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Effects of ageing on spermatozoal chromatin and its sensitivity to *in vivo* and *in vitro* oxidative challenge in the Brown Norway rat

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BACKGROUND: The goals of our study were to examine chromatin packaging and integrity in spermatozoa taken from the caput and cauda epididymides of young (4-month-old) and old (21-month-old) Brown Norway rats and to assess whether spermatozoal sensitivity to oxidative treatments is altered with age. METHODS: Oxidative treatments consisted of (i) *in vivo* oxidative challenge by systemic administration of the glutathione-depleting drug L-buthionine-[S,R]-sulphoximine (BSO) and (ii) *in vitro* oxidative challenge by incubating collected spermatozoa with hydrogen peroxide (H_2O_2). Chromatin parameters assessed included quantification of thiols, nuclear chromomycin A3 (CMA3) penetration, DNA breaks by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) and ease of DNA dissociation by acridine orange (AO) staining. RESULTS: In spermatozoa from older rats, we found decreases in thiols, CMA3 penetration and the percentage of cells that undergo DNA dissociation. Administration of BSO had oxidizing effects on the thiol groups. It also decreased CMA3 penetration and DNA dissociation and increased TUNEL staining. Furthermore, BSO treatment sensitized cauda epididymidis spermatozoa, from older animals, to H_2O_2. CONCLUSIONS: Overall, we show that spermatozoa from older rats have altered chromatin packaging and integrity and that spermatozoa from the cauda epididymidis are more responsive to combined *in vivo* and *in vitro* oxidative challenge than spermatozoa from young rats.

Key words: DNA damage/sperm biochemistry/sperm chromosomes/sperm quality

Introduction

Ageing in mammals is associated with a widespread decline in physiological function. Although it had been generally accepted that male fertility is immune to the effects of age, several recent studies have established that many aspects of male fertility are affected by ageing. Specifically, semen volume and spermatozoal motility have been found to decrease in ageing men, whereas the proportion of abnormal spermatozoa increases (Plas *et al*., 2000; Kidd *et al*., 2001). Increasing paternal age is also associated with numerical and structural chromosomal abnormalities in spermatozoa (Sartorelli *et al*., 2001; Kuhnert and Nieschlag, 2004). Furthermore, epidemiological evidence indicates that children born to older fathers have a greater incidence of genetic diseases; these diseases cover a wide spectrum that remains poorly defined, but some of the stronger correlations include schizophrenia, achondroplasia and Apert's syndrome (Kuhnert and Nieschlag, 2004; Baird *et al*., 2005).

The mechanisms that underlie the increases in genetic disease with age remain unclear, but they are a symptom of the ageing male reproductive system. Previous reports have found structural changes in the seminiferous tubules of the testes with age (Levy *et al*., 1999; Morales *et al*., 2004), and the high number of divisions that spermatogonia undergo over the course of a man's lifetime may also result in an accumulation of DNA mutations (Hurst and Ellegren, 1998; Crow, 1999).

Protamination of spermatozoa during spermiogenesis is more likely to play a major role in regulating its final DNA quality. During spermiogenesis, histones become hyperacetylated and are briefly replaced by transition proteins, which are then replaced by protamines (Wouters-Tyrou *et al*., 1998; Dadoune, 2003). Protamines are rich in free thiols, which can form disulphide bonds with other protamines (Balhorn, 1982), thus stabilizing the already compact spermatozoal chromatin. When spermatozoa begin their passage through the epididymis, the thiols have not yet become oxidized; this occurs during the journey in the epididymal lumen, where over the course of several days, the balance becomes shifted in favour of disulphides (Calvin and Bedford, 1971; Shalgi *et al*., 1989). Defective protamination has been shown to be associated

with poor spermatozoa quality and to be correlated with male-factor infertility (Bianchi *et al*., 1993; Nasr-Esfahani *et al*., 2004; Aoki *et al*., 2005).

The balance between oxidation and reduction has been established as a key component in maintaining the integrity of spermatozoa. Although some oxidation is critical to ensure disulphide bond formation within the nucleus of spermatozoa, excessive oxidation is a well-established hazard to spermatozoa, resulting in peroxidation of membrane lipids and in DNA damage (de Lamirande and Gagnon, 1995; Aitken and Krausz, 2001; Sikka, 2001). Disproportionate spermatozoa oxidation can occur from increased *in vivo* reactive oxygen species (ROS) production, such as during inflammation of the male reproductive tract, or as a result of certain medications, exposure to chemicals and smoking (Tirmenstein and Nelson, 1990; Bagchi *et al*., 1993; Ochsendorf, 1999; Sikka, 2001; Alberg, 2002; Bruno and Traber, 2005; Gurbay *et al*., 2005). Ageing is another example of increased systemic oxidant load. The results of several studies clearly establish that ageing is associated with increased production of ROS, primarily from the mitochondria electron transport chain (Harman, 1972; Bokov *et al*., 2004; Balaban *et al*., 2005). There is also evidence to suggest that levels of antioxidants, such as glutathione, are altered with age and that ROS removal may become impaired (Hazelton and Lang, 1985; Rebrin *et al*., 2004; Zubkova and Robaire, 2004; Morrison *et al*., 2005). Other than the *in vivo* sources of ROS, spermatozoa can also become damaged during and after ejaculation; ROS and ROS-producing bacteria from accessory gland infections can mix with the seminal fluids, or spermatozoa can become exposed to ROS in the course of laboratory manipulation during IVF (Alvarez and Storey, 1992; de Jager *et al*., 1996; Hughes *et al*., 1998; Comhaire *et al*., 1999; Roca *et al*., 2005).

The objective of this study was to assess the packaging of chromatin in spermatozoa, and to compare the susceptibility to oxidative stress of spermatozoa from the caput and cauda epididymides of young and old rats. To exacerbate the effects of ageing, we oxidatively challenged the rats by depleting the main cellular antioxidant, glutathione, using the drug L-buthionine-[S,R]-sulphoximine (BSO), which is an inhibitor of the rate-limiting enzyme of glutathione synthesis, γ-glutamylcysteine synthatase (Griffith and Meister, 1979; Martensson *et al*., 1991). We have previously shown that systemic BSO administration effectively decreases glutathione in male reproductive tissues (Zubkova and Robaire, 2004).

By treating the rats with BSO for 7 days, spermatozoa became exposed to the drug during the following two phases of their maturation: spermatozoa collected from the caput epididymidis were exposed from approximately step 16 of spermiogenesis and until their entry into the caput epididymidis (Clermont, 1972), whereas spermatozoa collected from the cauda epididymidis were exposed during their transport throughout the organ (Robaire and Hermo, 1988). This allowed the determination of the differential effects of BSO on spermatozoa at these two stages in their development.

We evaluated the sensitivity of spermatozoa to external oxidant exposure in addition to their susceptibility to systemic oxidative challenge. For this, we used hydrogen peroxide (H_2O_2), as this compound reacts with transition metal ions to rapidly form the highly oxidizing hydroxyl radical (OH^-) (Halliwell and Gutteridge, 2002). This experimental design also allows us to determine whether *in vivo* oxidizing challenge increases the sensitivity of spermatozoa to *in vitro* oxidative stress and whether this sensitization effect is amplified by ageing.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for BSO (Toronto Research Chemicals Inc., North York, ON, Canada), monobromobimane (mBBr), sold as Thiolyte® MB, (Calbiochem, San Diego, CA, USA) and the Apo-Direct™ kit (Pharmingen, San Diego, CA, USA).

Animals

Male Brown Norway rats were obtained through the National Institutes on Aging (Bethesda, MD, USA) from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Rats in the young group were 4 months old and those in the aged group were 21 months old. The animals were housed in a 14 h light : 10 h dark cycle; food and water were provided *ad libitum*. All animal studies followed the principles and procedures outlined in 'A Guide to the Care and Use of Experimental Animals' prepared by the Canadian Council on Animal Care (McGill protocol no. 4687).

BSO administration

The young and old rats were assigned randomly to either treated or control groups. Those in the treated groups received subcutaneous injections of 2 mmol/kg BSO dissolved in saline at 12 h intervals, for 7 days. Those in the control groups received injections of volume-adjusted saline. Animals were killed by CO_2 asphyxiation 2 h after their last injection.

Collection of spermatozoa

Spermatozoa from the caput and cauda epididymides of non-regressed testes were collected into a motility buffer (Slott *et al*., 1991) to increase yield. They were then washed once with hypotonic buffer (0.45% NaCl) to lyse any contaminating cells and twice with phosphate-buffered saline (PBS) (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0). The final suspension was divided into 1 ml aliquots of approximately $5–10 \times 10^6$ spermatozoa that were stored at –80°C.

mBBr thiol labelling

Thiol labelling was done to quantify the total thiol, free thiol and disulphide levels in the nuclei of spermatozoa. On the day of the assay, spermatozoa were thawed and both the control and BSO-treated samples were further separated into two groups of about $5–10 \times 10^6$ cells each; one group was incubated in PBS and the other in 5 mM H_2O_2 for 1 h at 25°C. The spermatozoa were then washed twice in PBS and sonicated on ice to separate the heads from the tails. Labelling with mBBr and analysis by flow cytometry were performed as described by Zubkova *et al*. (2005).

Chromomycin A3 staining

The CMA3 penetrability test was used to assess the extent of spermatozoal nuclear protamination and as an indicator of chromatin condensation. On the day of the assay, spermatozoa were thawed and both the control and BSO-treated samples were further separated into two

groups of about 5–10 × 10^6 cells each; one group was incubated in PBS and the other in 5 mM H_2O_2 for 1 h at 25°C. The spermatozoa were then washed twice in PBS and sonicated on ice to separate the heads from the tails. CMA3 labelling and analysis by flow cytometry were performed as described previously by Zubkova *et al*. (2005).

TUNEL assay

The TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay was used to quantify the extent of 3′-hydroxyl (OH) breaks in single- and double stranded DNA. On the day of the assay, spermatozoa were thawed and both the control and BSO-treated samples were further separated into two groups of about 5–10 × 10^6 cells each; one group was incubated in PBS and the other in 5 mM H_2O_2 for 1 h at 25°C. The cells were then washed in PBS twice, followed by sonication on ice. DNA strand breaks were analysed using the Apo-Direct™ kit with some modifications. Briefly, spermatozoa were resuspended in 70% ice-cold ethanol to a concentration of about 1–2 × 10^6 cells/ml and stored at −20°C for 3 days. They were then centrifuged for 5 min at 5000 × *g*, washed twice with 1 ml wash buffer each time and incubated in 100 µl staining solution [containing the reaction buffer, terminal deoxytransferase (TdT) enzyme, fluorescein isothiocyanate (FITC)-tagged deoxyuridine triphosphate nucleotides and distilled water, as per kit instructions] in the dark at 37°C for 1 h. The reaction was terminated by the addition of 1 ml Rinse buffer, after which the spermatozoa were centrifuged for 5 min at 5000 × *g* and washed one more time with Rinse buffer. They were then resuspended in 500 µl propidium iodide (PI)/RNase and stored in the dark overnight at 4°C. To verify the linearity of the reaction, we tested a set of positive controls, which were produced by pretreating the cells with DNase I (100 U/µl) for 5–15 min at 25°C. A negative control, consisting of cells stained with the staining solution lacking the terminal deoxytransferase enzyme, was included as a negative control with every group in the study.

Intensity of FITC staining was analysed spectrophotometrically using the BD FACSAria Cell Sorting System (BD Bioscience, San Jose, CA, USA) fitted with a 488 nm laser. For FITC detection, light emission was filtered through a 502 nm long pass filter, as well as a 530/30 nm band pass filter, whereas PI was detected using a 556 nm long pass filter, followed by a 575/26 nm band pass filter. Fluorescence was quantified by the BD FACSDiva software (BD Bioscience). A total of 10 000 events was analysed for every sample.

Acridine orange assay

The acridine orange (AO) assay was used to measure DNA denaturability in mature spermatozoa, which is an indicator of overall DNA quality (Evenson *et al*., 2002). On the day of the assay, spermatozoa were thawed and both the control and BSO-treated samples were further separated into two groups of about 5–10 × 10^6 cells each: one group was incubated in PBS and the other in 1 mM H_2O_2 for 1 h at 25°C. The cells were then washed in PBS twice, followed by sonication on ice.

The AO assay was performed based on the method described by Evenson *et al*. (1985). Briefly, a 200 µl aliquot of spermatozoa was placed into a 12 mm × 75 mm tube and was mixed with 400 µl of DNA denaturation buffer (0.1% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4). After 30 s, 1.2 ml of AO staining buffer (200 mM Na_2HPO_4, 0.1 M citric acid buffer, pH 6.0, 1 mM EDTA, 150 mM NaCl and 6 µg/ml AO) was added and the reaction was allowed to proceed for 3 min, after which nuclear fluorescence was analysed spectrophotometrically using a BD FACScan Analyzer System (BD Bioscience) fitted with a 488 nm argon-ion laser. Light emission was filtered using a 502 nm long pass filter, followed by a 530/30 nm band pass filter (for detecting green fluorescence), and also using a 670 nm long pass filter, followed by a 660/20 nm band pass filter (for detecting red fluorescence). The resulting fluorescence was quantified by the BD CellQuest Pro software (BD Bioscience). A total of 10 000 events was analysed for each sample.

The extent of DNA denaturation was quantified by the calculated parameter DNA fragmentation index (DFI), using the following formula: red fluorescence/(red + green fluorescence) × 1024. The percentage of cells with abnormal DFI was defined as percentage DFI.

Statistical analysis

Statistical analysis was performed using Systat v10.2 (Systat Software Inc., Richmond, CA, USA) and consisted of two-way ANOVA (to assess age and epididymal segment-specific changes), three-way ANOVA (to assess whether the effect of BSO treatment was dependent on age and/or segment) and four-way ANOVA (to assess whether the effect of H_2O_2 was dependent on age and/or segment and/or BSO). Results were considered significant at $P < 0.05$ ($n = 8$) and the figures show mean ± SEM values.

Results

A full statistical summary is provided in Table I.

mBBr thiol labelling

Consistent with previous observations (Calvin and Bedford, 1971; Evenson *et al*., 1989; Conrad *et al*., 2005), we noted a significant primary effect of segment on free thiols and disulphides (Figure 1B and C). Interestingly, age also had a significant effect, where all of the thiol parameters were lower in spermatozoa heads of older rats. Additionally, all thiol parameters had a significant interaction between age and segment, the decrease being consistently greater in the caput epididymidis region. Free thiols decreased by about 13% in spermatozoa from the caput epididymidis and did not change in spermatozoa from the cauda. Furthermore, disulphides decreased by 45 and 28% in spermatozoa from the caput and cauda epididymidis region, respectively.

Exposure to BSO did not change the overall thiol quantities, but it shifted the balance towards the disulphides. However, this effect was dependent on segment (segment × BSO), as the changes were observed primarily in spermatozoa from the caput epididymidis.

Incubation with H_2O_2 had a dramatic effect on all spermatozoa, decreasing total thiol levels and converting most of the free thiols to disulphides. The H_2O_2-induced changes in total thiols and free thiols were dependent on age (age × H_2O_2), with the effects being greater on spermatozoa from younger animals. Clearly, the shift was more pronounced in caput epididymal spermatozoa than in those from the cauda epididymidis, at which point most of the thiols were already in the form of disulphides. The treatment with H_2O_2 interacted with BSO (BSO × H_2O_2), where the BSO treatment pre-shifted the thiol balance, thus diminishing the H_2O_2-induced change.

Analysis by flow cytometry is a very powerful technique, because it determines the average fluorochrome intensity and allows for quantification of fluorescence in each individual spermatozoal head. Therefore, by analysing the raw flow cytometric data for mBBr, we noted that the spermatozoa from older animals composed not one, but two distinct populations

Table I. Complete summary of statistical analysis by ANOVA

	Total thiols	Free thiols	Disulphides	CMA3	TUNEL	DFI	Percentage DFI
Two-way ANOVA							
Age	<0.001	<0.001	<0.001	<0.001	0.0016	0.014	<0.003
Segment	0.648	<0.001	<0.001	<0.001	0.029	<0.001	<0.001
Age × segment	0.007	<0.001	<0.001	0.275	0.071	0.263	0.015
Three-way ANOVA							
BSO	0.163	<0.001	<0.001	0.044	<0.001	<0.001	<0.001
Age × BSO	0.472	0.397	0.654	0.157	0.696	0.011	0.007
Segment × BSO	0.080	<0.001	<0.001	0.025	0.029	<0.001	<0.001
Age × segment × BSO	0.156	0.255	0.054	0.680	0.084	0.764	0.073
Four-way ANOVA							
H_2O_2	<0.001	<0.001	<0.001	<0.001	0.551	<0.001	<0.001
Age × H_2O_2	0.002	<0.001	0.722	<0.001	<0.001	0.426	0.059
Segment × H_2O_2	0.128	<0.001	<0.001	<0.001	<0.001	<0.001	0.580
BSO × H_2O_2	0.954	<0.001	<0.001	0.097	0.164	0.006	<0.001
Age × BSO × H_2O_2	0.143	0.370	0.202	0.013	0.401	0.063	0.090
Age × segment × H_2O_2	0.485	<0.001	0.012	0.009	0.441	0.260	0.893
BSO × segment × H_2O_2	0.273	<0.001	<0.001	0.968	0.437	0.016	0.002
Age × segment × BSO × H_2O_2	0.656	0.383	0.310	<0.001	0.086	0.833	0.902

BSO, L-buthionine-[S,R]-sulphoximine; DFI, DNA fragmentation index.
Columns 2–8 summarize statistical analysis for the mBBr assay (consisting of total thiols, free thiols and disulphides), the CMA3 assay, the TUNEL assay and the acridine orange assay (consisting of DFI and percentage DFI), respectively.

(Figure 2). This finding was highly consistent among different samples, and clearly showed that about 5–10% of cells had considerably lower thiol staining. In fact, the observed decreases in the mean values of total, free thiols and disulphides in spermatozoa from older rats can be entirely attributed to this second population.

Chromomycin A3 staining

Spermatozoa from the caput epididymidis had more CMA3 staining than those from the cauda region (Figure 3). An age effect was also observed, where staining was decreased by over 10% in spermatozoa from both the caput and cauda epididymidis from older animals. BSO treatment significantly reduced CMA3 staining in spermatozoa; this decrease was segment-dependent (BSO × segment), where it was observed primarily in spermatozoa from the caput epididymidis.

Exposure to H_2O_2 had a dramatic effect. There was also an interaction with segment (H_2O_2 × segment), manifested by a 44–53% decrease in staining in all caput epididymidis spermatozoa and a 23–37% decrease in cauda epididymidis spermatozoa (except for spermatozoa extracted from BSO-treated older animals). The age × segment × BSO × H_2O_2 interaction was highly significant, drawing attention to the observation that in cauda epididymidis spermatozoa from old, BSO-treated animals, CMA3 fluorescence did not change after exposure to H_2O_2. This was unlike what was seen in spermatozoa from the other groups, in which H_2O_2 exposure consistently resulted in decreased CMA3 staining.

TUNEL assay

Relative FITC intensity was low in most of the spermatozoa samples, suggesting that rat spermatozoa have relatively few 3′OH breaks in their DNA (Figure 4). There was a significant difference in staining depending on age and also on the epididymal segment, where cauda spermatozoa from old animals showed more staining than those from the caput epididymidis. The interaction between age and segment, however, was not significant.

Administration of BSO resulted in increased average FITC staining in all spermatozoa samples. The increase was also segment-dependent (segment × BSO), with dramatically higher staining observed in cauda spermatozoa.

Exposure to H_2O_2 did not have a significant primary effect, which is probably due to the variability in mean FITC fluorescence within each group. However, the interaction between age × H_2O_2 and segment × H_2O_2 were highly significant. Spermatozoa from the caput epididymidis of young rats were most responsive to H_2O_2, whereas staining in caput epididymidis spermatozoa from old rats remained unchanged. Furthermore, we observed that the staining in BSO-treated spermatozoa from the cauda epididymides of old rats decreased to extremely low levels. This response was strikingly different from that observed for other spermatozoa samples, in which staining remained the same or increased.

AO assay

The DFI and DFI percentage parameters depended on both the age of the rat and the epididymal segment (Figure 5). Overall, denaturation parameters were greater in spermatozoa from the caput epididymidis than from the cauda and greater in spermatozoa from young than from old rats. However, although DFI was only slightly higher in spermatozoa from young animals, the DFI percentage was about double the value observed in old rats, indicating that although the extent of DNA denaturation was not vastly altered, the number of cells that responded to the denaturating treatment was about twice as great in the young rat as in the old. This is highlighted by the fact that the age segment interaction was significant only for DFI percentage.

Administration of BSO had a significant primary effect, which was also dependent on age (age × BSO) and segment (segment × BSO). It resulted in a decreased DFI percentage in both caput and cauda spermatozoa. The DFI also decreased, but only in spermatozoa taken from the caput epididymidis.

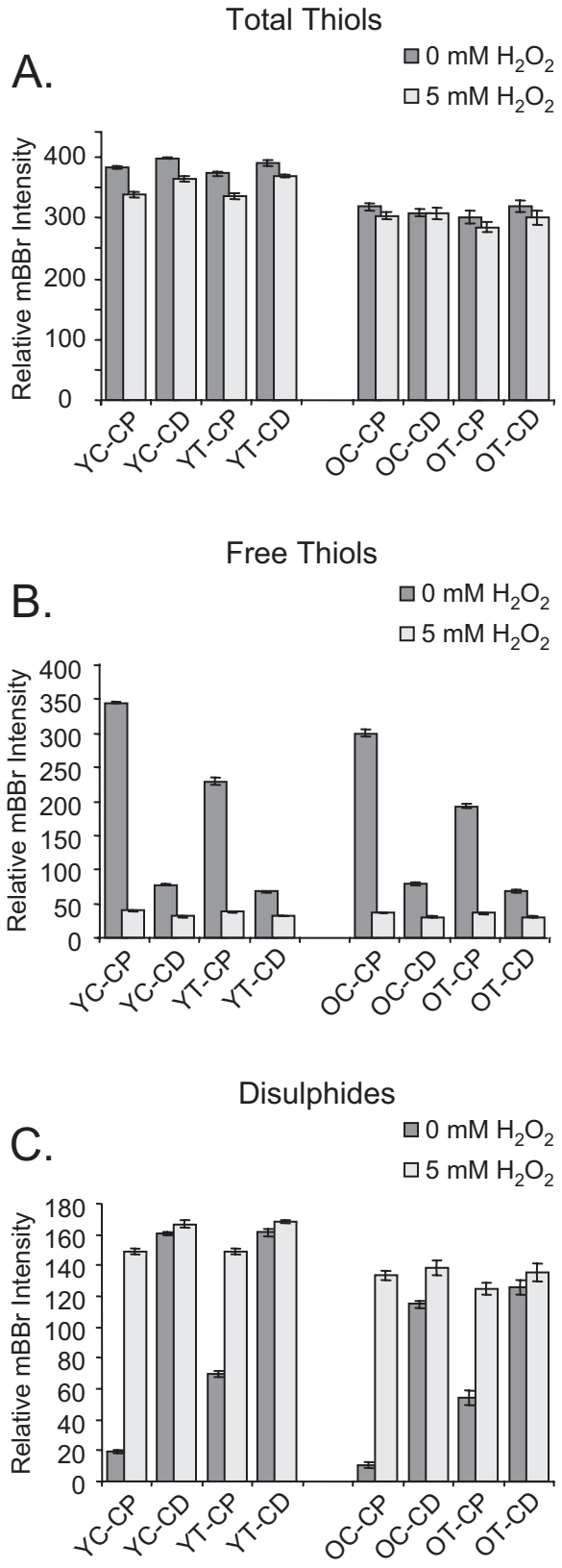

Figure 1. Quantification of total thiols (**A**), free thiols (**B**) and disulphides (**C**) in spermatozoal heads by monobromobimane (mBBr) staining. Data are mean ± SEM, $n = 8$. Statistical analysis for these figures is provided in Table I. YC, young control rats; YT, young rats treated with BSO; OC, old control rats; OT, old rats treated with BSO; CP, spermatozoa extracted from the caput epididymidis; CD, spermatozoa extracted from the cauda epididymidis.

After incubation with H_2O_2, spermatozoa became noticeably damaged and their susceptibility to denaturation increased, as was evident from increased DFI and DFI percentage values. Both of these parameters showed a significant interaction between BSO and H_2O_2; the former drug decreased denaturation, whereas the latter overcame this effect and increased it. Finally, the BSO × segment × H_2O_2 interaction was also significant, where caput spermatozoa were most affected by the combined treatments.

Discussion

Differences between spermatozoa from the caput and cauda epididymidis

Our first objective was to examine the effect of epididymal segment on chromatin of spermatozoa. Spermatozoa from the caput and cauda epididymidis differed, to a greater or lesser extent, in all measured parameters. Although the changes in thiol oxidation status in spermatozoal heads during epididymal passage have been reported previously (Calvin and Bedford, 1971; Evenson *et al*., 1989; Conrad *et al*., 2005), the other parameters have been less studied.

The decrease in CMA3 staining observed in spermatozoa from the cauda epididymis was most surprising. This finding is apparently inconsistent with the principle of the assay, which states that CMA3 competes with protamines for access to chromatin and that CMA3 binding is inversely proportional to the level of protamine deposition on the chromatin (Bianchi *et al*., 1993; Bizzaro *et al*., 1998). By this definition, CMA3 binding in caput and cauda epididymal spermatozoa should be identical, as protamine levels are not being altered at this stage of spermatozoal maturation (Dadoune, 2003). This was, indeed, what was found in an earlier study, where CMA3 staining was examined *in situ* in spermatozoa from the caput and cauda epididymides of mice (Sakkas *et al*., 1995). The apparent discrepancy between our results and those of Sakkas *et al*. (1995) may be attributable to (i) the fact that mouse spermatozoa have two protamines, whereas rat spermatozoa have only one; and (ii) the use of flow cytometry for detecting changes in fluorescence, which is more sensitive than the *in situ* method, may have allowed the identification of changes that would not otherwise have been possible.

Our results suggest that other factors, such as chromatin condensation, may affect the results of this assay. This puts in question the conclusions that have been reached by those studying human infertility, who found that men with reduced fertility have increased CMA3 staining in their spermatozoa and interpreted this as a sign of decreased protamination (McMurray and Kortun, 2003; Nasr-Esfahani *et al*., 2004); an alternative explanation may be that this reduced fertility is associated with less cross-linked or compact spermatozoa.

Passage through the epididymis also resulted in a dramatic change in spermatozoal denaturation parameters. Both DFI and DFI percentage were lower in the more mature spermatozoa, perhaps reflecting the more oxidized status of the thiols, which would impede strand dissociation. Interestingly, the DFI was only slightly lower in spermatozoa from the

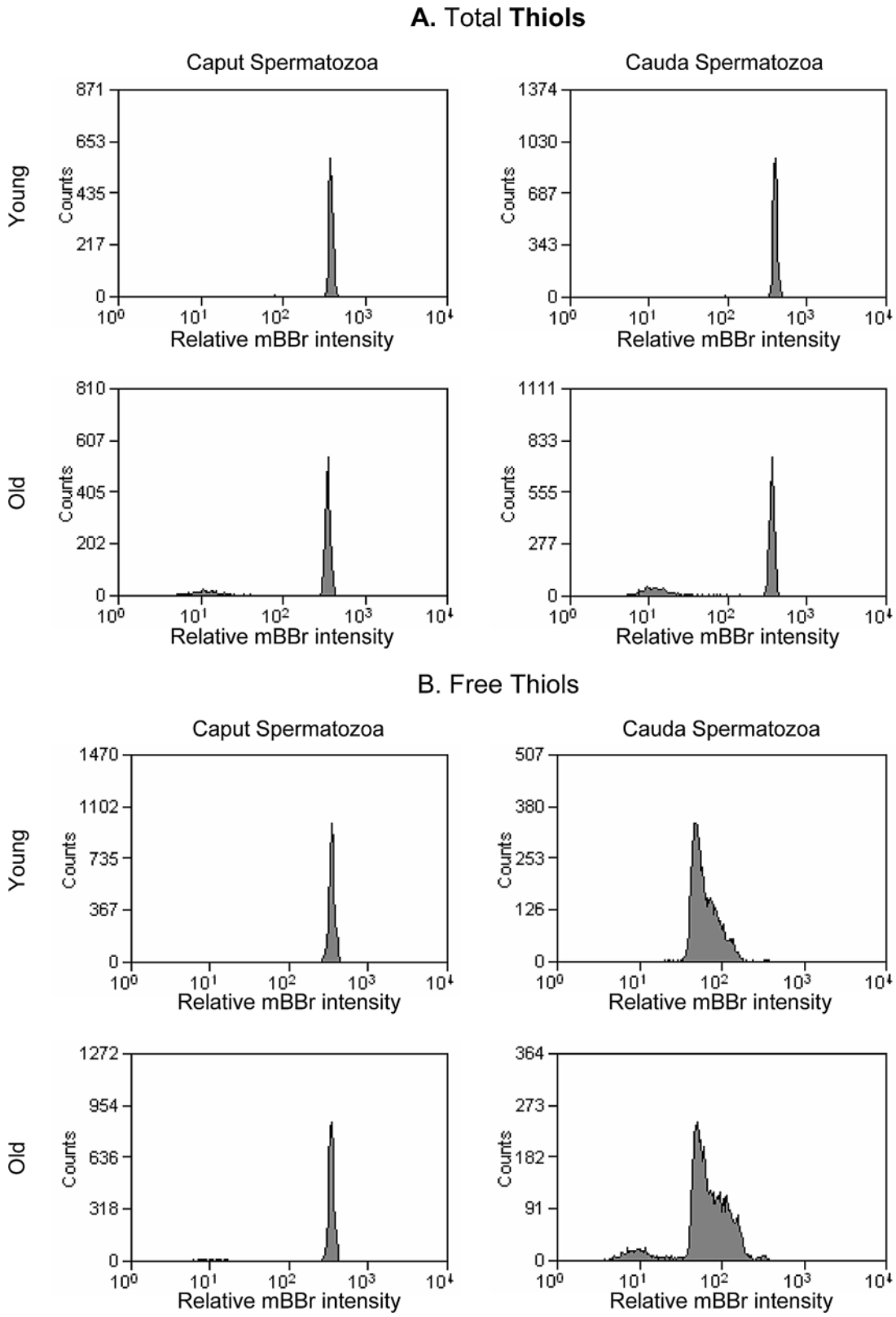

Figure 2. Flow cytometric histogram showing fluorescence intensity of monobromobimane (mBBr) staining of total thiols (**A**) and free thiols (**B**). The population of cells with low thiol levels is clearly visible to the left of the main spermatozoa population in old animals.

cauda epididymidis, whereas DFI percentage was dramatically reduced. This difference reflects the complementary nature of the two tests, where DFI percentage reflects the percentage of cells that have abnormal denaturation and DFI indicates the extent of the shift. It appears that the majority of spermatozoa from the caput epididymidis will undergo chromatin dissociation, but the extent of this dissociation is slight.

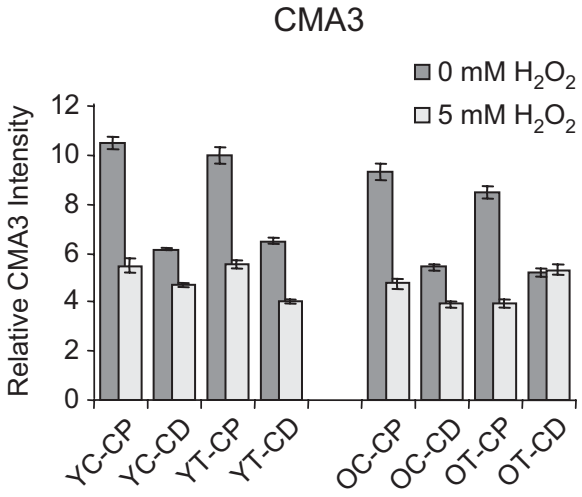

Figure 3. Association of CMA3 with spermatozoal nuclei. Data are mean ± SEM, $n = 8$. Statistical analysis for this figure is provided in Table I. YC, young control rats; YT, young rats treated with BSO; OC, old control rats; OT, old rats treated with BSO; CP, spermatozoa extracted from the caput epididymidis; CD, spermatozoa extracted from the cauda epididymidis.

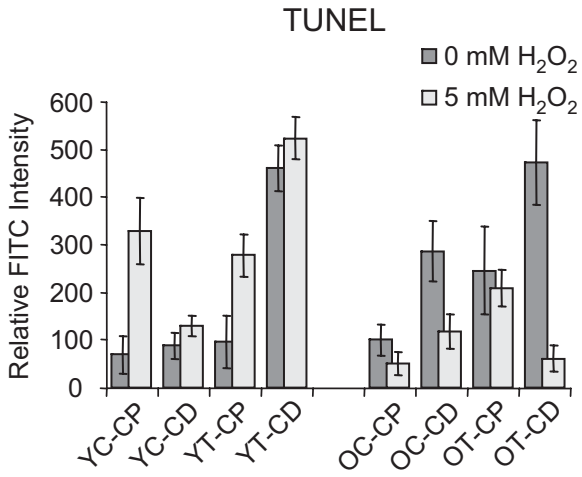

Figure 4. Measurement of DNA breaks containing 3′OH ends in spermatozoal nuclei by TUNEL staining. Data are mean ± SEM, $n = 8$. Statistical analysis for this figure is provided in Table I. YC, young control rats; YT, young rats treated with BSO; OC, old control rats; OT, old rats treated with BSO; CP, spermatozoa extracted from the caput epididymidis; CD, spermatozoa extracted from the cauda epididymidis.

Other differences between spermatozoa from the caput and cauda epididymidis include increased TUNEL staining in spermatozoa taken from the cauda region. However, as this change is observed only in spermatozoa from old animals, it will be discussed in greater detail in the following section, which focuses on changes observed with age.

Changes in spermatozoa with age

Our second objective was to evaluate how chromatin packaging in spermatozoa changes with age. When looking at thiol

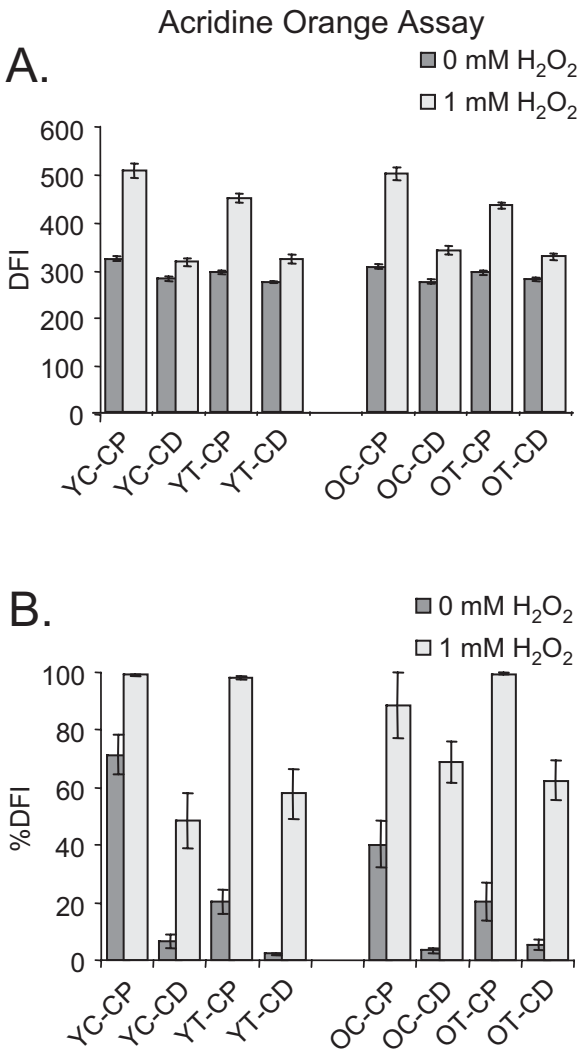

Figure 5. Measurement of DFI (**A**) and DFI percentage (**B**) in spermatozoal heads by the acridine orange (AO) assay. Data are mean ± SEM, $n = 8$. Statistical analysis for these figures is provided in Table I. YC, young control rats; YT, young rats treated with BSO; OC, old control rats; OT, old rats treated with BSO; CP, spermatozoa extracted from the caput epididymidis; CD, spermatozoa extracted from the cauda epididymidis.

groups in spermatozoa heads, we discovered the presence of a distinct spermatozoa population in older rats, which was not detected with the other assays. This might be because the sensitivity of the other assays was not as great, as the population constituted approximately 10% of the cells, or because the observed effect was specific to thiols and did not influence other parameters. However, all of the assays are based on flow cytometry, and their sensitivity would be expected to be similar. Therefore, the mBBr assay appears to measure a parameter that is not detected by the other tests, suggesting that it would be useful to further define the relationship between thiols in the spermatozoal heads and successful pregnancy outcomes by adding this simple but powerful assay with other tests routinely used in infertility clinics. It has a strong potential for becoming useful in the assessment of spermatozoa quality as mBBr does not bind to the chromatin directly and spermatozoa remain

motile and maintain their ability to capacitate and acrosome-react even after being stained with this dye (Cummins *et al*., 1986).

Other than the change in thiols, we also observed an age-dependent change in DFI percentage, where the DFI percentage was almost twice as high in spermatozoa from the caput and cauda epididymidis from young animals as in those from old. The DFI, on the contrary, was only slightly higher in the young. Combined, these results indicate that in young animals more of the spermatozoa were susceptible to chromatin dissociation, but the extent of this dissociation was not great. At this point, it is difficult to determine why the susceptibility of spermatozoal chromatin to dissociation is decreasing with age, but it is clear that some aspect of chromatin packaging is affected.

Another parameter that points to a change in chromatin packaging with age is the decreased CMA3 staining observed in epididymal spermatozoa from old rats. This finding once again raises the question as to whether this assay truly measures a change in protamine levels or whether decreased staining implies that the DNA is less accessible to the fluorochrome. It appears unlikely that the decrease in CMA3 staining would be explained by increased protamination, as total thiol levels were consistently lower in spermatozoa from older rats. Therefore, the results of this assay suggest that chromatin packaging in epididymal spermatozoa becomes altered with age.

We can only speculate as to what might be changing in chromatin packaging and what could be the reason for having a spermatozoal population with low thiols. The change in thiols might be because of problems with the protamines, as they are the main source of thiols in the spermatozoa head. Studies in humans have demonstrated that some types of male infertility have increased histone-to-protamine ratios (Silvestroni *et al*., 1976; Zhang *et al*., 2006), as well as abnormal protamine deposition (Balhorn *et al*., 1988; Carrell and Liu, 2001; Aoki *et al*., 2005) and decreased protamine RNA (Steger *et al*., 2001; Morrison *et al*., 2005). It is possible that the changes we observed in spermatozoa of ageing rats have the same origin as the problems described in these studies. However, there are several other possibilities, such as abnormal protamine dephosphorylation or sloughing of immature spermatozoa from the seminiferous tubes that could be causing the observed phenotype.

In this study, we used the TUNEL assay coupled to flow cytometry to assess spermatozoa DNA quality. Traditionally, the TUNEL assay is referred to as an apoptosis assay, but this definition is limiting, as the TdT enzyme will label 3′OH DNA termini with FITC-dUTP in any cell that contains this type of DNA breaks, not only in those undergoing apoptosis. Some authors have stated that mature spermatozoa staining positive for TUNEL are in the process of undergoing apoptosis (Oosterhuis *et al*., 2000; Anzar *et al*., 2002; Oosterhuis and Vermes, 2004), but in fact mature spermatozoal DNA is not accessible for transcription, and spermatozoa do not have the ability to synthesize proteins *de novo* (Diez-Sanchez *et al*., 2003), making upregulation of apoptotic mediators and apoptosis itself a virtual impossibility. Therefore, when applied to spermatozoa, this assay measures DNA integrity and not cellular apoptosis. Over the last few years, it has gained popularity as a tool for assessing chromatin quality in spermatozoa from humans (Sun *et al*., 1997; Vicari *et al*., 2002; Muratori *et al*., 2003; Erenpreiss *et al*., 2004; Said *et al*., 2005).

In this report, we are the first to adapt the TUNEL assay for evaluating rodent spermatozoa or to use it on caput spermatozoa from any source. Our results for the young and old control animals show that there is more 3′OH DNA staining in spermatozoa from cauda epididymides in older rats. This suggests a decrease in DNA quality with age and further implicates the epididymis as the site where antioxidant protection becomes compromised. These results support the findings of our earlier study, where we found a significant age effect on the antioxidant enzymes in the cauda epididymidis (Zubkova and Robaire, 2004).

The increase in TUNEL, as well as the presence of a low thiol staining spermatozoal population in spermatozoa from older animals, suggests the possibility of an increase in immature spermatozoa becoming released from the seminiferous epithelium of the testes with age. An even more serious concern in older animals is that, because of the apparent increase in oxidative challenge and/or decrease in antioxidants in the epididymis, spermatozoal chromatin may be accumulating the 8-OH-2′deoxyguanosine (8-OHdG) adduct, which, upon replication, has been shown to become mutagenic (Kamiya, 2003).

Changes in spermatozoa after BSO and H_2O_2

From the end-points measured in this study, the most evident response to BSO was the dramatic oxidizing shift in caput epididymidal spermatozoa from a predominantly free thiol state to disulphides, probably attributable to the systemic oxidative challenge induced by BSO. This increase in disulphides might also be the reason behind the decrease in CMA3 penetration and the decreased DFI and DFI percentage, particularly because changes in these parameters were observed only in spermatozoa from the caput, but not the cauda epididymidis, where disulphide levels are already high. The changes in TUNEL staining, however, were probably not dependent on thiol oxidation but were due to direct effects of increased oxidants on DNA. It would then appear reasonable that spermatozoa are more susceptible to DNA damage during their transport through the epididymis than during the final steps of spermiogenesis, as there is no evidence for DNA repair in epididymal spermatozoa (Parvinen and Hecht, 1981; Van Loon *et al*., 1991; McMurray and Kortun, 2003). Overall, the response of spermatozoa from young and old rats to BSO treatment was very similar in all assays. Therefore, it appears that the reaction to glutathione depletion is not greatly altered by age but rather is dependent on the stage of maturation of spermatozoa.

The incubation of spermatozoa in H_2O_2, on the contrary, generated striking responses. Some parameters, such as the thiol measurements and AO assay results, changed in a manner that is consistent with the oxidizing and DNA-damaging properties of H_2O_2 (Halliwell and Gutteridge, 2002; Bennetts and Aitken, 2005; Zubkova *et al*., 2005). Other parameters, specifically the TUNEL and CMA3 assays, showed an unexpected effect of H_2O_2 on BSO-treated spermatozoa taken from the cauda epididymides of old rats. In this group, TUNEL staining

dropped to very low levels, which is inconsistent with results we obtained for the other spermatozoa groups, with previous reports on the effect of oxidants on spermatozoa (Said *et al*., 2005; Smith *et al*., 2005) or with what is known about the DNA-damaging effect of H_2O_2 in general (Halliwell and Gutteridge, 2002). This unusual response might be because of the oxidative damage reaching a critical level, at which point the 3′OH DNA groups become altered and can no longer bind the fluorochrome.

The increase in CMA3 staining in H_2O_2- and BSO-treated spermatozoa from the cauda epididymides of old rats was also an uncharacteristic response, as in this and previous studies, we observed that thiol oxidation corresponded to decreased CMA3 penetrability (Zubkova *et al*., 2005). No other research groups have looked at the effect of oxidants on CMA3 staining, making it difficult to explain the observed response. It is possible, however, that similar to the TUNEL assay, altered CMA3 staining may be because of oxidative damage reaching a critical point, no longer condensing the chromatin, but creating a more fragmented structure more prone to absorbing fluorochrome.

Conclusion

In summary, we have used clinically relevant assays to examine spermatozoal chromatin packaging and integrity. The results of these assays show that the chromatin was altered during spermatozoal passage along the epididymis, where it became more compact and nuclear thiols were oxidized. Furthermore, we found that the measured parameters differed between young and old animals, demonstrating a shift towards greater chromatin compactness and decreased quality with age. Finally, although spermatozoa from young and old animals responded similarly to *in vivo* oxidative challenge, it is clear that spermatozoa from the cauda epididymidis of old rats have a different susceptibility to the combined effects of *in vivo* and *in vitro* oxidative challenges than did the spermatozoa from young animals.

The results of this study may have implications for older men who wish to father children. If their spermatozoa are more sensitive to the effects of oxidants, dietary antioxidant supplementation might be of benefit, because this has been found to improve spermatozoal quality in several trials (Lenzi *et al*., 1993; Vezina *et al*., 1996; Zabludovsky *et al*., 1999; Keskes-Ammar *et al*., 2003). In addition to this, the finding of a distinct population in spermatozoa from older males is particularly relevant to patients undergoing IVF and especially intra-cytoplasmic sperm injection—a technique in which the natural spermatozoa selection processes are entirely bypassed. Further examination of the changes that occur in spermatozoal quality with age may unveil a common origin with some of the problems seen in male infertility.

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