Protection of sperm DNA against oxidative stress *in vivo* by accessory sex gland secretions in male hamsters

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Reactive oxygen species scavengers present in male accessory sex gland secretions might afford antioxidant protection to sperm DNA. This study was conducted to determine whether accessory sex gland secretions protect the genome and function of spermatozoa against oxidative damage in the uterus. Male golden hamsters were divided into four experimental groups: (i) all accessory sex glands removed; (ii) ampullary glands removed; (iii) ventral prostate gland removed and (iv) sham-operated controls. Ejaculated spermatozoa recovered from uteri 15–30 min after mating with experimental males and caput and cauda epididymal spermatozoa obtained from intact males were incubated in 0–20 mmol NADPH l⁻¹ for 2 h. These spermatozoa and untreated uterine spermatozoa were processed for two types of comet assay (single cell gel electrophoresis): alkaline comet assay (pH > 13) which revealed single-strand DNA breakage and neutral comet assay (pH 9) which revealed double-strand DNA breakage. In comparison

with the sham-operated controls, spermatozoa that had not been exposed to accessory sex gland secretions had a higher incidence and more extensive single-strand DNA damage with increasing concentrations of NADPH. Spermatozoa from hamsters without ampullary glands and from hamsters without the ventral prostate glands were similar to those of the control group. After incubation with NADPH, the capacity of spermatozoa from hamsters without accessory glands and from sham-operated controls to fuse with oocytes *in vitro* was reduced. However, only hamsters without accessory glands showed a negative correlation between single-strand DNA damage and sperm–oocyte fusion. Cauda epididymal spermatozoa were less susceptible to NADPH treatment compared with caput epididymal spermatozoa. The results of the present study showed that male accessory sex gland secretions can preserve the integrity of the sperm genome.

Introduction

In golden hamsters, the main male accessory sex glands include the ampullary glands, ventral prostate gland, dorso-lateral prostate glands, coagulating glands and seminal vesicles. The secretions of these glands contain antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, and free radical scavengers, such as vitamins C and E, hypotaurine, taurine, uric acid and albumin. These small molecular scavengers are thought to contribute significantly to antioxidant protection of spermatozoa (van Overveld *et al.*, 2000). In addition, albumin present in human seminal plasma protects spermatozoa against peroxidative damage by absorbing lipid peroxides from the plasma membrane of spermatozoa (Twigg *et al.*, 1998).

Reactive oxygen species (ROS) are free radicals, such as superoxide anions, hydroxyl radicals and H₂O₂. ROS may influence the structural integrity and function of

spermatozoa, such as motility, capacitation and sperm–oocyte fusion (Jones and Mann, 1976; Aitken, 1995; Griveau *et al.*, 1995; de Lamirande and Gagnon, 1995a,b). A fine balance to maintain an optimal amount of ROS is affected by ROS generated during sperm maturation in the epididymis and antioxidant factors in secretions of the male reproductive system. Spermatozoa experience adverse oxidative stress once this balance is lost. ROS have been shown to reduce male fertility (Iwasaki and Gagnon, 1992), cause peroxidative damage to the sperm plasma membrane (Hughes *et al.*, 1996) and induce both DNA strand breakages and oxidative base damage in human spermatozoa (Kodama *et al.*, 1997). In addition, a decrease in the total antioxidant capacity of seminal plasma has been correlated with a reduction in sperm quality factors, such as concentration, motility and morphology (Lewis *et al.*, 1994; Palan and Naz, 1996; Smith *et al.*, 1996; Sharma *et al.*, 1999).

It has been shown that removal of all or some of the accessory sex glands in the golden hamster reduces male fertility with respect to a decrease in preimplantation cleavage, implantation rate and an increase in post-implantation abnormality or loss of embryos (O *et al.*, 1988; Jiang *et al.*,

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2001; Chan *et al.*, 2001). Oocyte activation, sperm decondensation and DNA synthesis during the first cell cycle were also adversely affected (Ying *et al.*, 1998; 1999a,b). We proposed that damage to sperm genomic integrity might be one of the causes.

Comet assay, also known as single cell gel electrophoresis, has been applied to detect the integrity of sperm DNA. This is a rapid, simple, visual and sensitive technique for measuring alkali labile sites and DNA strand breakages in individual mammalian cells (Ostling and Johanson, 1984; Singh *et al.*, 1988; Olive *et al.*, 1990). The introduction of an alkaline (pH > 13) condition to unwind, denature and separate by electrophoresis makes the relationship of single-strand DNA (ssDNA) breakage more obvious. During electrophoresis, the fragmented DNA migrates to form a comet-like image. The 'head' consists of intact DNA, whereas the 'tail' contains fragmented DNA. Furthermore, the assay can detect different types of DNA damage according to the pH of the buffers used. In highly alkaline buffer (pH > 13), the assay can expose nicks, apurine/aprimidine sites and other alkaline labile sites, and detect the ssDNA damage. In neutral buffer (pH 9), the assay can be applied to analyse double-strand DNA (dsDNA) breaks.

The aim of the present study was to apply the comet assay and sperm–oocyte fusion test to assess the susceptibility of sperm DNA to ROS damage and functional integrity in relation to bilateral ablation of male accessory sex glands in the golden hamster.

Materials and Methods

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Tyrode's albumin lactate pyruvate (TALP, pH 7.4 (pH 7.7 before gassing), 285–290 mOsm) medium was prepared with Milli-Q water and composed of 114.0 mmol NaCl l⁻¹ (BDH, Poole), 3.2 mmol KCl l⁻¹, 2.0 mmol CaCl₂ · 2H₂O l⁻¹, 0.4 mmol NaH₂PO₄ · H₂O l⁻¹, 5.0 mmol D-glucose l⁻¹ (E. Merck, Darmstadt), 0.5 mmol MgCl₂ · 6H₂O l⁻¹, 25.0 mmol NaHCO₃ l⁻¹, 10.0 mmol sodium lactate l⁻¹, 0.1 mmol sodium pyruvate l⁻¹, 0.1 mmol sodium penicillin G l⁻¹, 0.01 mg phenol red ml⁻¹ and 3.0 mg BSA ml⁻¹. Modified TALP (mTALP) medium for capacitation contained 0.5 mmol taurine l⁻¹, 0.05 mmol adrenaline l⁻¹ (CALBIOCHEM, San Diego, CA) and 15 mg BSA ml⁻¹. PBS (10 × PBS, pH 7.4) composed of 80.1 g NaCl, 2.0 g KCl, 11.5 g Na₂HPO₄ (Fisher, Fairlawn, NJ) and 2.0 g KH₂PO₄ (WAKO, Osaka) in 1 l Milli-Q water, was diluted 10-fold just before use.

Animal model

Animals were maintained and handled in compliance with a protocol approved by the Committee on the Use of Live Animals for Teaching and Research of The University of Hong Kong. Randomly bred Syrian hamsters (*Mesocricetus auratus*) were supplied by and housed in the Laboratory Animal Unit (Faculty of Medicine, The University of Hong Kong) under a 14 h light:10 h dark photoperiod, lights on at

11:00–01:00 h, at 22°C. Food and tap water were available *ad libitum*. Vaginal secretions of 6–8-week-old female hamsters were checked daily for at least two normal consecutive oestrous cycles before mating.

The main male accessory sex glands were surgically ablated from 6–8-week-old male hamsters as described by Chow *et al* (1986) to give the following four groups: (i) bilateral excision of ampullary glands (AGX, *n* = 8); (ii) excision of ventral prostate (VPX, *n* = 8); (iii) bilateral excision of the ampullary glands, ventral prostate, dorsolateral prostates, coagulating glands and seminal vesicles (TX, *n* = 10) and (iv) sham-operated control hamsters (SH, *n* = 8). The animals were allowed to recuperate for 1 month and the success of surgery was confirmed post-mortem.

Preparation of spermatozoa

Collection of epididymal spermatozoa. Eight- to twelve-week-old male hamsters of proven fertility were killed with an overdose of sodium pentobarbitone injected i.p. (Boehringer Ingelheim, Artarmon). The caput and cauda epididymides were removed. After clearing blood and connective tissue, small incisions were made to release spermatozoa. The spermatozoa were washed twice in TALP medium and centrifuged at 300 *g* for 10 min to form a pellet.

Collection of uterine spermatozoa. Each normal cyclic female hamster was mated with one operated male for 15 min on the day of oestrus and killed with an overdose of sodium pentobarbitone within 30 min after mating. The uterine horns were removed and cleaned of blood and connective tissue. Spermatozoa collected from the uteri of two females by flushing with TALP medium were pooled together, washed and centrifuged twice at 300 *g* for 10 min.

Treatment of spermatozoa with β-NADPH

Uterine spermatozoa ejaculated by males from all four experimental groups were subjected to comet assay. These spermatozoa, together with caput and cauda epididymal spermatozoa from intact males were incubated at a final concentration of 1 × 10⁶ cells ml⁻¹ for 2 h in 0, 1.25, 2.5, 5.0, 10.0 and 20.0 mmol NADPH l⁻¹ at 37°C in 5% CO₂. After incubation, the treated spermatozoa were centrifuged twice at 300 *g* for 10 min to form a pellet and resuspended in fresh medium for comet assay and sperm–oocyte fusion.

Sperm–oocyte fusion

Female hamsters were killed by an overdose of sodium pentobarbitone on the day of oestrus. Oocytes were recovered from the oviducts by flushing with BSA-free TALP. Cumulus cells and zonae pellucidae were removed by incubation in 0.1% (w/v) hyaluronidase and 0.05% (w/v) trypsin. Oocytes were washed twice with TALP containing BSA. The zona pellucida-free oocytes were dispensed into the prepared sperm droplets (five oocytes per droplet of spermatozoa, which was repeated three times per concen-

tration of NADPH treatment as described previously). After incubation at 37°C in 5% CO₂ for 6 h, the oocytes were collected and washed free of loosely adhered spermatozoa, transferred on to silanized slides (DAKO, Glostrup), air-dried overnight and fixed in 70% ethanol for 30 min. The slides were treated with 0.1% (v/v) Tween-20 and Triton-X100 in PBS for 20 min in a humid chamber followed by three washes in PBS. The oocytes were stained with 10 µg propidium iodide ml⁻¹ (Molecular Probes, Eugene, OR) for 10 min and observed under an epifluorescence microscope (Zeiss) fitted with 510–560 nm excitation filter, 580 nm dichromatic mirror and 590 nm emission filter. The number of decondensed sperm heads per oocyte was counted.

Assessment of sperm DNA damage by the comet assay

The comet assay was adapted from Shen and Ong (2000) and was performed in the dark. Spermatozoa were resuspended in PBS at $6\text{--}8 \times 10^5$ cells ml⁻¹. Ten microlitres of sperm suspension was mixed with 75 µl (0.5%, w/v) agarose SFRTM (Amresco, Solon, OH). After mixing thoroughly, 50 µl of the sperm suspension was pipetted on to a CometSlide (Trevigen, Gaithersburg, MD) and allowed to gel. The slides were submersed carefully in cold lysis buffer (2.5 mol NaCl l⁻¹, 100.0 mmol EDTA l⁻¹, 10.0 mmol Tris-HCl l⁻¹, 10% (v/v) dimethyl sulphoxide and 1% (v/v) Triton-X100, pH 10) for 1 h at 4°C. For the neutral comet assay, the slides were pretreated with buffer (2.5 mol NaCl l⁻¹, 100.0 mmol EDTA l⁻¹, 10.0 mmol Tris-HCl l⁻¹, pH 7.4) containing 10 µg RNase ml⁻¹ (Amresco) and incubated for 2 h at 37°C. The slides were then transferred to another buffer (2.5 mol NaCl l⁻¹, 100.0 mmol EDTA l⁻¹, 10.0 mmol Tris-HCl l⁻¹, pH 7.4) containing 10 µg DNase-free proteinase K ml⁻¹ (Amresco) and incubated at 37°C overnight. For the alkaline comet assay, slides were handled in the same way as for the neutral comet assay, without pre-treatment.

After enzyme treatment, the slides were transferred into the electrophoresis chamber (Hoefer Pharmacia Biotech, San Francisco, CA) filled with neutral buffer (300 mmol sodium acetate l⁻¹ and 100 mmol Tris-HCl l⁻¹, pH 9) or alkaline buffer (300 mmol NaOH l⁻¹ (E. Merck), 1 mmol EDTA l⁻¹, 0.2% (v/v) dimethyl sulphoxide and 0.1% (w/v) 8-hydroxyquinoline, pH > 13). The slides were left side by side in the respective buffer to allow unwinding of the DNA for 20 min, before electrophoresis at 0.96 V cm⁻¹, 100 mA (for neutral comet assay) or 250 mA (for alkaline comet assay) for 20 min at room temperature. After electrophoresis, the slides were neutralized in 0.4 mol Tris-HCl l⁻¹ (pH 7.4) for 15 min, fixed in 100% ethanol for 15 min and then air-dried. The dried slides were treated in 10 mmol NaH₂PO₄ l⁻¹ and 5% (w/v) sucrose for 10 min and finally stained with 0.25 mmol YOYO[®]-1 l⁻¹ (Molecular Probes) in 5% (v/v) dimethyl sulphoxide and 5% (w/v) sucrose for 10 min.

CometSlides were viewed at $\times 250$ under a Zeiss fluorescence microscope. The incidence of spermatozoa with a comet was scored from each circle of the CometSlide (two

circles) and expressed as the number of comets per 10 000 spermatozoa. The comets were captured with a cooled CCD camera (KX Series Imaging System, Apogee Instruments Inc, Tucson, AZ) connected to the Zeiss fluorescent microscope and the images were evaluated for tail moment from 50 randomly selected comets using a Euclid Comet Analysis Software (Euclid Analysis, St Louis, MO). DNA damage was quantified as tail moment, which was defined as the fraction of DNA in the tail multiplied by the distance of fragmented DNA from the centre of the head (Olive *et al.*, 1990).

Statistical analysis

All results are presented as mean $\pm$ SEM. The incidence of DNA damage and sperm function (expressed as the number of decondensed spermatozoa per oocyte) was arcsine and square root transformed, respectively, before statistical analyses (Prism software version 3.0, GraphPad, San Diego, CA). All results, including transformed or original data were analysed by one-way ANOVA followed by Dunnett's post-test. Two-way ANOVA followed by Bonferroni post-test was also applied to analyse the response of caput and cauda epididymal spermatozoa as well as uterine spermatozoa to NADPH incubation and sperm-oocyte fusion test. The distribution of comet patterns in relation to increasing amounts of NADPH in caput spermatozoa was analysed by linear regression. Moreover, the relationship between the observed amounts of DNA damage and sperm-oocyte fusion was analysed by correlation. $P < 0.05$ was considered significant. Each experiment was repeated at least five times.

Results

Effects of deletion of male accessory sex glands on uterine sperm DNA damage

The incidence of DNA damage in uterine spermatozoa is shown (Fig. 1a). The incidence of both ssDNA and dsDNA breakage was significantly different among the four experimental groups (alkaline assay: $F = 5.935$, $P < 0.05$; neutral assay: $F = 9.745$, $P < 0.05$). Spermatozoa from the TX group showed a significant increase in the incidence of ssDNA and dsDNA breakage compared with the SH group ($P < 0.05$). Moreover, the total incidence of spermatozoa from the TX group with DNA damage (ssDNA and dsDNA damage) was higher than that of spermatozoa from the SH group (TX = 2.58%; SH = 0.69%), whereas spermatozoa from both AGX and VPX groups did not show any significant difference compared with spermatozoa from the SH group. The extent of DNA damage (tail moment) in uterine spermatozoa is shown (Fig. 1b). A significant difference in alkaline comet assay was found in the experimental groups ($F = 4.016$, $P < 0.05$). Spermatozoa from the TX group showed a greater extent of ssDNA damage compared with control spermatozoa ($P < 0.05$). However, spermatozoa from both AGX and VPX groups were not different from those of the control group.

Fig. 1. The (a) incidence and (b) extent of DNA damage in spermatozoa of the male golden hamster after the removal of the accessory sex glands (TX, ■), the removal of the ampullary glands (AGX, ▨), the removal of the ventral prostate (VPX, ▩) and in sham-operated controls (SH, □). Asterisks indicate significant increases in the incidence of (a) single-strand DNA damage (alkaline assay) and double-strand DNA damage (neutral assay), and the extent of (b) single-strand DNA damage (alkaline assay) compared with sham-operated controls ($P < 0.05$; one-way ANOVA followed by Dunnett's post-test).

Susceptibility of epididymal and uterine spermatozoa to induced ROS damage

Spermatozoa from the caput and cauda epididymides and uterine spermatozoa ejaculated by males from the control and experimental groups were incubated with NADPH followed by comet assay to assess the response of epididymal spermatozoa to ROS at different maturation stages and the protective effects of male accessory sex gland secretions against the induced ROS.

Epididymal spermatozoa from intact males of proven fertility

After NADPH incubation, a significantly higher incidence of ssDNA damage was found in spermatozoa from the caput compared with the cauda epididymidis ($F = 227.50$,

$P < 0.001$) (Table 1). Spermatozoa from the caput epididymidis showed no significant change in the incidence of ssDNA damage after incubation with various concentrations of NADPH. The comet from caput epididymal spermatozoa was categorized into three patterns (Fig. 2). Pattern 1: comets only with comet head (intact DNA) but no tail (fragmented DNA); pattern 2: comets with various head and tail sizes; and pattern 3: no comet head (intact DNA). Even though NADPH incubation did not affect the incidence of ssDNA damage in caput epididymal spermatozoa, a higher percentage of pattern 3 (ssDNA breakage) was evident at higher NADPH concentration ($R^2 = 0.4220$, $P < 0.001$).

Uterine spermatozoa from males with all or some of the accessory sex glands removed

The incidence of ssDNA breakage in uterine spermatozoa from experimental groups incubated with increasing concentrations of NADPH is shown (Fig. 3a). There was a significant dose-dependent increase in the incidence of ssDNA damage in spermatozoa from the experimental groups ($F = 49.2571$, $P < 0.001$); however, data on spermatozoa from the TX group showed significance only when compared with the control group ($P < 0.001$). Spermatozoa from both the AGX and VPX groups showed a significant increase in the incidence of ssDNA damage when compared with the control group ($P < 0.05$), but a dose-response mode was not observed. The extent of ssDNA damage in uterine spermatozoa from the different experimental groups in response to incubation with NADPH (Fig. 3b) showed a significant dose-dependent increase among the four groups ($F = 12.1161$, $P < 0.001$). Spermatozoa from the TX group showed a larger dose-dependent increase compared with that from the SH group ($P < 0.05$). However, neither AGX nor VPX groups showed any significant difference from the SH group.

It was worth noting that the extent of ssDNA damage among spermatozoa from SH, AGX, VPX and TX groups were significantly different after 2 h of incubation without NADPH (SH = 48.83 ± 2.95 ; AGX = 51.60 ± 2.21 ; VPX = 61.20 ± 6.80 ; TX = 64.25 ± 2.29), which is similar to the response of spermatozoa that were not subjected to incubation (Fig. 1b). However, after treatment with up to 10 mmol NADPH l^{-1} , the extent of ssDNA damage in all samples was similar (SH = 71.86 ± 3.48 ; AGX = 69.83 ± 3.83 ; VPX = 66.60 ± 6.80 ; TX = 76.93 ± 3.39).

DNA damage and sperm-oocyte fusion

After incubation with an increasing amount of NADPH, uterine spermatozoa recovered from SH and TX groups showed no difference in their ability to fuse with oocytes. However, a dose-dependent decrease in the number of decondensed spermatozoa per oocyte was noted (Fig. 4; SH: $R^2 = 0.6071$, $P < 0.001$; TX: $R^2 = 0.7061$, $P < 0.001$). A linear correlation was found between ssDNA damage and sperm-oocyte fusion. This result showed that the induction

Table 1. Incidence of DNA damage in hamster caput and cauda epididymal spermatozoa after NADPH treatment

NADPH (mmol l ⁻¹)	Number of animals	Number of comets per 10 000 spermatozoa	
		Caput epididymidis***	Cauda epididymidis
0	5	153 ± 25	8 ± 3
1.25	5	201 ± 35	13 ± 3
2.5	5	200 ± 43	11 ± 4
5.0	5	191 ± 16	10 ± 2
10.0	5	216 ± 24	10 ± 2
20.0	5	130 ± 10	10 ± 3

Values are mean ± SEM.

***Significantly higher compared with cauda epididymal spermatozoa in all concentrations of NADPH ($P < 0.001$; two-way ANOVA followed by Bonferroni post-test).

Fig. 2. Comet patterns in hamster caput epididymal spermatozoa after NADPH treatment. Asterisks indicate that caput epididymal spermatozoa showed a significantly higher percentage in comet pattern 3 after NADPH treatment ($R^2 = 0.4220$; $P < 0.001$). ■: pattern 1, comets only with comet head (intact DNA) but no tail (fragmented DNA); ▨: pattern 2, comets with various head and tail sizes; □: pattern 3, no comet head (intact DNA).

of ssDNA damage by NADPH results in a significant negative correlation with sperm–oocyte fusion in spermatozoa from the TX group ($r = -0.9579$; $P < 0.05$). However, such a relationship was not found in the SH group ($r = -0.9069$).

Discussion

The present study demonstrated that DNA damage was more extensive in spermatozoa that were not exposed to

Fig. 3. The (a) incidence and (b) extent of sperm DNA damage in male golden hamsters after NADPH treatment. Spermatozoa from experimental hamsters after the removal of the male accessory sex glands (●) showed a significantly higher incidence and extent of single-strand DNA damage compared with spermatozoa from sham-operated control hamsters (▽) (** $P < 0.001$; * $P < 0.05$; two-way ANOVA followed by Bonferroni post-test). However, spermatozoa from experimental hamsters after the removal of ampullary glands (□) and after the removal of the ventral prostate (▼) were not different from spermatozoa from sham-operated control hamsters (▽).

male accessory sex gland secretions. Mature epididymal spermatozoa were less susceptible to NADPH treatment, and male accessory sex gland secretions afford protection to uterine sperm from DNA breakage after NADPH treatment, indicating that male accessory sex gland secretions might have a role in preserving sperm genomic integrity in the female genital tract.

During sperm maturation, chromatin is packed through increasing disulfide bond formation upon oxidation of protamine thiols. The thiol–disulfide status is important in DNA stability (Kosower *et al.*, 1992). DNA-associated protamines deficient in disulfide could lead to ssDNA damage. In the present study, after incubation for 2 h without NADPH, spermatozoa from the caput epididymidis

Fig. 4. The sperm–oocyte fusion test in uterine spermatozoa from male golden hamsters after NADPH treatment. No significant difference was found between sham-operated control hamsters (SH, ■) and experimental hamsters with all male accessory sex glands removed (TX, ▲) ($P > 0.05$). A dose-dependent decrease in the number of decondensed spermatozoa per oocyte was observed (SH: $R^2 = 0.6071$; $P < 0.001$; TX: $R^2 = 0.7061$; $P < 0.001$).

appeared to be more vulnerable to denaturation and suffer more extensive ssDNA damage than did spermatozoa from the cauda epididymidis, which might be due to a difference in chromatin condensation (Weissenberg *et al.*, 1994). However, changes in the redox status of mammalian spermatozoa seem to have great impact on the completion of chromatin compaction. Fisher and Aitken (1997) reported that when induced by NADPH, spermatozoa from the caput epididymidis have a higher spontaneous capacity for superoxide anion production than do spermatozoa from other regions of the epididymis. This higher capacity appears to be mediated by a membrane-bound NADPH oxidase, which may generate hydrogen peroxide acceptors for phospholipid hydroperoxide glutathione peroxidase (PH-GPx or GPx4) during induction of sperm chromatin condensation (ROS-PH-GPx system) (Aitken and Vernet, 1998). Too little ROS may lead to a failure of chromatin condensation and an increase in susceptibility to DNA damage (Bianchi *et al.*, 1993). In contrast, too much ROS overwhelms intracellular GPx and PH-GPx leading to oxidative stress and DNA damage (Hughes *et al.*, 1996). The present study also observed a dose-dependent increase in the extent of ssDNA damage in spermatozoa from the caput epididymidis after NADPH incubation, but this dose-dependent effect is not evident in the cauda epididymidis. It is possible that this finding resulted from poorly packaged chromatin (rich in thiol) and a high capacity of superoxide anion production by NADPH oxidase in the plasma membrane of caput epididymal spermatozoa. Thus, the results of the present study indicate that the immature caput epididymal spermatozoa are more vulnerable to NADPH-induced ssDNA damage than are mature spermatozoa from the cauda epididymidis.

DNA damage of human ejaculated spermatozoa might

arise from three sources: underprotamination during spermiogenesis, abortive apoptosis during spermatogenesis (Sakkas *et al.*, 1999) and oxidative stress (Alvarez *et al.*, 1987; Aitken and Fisher, 1994). The main cause of oxidative stress may be a high rate of ROS generated in association with the retention of excess residual cytoplasm in the mid-piece of spermatozoa and antioxidant depletion (Aitken and Krausz, 2001). The types of ROS-induced DNA damage in spermatozoa in the experimental systems included modification of all bases and the production of base-free sites, deletions, frameshifts, ssDNA breaks, DNA protein crosslinks and chromosomal rearrangements (Halliwell and Aruoma, 1991) in both mitochondria and nuclear DNA. However, apoptosis was characterized by a dsDNA break (Allen *et al.*, 1997), but not a ssDNA break.

Antioxidants, such as epididymal secretory GPx, superoxide dismutase and seminal plasma contents in the reproductive tract, play an important role in oxidative stress (Perry *et al.*, 1993; Vernet *et al.*, 1996; Twigg *et al.*, 1998; van Overveld *et al.*, 2000). Previous observations of a higher embryonic wastage and reduced fertility in terms of a decrease in implantation rate and an increase in post-implantation loss of embryos after removal of all or some male accessory sex glands in the golden hamster (O *et al.*, 1988), structural abnormalities in implanted embryos (Jiang *et al.*, 2001) and delay in sperm chromatin decondensation and DNA replication in the first cell cycle (Ying *et al.*, 1998, 1999a) could be attributed to damage to DNA of spermatozoa in the absence of contact with secretions from paternal accessory sex glands. The results of the present study show that compared with the control hamsters, more spermatozoa from the TX group experienced ssDNA and dsDNA damage, but the extent of damage was higher only in the ssDNA type. However, removal of ampullary glands and the ventral prostate gland does not appear to have any effect, indicating that elements with a potential to protect spermatozoa from oxidative damage may originate from the dorsolateral prostate glands, coagulating glands or seminal vesicles. Moreover, a significantly higher dose-dependent response in incidence and extent of ssDNA damage was found in uterine spermatozoa ejaculated from hamsters with all male accessory sex glands removed (TX group) when incubated with NADPH compared with the control hamsters (SH group). However, this dose-dependent response was not observed in uterine spermatozoa ejaculated from hamsters with either ampullary glands or ventral prostate removed. These results further indicate that the ventral prostate gland and ampullary glands do not afford any protection to spermatozoa against NADPH-induced DNA damage.

Oxidative stress has an influence on sperm motility, capacitation and fusion with the oocyte (Aitken *et al.*, 1989, 1993). The present study demonstrated that NADPH induces a comparable dose-dependent decrease in sperm-oocyte fusion in spermatozoa from both TX and SH groups. This finding is in agreement with previous findings in which a lack of effect of deletion of male accessory sex glands on

the fertilizing ability of spermatozoa *in vivo* and *in vitro* was reported (O *et al.*, 1988; Ying *et al.*, 1999b). However, the negative linear correlation between induced ssDNA damage and sperm-oocyte fusion rate in the TX group does indicate that ssDNA damage can reduce the ability of the spermatozoa to fuse with the oocytes (Morris *et al.*, 2002). Whether this is a direct effect of NADPH remains to be established.

The results of the present study also show that uterine spermatozoa ejaculated by hamsters with all male accessory sex glands removed are more vulnerable to NADPH treatment in comparison with cauda epididymal spermatozoa. This difference might be related to uterine factors that are harmful to spermatozoa, such as nitric oxide (Norman and Cameron, 1996); amino acids (Fahning *et al.*, 1967), which have been reported to stimulate production of H₂O₂ in bovine spermatozoa through the action of an amino acid oxidase (Lapointe and Sirard, 1998); leucocytes, mainly eosinophils (Perez *et al.*, 1996), could also produce large quantities of H₂O₂ upon stimulation by seminal plasma (Hansen *et al.*, 1987) and other ROS that were toxic to spermatozoa (Thompson *et al.*, 1992; Williams *et al.*, 1993). In addition, oestrogen has pro-oxidant and antioxidant effects. At oestrus, under the influence of oestrogen, endometrial epithelial NADPH oxidase produces NADPH (Moulton and Barker, 1971; Hilf *et al.*, 1972; Swanson and Barker, 1983), superoxide anions (Laloraya *et al.*, 1991; Jain *et al.*, 1999, 2000) and hydrogen peroxide (Riley and Behrman, 1991). Optimal amounts of these oxidants were balanced for sperm capacitation by the oestrogen-stimulated antioxidant system, which mainly included superoxide dismutase, peroxidase containing eosinophilic peroxidase and endogenous peroxidase associated with epithelial cells (Hosoya and Saito, 1981; Anderson *et al.*, 1986; Riley and Behrman, 1991), and glutathione peroxidase (GPx)/reductase (GR) system (Ohwada *et al.*, 1996; Diaz-Flores *et al.*, 1999; Kaneko *et al.*, 2001). It has been reported that in the hamster, hydrogen peroxide is needed to control oxidation of sperm membrane thiol groups during capacitation (Bize *et al.*, 1991). It has been suggested that the combined effects of these uterine factors render the spermatozoa more vulnerable to NADPH treatment.

The present study demonstrated that spermatozoa incubated with NADPH and untreated spermatozoa showed the same incidence and extent of ssDNA. This finding indicates that our experimental procedure does not induce additional ssDNA damage, that the dose-dependent increase in both the incidence and extent of ssDNA damage after incubation with NADPH is indeed a *bona fide* induction by NADPH treatment (Aitken *et al.*, 1997). The presence of NADPH oxidase in the sperm membrane was challenged by Richer and Ford (2001); they were not able to detect NADPH oxidase in the sperm membrane. However, in the present study, the addition of 50 U superoxide dismutase ml⁻¹ into flushing and incubation media resulted in a significant reduction in ssDNA damage in the TX after NADPH incubation (H. Chen, P. H. Chow, A. L. M. Cheung and W. S. O,

unpublished). This finding indirectly confirms the presence of NADPH-oxidase in the sperm membrane, as ROS generated by it could be counteracted by superoxide dismutase *in vitro*. Furthermore, Vernet *et al.* (2001) proved by RT-PCR that, in rat epididymal spermatozoa, this proposed membrane-bound NADPH oxidase is indeed an NADPH oxidoreductase complex in the sperm plasma membrane and is quite distinct from a similar system in the leucocyte.

In summary, the present study demonstrated that mature epididymal spermatozoa are less susceptible to NADPH-induced oxidative damage. Male accessory sex gland secretions do afford protection to spermatozoa against natural and induced oxidative DNA damage. The protective factor(s) appears to come from the coagulating glands, dorsolateral prostate glands and seminal vesicles; the absence of ampullary glands and the ventral prostate gland has little affect on fertility *in vivo*. Thus, even though spermatozoa may suffer oxidative genomic damage, the embryos may have adequate mechanism to repair the damage to allow full development.

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