the packaging and protection of the nuclear genome. During animal spermiogenesis, the cell nucleus commences a progressive condensation process that results in the replacement of sperm nuclear histones to transitional proteins and ultimately, protamines. Although sperm nuclear condensation is considered an essentially conserved process, the diversity of spermatic protein composition and structure is vast. These proteins vary from species to species and even within populations of the same species (Ausió 1995).

The protamines of all eutherian species thus far studied are cysteine-rich molecules that become extensively cross-linked by disulfide bonds during epididymal transit; this covalent cross-linking stabilises eutherian sperm nuclei (Balhorn 1989). By contrast, the metatheria have a very different structural organisation of the sperm head in that all species, except in genus *Planigale* (Retief *et al*. 1995), lack cysteine residues and, hence, disulfide bonds within their chromatin (Bedford 1991). It is thought that the disulfide bonds formed between and within the eutherian protamines make the spermatozoa highly resistant to a variety of agents, such as acids, proteases, DNAse and detergents, whereas marsupial spermatozoa are more susceptible to such exposure (Cummins 1980). Such comparative difference in sperm DNA packaging is also likely to have implications in regards to the protection of sperm chromatin during transport and fertilisation in the female reproductive tract and during manipulative procedures such as *in vitro* incubation or cryopreservation (Zee *et al*. 2009).

In this study, we try to elucidate whether the difference in the protamine structure in spermatozoa between eutherian and metatherian mammals confers any detectable difference in stability and resistance to sperm chromatin when the spermatozoa are exposed to dose-response oxidative DNA damage. For this purpose, semen samples from three eutherian species (*Sus domesticus*, *Homo sapiens*, *Mus musculus*) and three metatherian species (*Phascolarctos cinereus*, *Macropus giganteus*, *Vombatus ursinus*) were treated with increasing concentrations of hydrogen peroxide (H_2O_2), a known producer of single-stranded DNA breaks (ss-Bs; Enciso *et al*. 2009).

Materials and methods

Sperm preparation

Sus domesticus

Two normozoospermic samples from two fertile adult boars aged 24 months were used in the present study. All specimens were collected using the gloved hand method.

Homo sapiens

Two normozoospermic semen samples were collected from two fertile men after an abstinence period of 48–72 h. All specimens were collected by masturbation and allowed to liquefy completely for 15–30 min at 37°C.

10.1071/RD10269 1031-3613/11/050633

Mus musculus

Two normozoospermic semen samples were collected from two fertile adult male mice from a heterogeneous population. The epididymis of each mouse was dissected and spermatozoa recovered from the *cauda* epididymides were allowed to swim out of dissected tubule for 10 min into 0.2 mL prewarmed phosphate-buffered saline (PBS; 37°C; Merck, Darmstadt, Germany).

Phascolarctos cinereus

Two normozoospermic semen samples were collected from two fertile adult male koalas donated from Dreamworld (Queensland, Australia). Semen samples were collected and frozen as described by Johnston *et al*. (2006).

Macropus giganteus

Two normozoospermic semen samples were collected from two fertile adult male eastern grey kangaroos from Amby (Queensland, Australia). Semen samples were collected and frozen as described by McClean *et al*. (2008).

Vombatus ursinus

Two normozoospermic semen samples were collected from two fertile adult male common wombats located at Western Plains Zoo (Dubbo, NSW, Australia). Semen samples were collected and frozen as described by Johnston *et al*. (2006).

Straws from all marsupial species were thawed in a thermal bath at 37°C for 30 s.

For all eutherian species and *P.cinereus*, only semen samples that laid within the normal ranges of sperm DNA fragmentation established in the literature for fertile males were analysed (Evenson *et al*. 1999; López-Fernández *et al*. 2008; Sarabia *et al*. 2009; Johnston *et al*. 2007); this information for *V. ursinus* and *M. giganteus* is currently not available. All eutherian and metatherian semen samples were diluted to a concentration of 10 million spermatozoa mL^{-1} in PBS (Merck, Darmstadt, Germany).

Oxidative damage

Induction of ss-Bs was performed using H_2O_2 (Sigma, St Louis, MO, USA) Each semen sample, containing 10 million spermatozoa mL^{-1} in PBS, was divided into four 200-μL aliquots and exposed to increasing concentrations of H_2O_2 (0.003%, 0.03% and 0.3% v/v) for 30 min at room temperature.

DNA damage: two-tailed comet assay

The two-tailed comet assay (TTC) was performed as described by Enciso *et al*. (2009). This assay combines neutral and alkaline comet assay protocols providing information about both ss-Bs and double-stranded DNA breaks (ds-Bs). Briefly, each semen sample was diluted into freshly prepared 1% low melting point agarose and used to form a microgel on a precoated slide. Slides were then submerged sequentially in two lysing solutions, rinsed in Tris/Borate/EDTA (TBE) buffer and transferred to an electrophoresis tank. Electrophoresis was performed in fresh TBE buffer. Sperm DNA was then unwound in an alkaline solution and transferred back to the electrophoresis tank, where electrophoresis was performed in fresh alkaline buffer. Finally, the slides were rinsed once in a neutralisation buffer, washed briefly in TBE buffer, dehydrated in increasing concentrations of ethanol and air dried.

The DNA was stained with SYBR Green I (Molecular Probes, Leiden, The Netherlands) at a 1 : 3000 dilution in Vectashield (Vector Laboratories, Burlingame, CA, USA). Comets were assessed by visual scoring using a Leica DMLA model motorised epifluorescence microscope (Leica Microsystems, Barcelona, Spain). The frequency of sperm cells with single-strand (single-strand DNA fragmentation index; ss-DFI) and double-strand DNA damage (double-strand DNA fragmentation index; ds-DFI) was determined by measuring at least 500 spermatozoa per slide, as described by Enciso *et al*. (2009).

Induction of ds-Bs in P. cinereus *spermatozoa*

In order to confirm the ability of the TTC to detect ds-Bs in koala spermatozoa, the *Alu*I restriction enzyme was used to induce ds-Bs in koala sperm nucleoids, as described for human spermatozoa by Enciso *et al*. (2009).

Statistical analysis

A two-way ANOVA (factors: H_2O_2 treatment and taxa) was carried out to statistically to compare the mean ss-DFIs of the eutherian and metatherian groups across different concentrations of H_2O_2. Dunnett's test was used to assess the post hoc significance of each value in the different concentrations *v.* control. A significance level of 5% ($\alpha = 0.05$) was used for all statistical tests performed.

Results

Figure 1*a* shows the results of the TTC when conducted on human spermatozoa. The comet tail orientated along the *x*-axis (first electrophoresis) is representative of migrated fragmented ds-DNA, whereas the comet tail orientated along the *y*-axis (second electrophoresis) is composed of ss-DNA that was immobilised DNA following the first electrophoresis (Enciso *et al*. 2009). Based on the behaviour of the DNA fragments in the electrophoresis gel and according to the principle that the larger the tail and/or the higher the DNA density in the tail, the greater the extent of the DNA damage, the TTC, as exemplified by the koala, produced six different types of DNA migration patterns (see Fig. 1*b*). In a normozoospermic koala semen sample, only undamaged condensed, undamaged decondensed and single-strand DNA damaged types (i.e. 1, 2 and 3 in Fig. 1*b*) are found; none of the comet types containing ds-Bs is present. To confirm that the TTC protocol used was able to detect spermatozoa containing double-strand DNA damage, samples were treated with an active producer of ds-Bs, namely the *Alu*I restriction enzyme. Results from this treatment confirmed the presence of the comet types containing ds-Bs (i.e. 4, 5 and 6 in Fig. 1*b*) in the semen samples damaged enzymatically to produce such breaks. This treatment confirmed the efficacy of the TTC to detect ds-Bs in koala spermatozoa.

The effect of H_2O_2 on the ss-DFI of each species is shown in Fig. 2*a*.

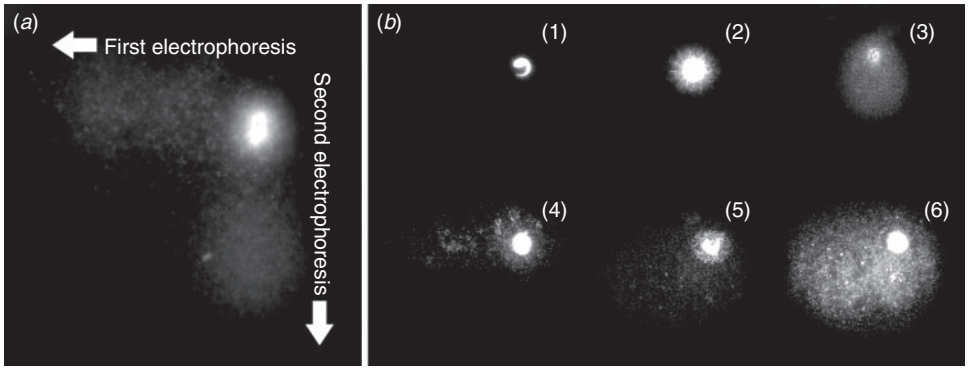

Fig. 1. Two-tailed comet (TTC) assay. (*a*) Results of the TTC for human spermatozoa, showing the two characteristic masses of DNA according to the distribution of double- or single-stranded DNA damage. (*b*) Six TTC types were found in a semen sample from *Phascolarctos cinereus*: (1) undamaged condensed; (2) undamaged decondensed; (3) single-strand (ss) DNA damage; (4) double-strand (ds) DNA damage; (5) low levels of ss-DNA and ds-DNA damage; and (6) high levels of ss-DNA and ds-DNA damage. Images from different fields were grouped in this figure.

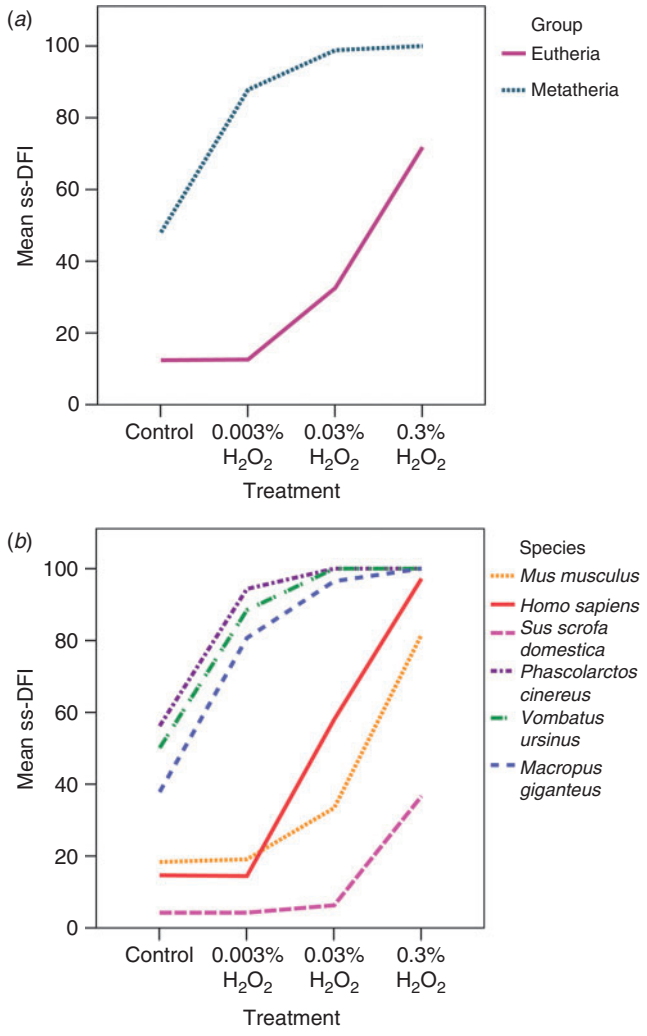

Fig. 2. Mean single-stranded DNA fragmentation index (ss-DFI) of H_2O_2-treated samples analysed. (*a*) Mean ss-DFI of the eutherian and metatherian groups analysed. (*b*) Mean ss-DFI of each of the animal species analysed.

In both metatherian and eutherian species, the frequency of spermatozoa containing single-stranded DNA damage increased with H_2O_2 concentration. The ss-DFI of all marsupial spermatozoa was significantly higher ($P < 0.05$, two-way ANOVA) than in eutherian samples across all H_2O_2 doses examined (Fig. 2*a*). At the lowest H_2O_2 dose, there was no adverse effect detected on eutherian sperm nuclei. Differences for the interspecific production of ss-Bs in each species are shown in Fig. 2*b*. Of the eutherian species examined in the present study, boar spermatozoa showed a marked resistance to H_2O_2 exposure, except at the highest concentration used (Fig. 2). No significant increase in the level of ds-Bs of any species was found after H_2O_2 treatment.

The resistance of spermatozoa to H_2O_2 exposure was found to be directly related to the proportion of protamine cysteine residuals (disulfide bonding) present in the spermatozoa of the species studied (Fig. 3). The number of cysteine residues found in protamine 1 was negatively associated with the damaging effect of H_2O_2.

It is also important to note that the control ss-DFI of the marsupial group was higher than the one determined for the eutherian species analysed (Fig. 1).

Discussion

The results of the present study confirm that marsupial sperm nuclei are much more sensitive to oxidative attack than those of eutherian species. A significantly higher proportion of DNA-damaged spermatozoa were found in all marsupial species analysed for all H_2O_2 doses used compared with eutherian species. Several authors have already shown a higher vulnerability of marsupial sperm nuclei when exposed to different treatments (Cummins 1980; Balhorn 1989). Bennetts and Aitken (2005) also provided evidence of the higher vulnerability of marsupial spermatozoa towards oxidative stress when compared to eutherian spermatozoa. It has been proposed that this vulnerability is associated with a lack of cysteine residues in sperm protamines. In eutherian mammals, the protamines are cysteine-rich molecules that become oxidised to form a

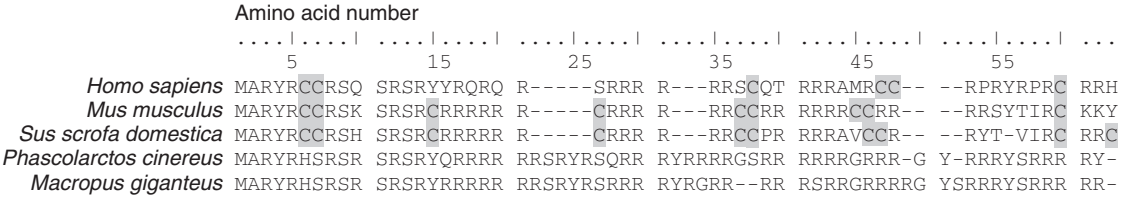

Fig. 3. Distribution of cysteine residues within protamine 1 amino acid sequences. Aligned P1 protamine protein sequences of *Homo sapiens*, *Mus musculus*, *Sus scrofa domestica*, *Phascolarctos cinereus* and *Macropus giganteus*. The single letter nomenclature for amino acids follows the International Union of Pure and Applied Chemistry- International Union of Biochemistry (IUPAC-IUB) rules (IUPAC-IUB 1970). Taken from the GenBank database (accession numbers: *Homo sapiens*, NP_002752; *Mus musculus*, NP_038665; *Sus scrofa domestica*, 0905175A; *Phascolarctos cinereus*, AAB88911; *Macropus giganteus* AAA74604).

three-dimensional network of disulfide bonds between, and within, protamine molecules in the sperm nucleus (Bedford 1991). Eutherian sperm protamines are thought to help stabilise and protect the DNA from damage (Bennetts and Aitken 2005).

The differences in response of metatherian and eutherian spermatozoa to oxidative damage found in the present study are independent of freeze–thawing process experienced by marsupial samples. Several studies have shown that the chromatin structure of spermatozoa remains unaltered after the cryopreservation process in a wide range of animal species, including koala (Johnston *et al*. 2007). In terms of their susceptibility towards oxidative stress, we assumed that if DNA remains unaltered, the susceptibility towards oxidative stress would also remain unaltered. The differential resistance to oxidative stress described here could be explained by the different mechanism used by each taxa for DNA packaging. The use and lack of disulfide bonding in eutherian and metatherian spermatozoa, respectively, is likely to have an evolutionary significance associated with sperm transport and fertilisation biology. In eutherian species, the development of highly resistant sperm chromatin has been proposed as an adaptation to prevent damage to the sperm nucleus during migration of the spermatozoa along extensive male and female reproductive tracts and in the penetration of the thickened zona pellucida. Moreover, because the eutherian zona pellucida is far more resistant to proteolysis than that of marsupials, it is possible that eutherian spermatozoa have evolved a strategy for zona penetration based on a cutting or thrusting action requiring stabilisation of the DNA (Bedford 1991).

Apart from the differences found in the spermatozoa of both mammalian taxa, there were also some differences in the resistance to oxidation between the sperm nuclei of the different species studied, particularly within the eutherian group. Boar spermatozoa showed a marked resistance to oxidative stress, greater than that of the other eutherian spermatozoa analysed. This stability may be also related to the amount of protamine cysteine residuals susceptible for disulfide bonding. Interspecific differences in nuclear sperm stability are thought to be related to the number and/or the efficiency of the disulfide bonds, which may be determined by the type(s) of protamine(s) present (Bennetts and Aitken 2005). In the species analysed in the present study, the number of protamine 1 cysteine residues was negatively associated with the susceptibility of the spermatozoa to oxidative stress. Under the experimental conditions described herein, human sperm chromatin was shown to be more sensitive than that of mice or boar. The TTC revealed a slight increase in the ss-DFI in the human spermatozoa exposed to 0.003% H_2O_2, and a very high level of DNA damage when these spermatozoa were exposed to 0.03% H_2O_2, compared with the moderate DNA damage produced in the mouse and the absence of any damage in boar spermatozoa. Similar differences in sensitivity to H_2O_2 between human and mouse spermatozoa were also reported by Bennetts and Aitken (2005). Another recent study evaluated the susceptibility of sperm chromatin from different eutherian species to DNAse I and H_2O_2 (Villani *et al*. 2010). The results of that study revealed a different sensitivity to DNAse I treatment among the species studied, in agreement with the number of protamine 1 cysteine residues rationale described above. However, no major differences between species were found after H_2O_2 treatment (Villani *et al*. 2010). This may be due to the different H_2O_2 treatments and concentrations used, as well as to the different techniques used to detect DNA damage.

It is also important to note the differences found in the ss-DFI of untreated samples between the eutherian and metatherian groups. The extensive content of single-strand breaks in the untreated marsupial samples is likely to be a structural feature of the normal metatherian nucleus as described in the koala sperm nucleus by Zee *et al*. (2009). This feature may be related to the absence of ds-Bs in normozoospermic koala samples found in the present study. Nevertheless, the enzymatic treatment performed in the normozoospermic koala samples confirmed the ability of the TTC protocol to detect not only ss-Bs, but also ds-Bs in spermatozoa in this species and hence the lack of ds-Bs in the normozoospermic samples analysed.

In conclusion, the present study has revealed, using the TTC assay, that the DNA of wombat, kangaroo and koala spermatozoa was significantly more sensitive to oxidative stress than the spermatozoa of eutherian species. Such susceptibility is consistent with the lack of disulfide cross-linking in marsupial sperm chromatin and suggests that the oxidation of thiols to disulfides for chromatin condensation during epididymal transit in eutherian mammals provides stability and protects these cells against adverse environments. In addition, the present study demonstrates the use of TTC for spermatozoa in a wide variety of species. The TTC assay is a very sensitive and highly effective technique for the simultaneous evaluation of both single- and double-strand breaks in the spermatozoa of eutherian and metatherian species.

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Manuscript received 19 October 2010, accepted 11 January 2011