Changes in spermatozoal chromatin packaging and susceptibility to oxidative challenge during aging

Ekaterina V. Zubkova, B.Sc.,[a] *Michael Wade, Ph.D.,*[c] *and Bernard Robaire, Ph.D.*[b]

[a] Departments of Pharmacology and Therapeutics and [b] Obstetrics and Gynecology, McGill University, Montreal, Quebec, Canada; and [c] Environmental Health Science Bureau, Health Canada, Ottawa, Ontario, Canada

Objective: Our goal was to test the hypothesis that spermatozoal chromatin packaging changes with age and that aging affects the susceptibility of spermatozoal DNA to oxidative damage.
Design: Laboratory study.
Setting: Academic facility.
Patient(s): Young (4 months) and old (21 months) Brown Norway rats.
Intervention(s): Spermatozoa were collected from the cauda epididymidis and were incubated in saline or H_2O_2.
Main Outcome Measurement(s): Thiols levels, chromatin condensation, DNA susceptibility to acid-induced DNA denaturation, and DNA damage were evaluated using monobromobimane, chromomycin A3 (CMA3), acridine orange, and polymerase chain reaction, respectively.
Result(s): Spermatozoa from old rats had 25% fewer disulfides but similar levels of free thiols as compared with young. The CMA3 staining was decreased by 13% with age. Levels of chromatin denaturation and DNA damage were similar in control groups. After exposure to oxidant, free thiols became oxidized by about 20% irrespective of age, but CMA3 staining changed little. The acridine orange assay, however, showed a trend for greater chromatin denaturation in spermatozoa from old rats after oxidant treatment. Furthermore, the DNA from spermatozoa of old rats was significantly more susceptible to developing DNA breaks and modification after oxidative challenge.
Conclusion(s): Spermatozoal chromatin packaging changes with aging and vulnerability to oxidative damage increases. (Fertil Steril® 2005;84(Suppl 2):1191–8. ©2005 by American Society for Reproductive Medicine.)

Key Words: Spermatozoa, chromatin, DNA, aging, oxidative challenge, rat, male reproductive system

Delayed parenthood is becoming an increasingly frequent option in today's society (1). While the decline in a woman's fertility with age is well studied and the consequences are well documented, it is only recently that issues relating to paternal age have started to be recognized (2). Epidemiologic studies on the effect of paternal age on offspring development have linked increased age with a number of genetic diseases, such as dwarfism, schizophrenia, Alzheimer's disease, cardiac defects, and cancers (3–7). Furthermore, both clinical studies and animal models show that the quality of spermatozoa and ejaculate change with advancing age, leading to decreased motility, abnormal morphology, decreased semen volume, and altered pregnancy outcome (8–12). Such studies offer strong evidence that spermatozoa produced in aged individuals differ from those of young ones.

The role of spermatozoa is to successfully deliver an intact set of paternal chromosomes to the oocyte. Therefore, the integrity of a spermatozoon's DNA is a critical issue in male fertility. Several studies have shown that damaged DNA in spermatozoa (such as increased fragmentation) is associated with decreased fertility or problems in the health of the offspring (13, 14).

There are several characteristics of spermatozoa and their microenvironment that protect chromatin integrity beyond what is seen in somatic tissues. The first level of defense is the privileged milieu created by the blood-testis and the blood-epididymis barriers, which have the potential of stopping many toxic and damaging agents from reaching spermatozoa (15–18). Additionally, fluids that bathe spermatozoa are rich in protective agents such as antioxidants (19–22). Lastly, the highly compacted nature of the chromatin in the nuclei of spermatozoa provides additional protection.

One of the distinctive features of the structure of spermatozoa is the manner in which its chromatin is packaged. Spermatozoal DNA is packaged with protamines in place of somatic histones. This packaging greatly condenses the DNA, precluding RNA transcription, and results in lower accessibility to DNA-damaging agents (23). The stability of the nucleus is further enhanced by inter- and intramolecular protamine disulfide bonds that form during epididymal transit (24).

Protamines are unique to spermatozoa; they replace histones during spermatogenesis, when these proteins align lengthwise along the major groove of the DNA helix (25, 26), condensing it into a toroidal structure (27). During the process of spermatozoal maturation in the epididymis, the

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Reprint requests: Bernard Robaire, Ph.D., Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montréal, QC, Canada H3G 1Y6 (FAX: 514-398-7120; E-mail: bernard.robaire@mcgill.ca).

cysteines become progressively oxidized, thus forming inter- and intraprotamine disulfide bonds and further stabilizing the chromatin (28, 29). Numerous studies have shown an association between abnormal protamine deposition and infertility (30–32). However, the effect of aging on spermatozoa thiol bond formation and protamine deposition has never been investigated. Therefore, the first goal of our study is to evaluate what effect aging has on chromatin packaging in spermatozoa.

Assessing the damage induced by oxidative radicals in spermatozoa obtained from individuals of increasing age is of particular interest because spermatozoa encounter this type of stress during maturation in the male reproductive tract, upon ejaculation if leucocytes are present in semen (33, 34), during capacitation (35, 36), and during preparation for in vitro fertilization (37). Exposure to oxidative radicals can cause changes in spermatozoa motility, lipid and protein structure, and DNA integrity, and it strongly correlates with male-factor infertility (38–40). Therefore, we compared the susceptibility of chromatin from young and old males with oxidative challenge. We chose H_2O_2 as the oxidative stressor for its membrane permeability and readiness to form the highly reactive hydroxyl radical ($OH^{\bullet}$) (41).

Using the Brown Norway rat, a well-established model to study male reproductive aging (42–44), we found that aging correlates with changes in spermatozoal chromatin packaging and that the DNA becomes more susceptible to oxidative damage.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except for monobromobimane (mBBr), marketed as Thiolyte MB (Calbiochem, San Diego, CA), and the GeneAmp XL polymerase chain reaction (PCR) kit (Applied Biosystems, Foster City, CA), which includes the *Thermus thermophilus* DNA polymerase.

Animals

Adult male BN RIJ rats, aged 4 and 21 months, were obtained through the National Institutes on Aging (Bethesda, MD) from Harlan Sprague-Dawley (Indianapolis, IN). The rats were housed on a 14 hours light/10 hours dark cycle and were provided with food and water ad libitum. The animal studies were conducted in accordance with 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).

Collection of Spermatozoa

Cauda epididymides from nonregressed testes were excised and finely chopped to release spermatozoa. To achieve maximum yields, spermatozoa were collected into a motility buffer (45). They were washed twice with hypotonic buffer (0.45% NaCl) to lyse any contaminating cells, then washed twice with PBS (1 mmol/L KH_2PO_4, 10 mmol/L Na_2HPO_4, 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.0) and finally divided into control, low-dose (2.5 mmol/L H_2O_2), and high-dose (5 mmol/L H_2O_2) treatment groups. Each sample was then incubated for 1 hour at room temperature, washed twice with PBS, divided into vials containing approximately 5 million spermatozoa for the corresponding assays, and frozen at −80°C.

Monobromobimane Thiol Labeling

Thiol labeling was done according to Seligman et al. (46) with minor modifications. Briefly, spermatozoa from each treatment group were divided into two samples: one that was preincubated with 1 mmol/L of 1,4-dithiothreitol (DTT) to reduce disulfides to free thiols and one that was not. A 50-mmol/L stock solution of mBBr was prepared in acetonitrile and added to the spermatozoa suspension for a final mBBr concentration of 0.5 mmol/L, and the sample was incubated in the dark for 10 min. During this time, mBBr, a fluorescent probe, reacted and bound to free thiols. Spermatozoa were then washed in PBS, sonicated on ice to detach heads from tails, and stored at 4°C (for 3 days) in the dark until analysis.

Analysis of spermatozoa was done using a fluorescence-activated cell sorter (FACS) Vantage flow cytometer (BD Biosciences, Missisauga, Ontario, Canada) equipped with an argon ion laser (488-nm line excitation, for FSC and SSC profile). Blue fluorescence emission of mBBr was detected by 355 UV laser excitation and quantified (in arbitrary units) after passage through a 424/44 bandpass filter using Cellquest Pro (BD Biosciences). A total of 20,000 sperm were analyzed for each sample.

Disulfide concentrations were calculated by subtracting free thiols from total thiols for the corresponding sample and then dividing the value by two.

Chromomycin A3 Staining

Our flow cytometry–based chromomycin A3 (CMA3) quantification was adapted from the slide-based method (47). The CMA3 was dissolved in McIlvaine's buffer (17 mL of 0.1 mol/L citric acid mixed with 83 mL of 0.2 mol/L Na_2HPO_4 and 10 mmol/L $MgCl_2$, pH 7.0) to a concentration of 0.25 mg/mL. Chromatin was labeled as follows: Spermatozoa were incubated in the CMA3/McIlvaine's buffer for 20 min at 25°C in the dark. They were then washed in PBS, sonicated on ice to detach heads from tails, and stored at 4°C in the dark until analysis.

Flow cytometry analysis was done using a MoFlo High Performance Cell Sorter (DakoCytomation, Fort Collins, CO) equipped with an I90 argon ion laser tuned to 457-nm line excitation (for FSC and SSC profile and also for excitation) and a 460/10 filter. The resulting fluorescence was detected with a 580/30 bandpass filter and quantified

(in arbitrary units) using Summit v.3.1 software (DakoCytomation). A total of 20,000 sperm were analyzed for each sample.

Acridine Orange Assay for Sperm DNA Denaturation

To assess susceptibility of sperm DNA to acid-induced denaturation, the acridine orange (AO) assay was performed using a method described previously (48, 49). On the day of assay sperm samples were thawed and sonicated on ice, as discussed previously, to remove tails. A 300-μL aliquot of spermatozoa in TNE (10 mmol/L Tris, 150 mmol/L NaCl, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4) was mixed with 400 μL of solution containing 0.08 N HCl, 150 mmol/L NaCl, and 0.1% Triton X-100, pH 1.2, and incubated at room temperature for 30 sec to denature any uncondensed sperm DNA. Spermatozoa were then stained by the addition of 1.2 mL of AO solution (200 mmol/L Na_2HPO_4, 0.1 mol/L citric acid buffer, pH 6.0, 1 mmol/L EDTA, 150 mmol/L NaCl, and 6 μg/mL AO).

Stained spermatozoa were analyzed using a FACS Calibur flow cytometer (BD Biosciences) fitted with an argon ion laser (488-nm line excitation), and green fluorescence emission of AO was reflected by a 550 dichroic longpass filter and quantified after passage through a 530/30-nm bandpass filter. Red fluorescence of AO was detected after sequential passage through the 550 dichroic filter and a 670 nm longpass filter. Degree of spermatozoa DNA denaturation (DD) was determined by the intensity of red fluorescence (denatured DNA, arbitrary units) divided by the sum of red and green intensity and was calculated from raw data using WinList cytometry software (Verity Software, Topsham, ME). Cells with abnormal DD within each sample were identified from the histogram plots of DD as the cells with DD values are clearly skewed higher than the tight cluster of DD values of normal cells. A total of 10,000 sperm were analyzed for each sample.

Extraction of Genomic DNA

Spermatozoa were resuspended in 1 mL STE buffer (50 mmol/L NaCl, 10 mmol/L Tris HCl, pH 8.0, 1 mmol/L EDTA) with 50 μL of 20% SDS, 40 μL of 0.5 mol/L DTT, and 200 U of proteinase K and incubated at 55°C for 6 hour with shaking. Organic extraction was done by first mixing DNA with one volume phenol, followed by extraction with one volume of phenol/chloroform/isoamyl alcohol (25:24:1), followed by extraction with one volume chloroform. The DNA was then ethanol precipitated and resuspend in TE buffer (10 mmol/L Tris-Cl, 1 mmol/L EDTA, pH 7.4).

Quantitative PCR

Quantitative PCR was used to establish the extent of bulky DNA adducts and single/double strand breaks in genomic DNA. This assay works on the principle that increased bulky adduct formation or DNA breaks would prevent the polymerase enzyme from completing amplification of the complimentary strands (50). Therefore, a sample with greater DNA damage would result in less PCR product being amplified. This is a powerful assay, which can be performed using nanogram quantities of DNA, and damage can be assessed in individual genes. By using primers for the clusterin gene and the mitochondrial genome, the quantitative PCR technique can be applied to both genomic and mitochondrial DNA.

The assay was done based on the method of Ayala-Torres et al. (50) Briefly, PCR conditions were as follows: 5 ng template DNA, 3.3× buffer, 1 mg/mL bovine serum albumin (BSA), 10 mmol/L dNTP, 25 mmol/L $Mg(OAc)_2$, 10 pmol of each primer, and one unit of *Thermus thermophilus* DNA polymerase. The PCR was initiated in a GeneAmp 2400 thermocycler (Perkin Elmer, Boston, MA) with a 75°C hotstart, followed by denaturation for 1 minute at 94°C and 25 cycles of the subsequent profile: 15-second denaturation at 94°C and 12-minute primer extension at 68°C. The PCR was completed with a 10-minute extension at 72°C.

We applied these conditions to amplify both a 12.5-kb clusterin gene fragment (using primers 5′-AGA CGG GTG AGA CAG CTG CAC CTT TTC-3′ and 5′-CGA GAG CAT CAA GTG CAG GCA TTA GAG-3′) and a 13.4-kb mitochondrial genome fragment (using primers 5′-GGC AAT TAA GAG TGG GAT GGA GCC AA-3′ and 5′-AAA ATC CCC GCA AAC AAT GAC CAC CC-3′). Quantitative conditions were confirmed by amplifying samples containing 50% DNA (data not shown).

Statistical Analysis

Data were initially analyzed using two-way analysis of variance (ANOVA) (for age and treatment), followed by the Holm–Sidak multiple comparison test for pairwise analysis ($P<.05$). All statistics were done with SigmaStat (Version 3.0, Systat Software, Richmond, CA). The groups had n = 6 each and results are expressed as mean ± SEM.

RESULTS

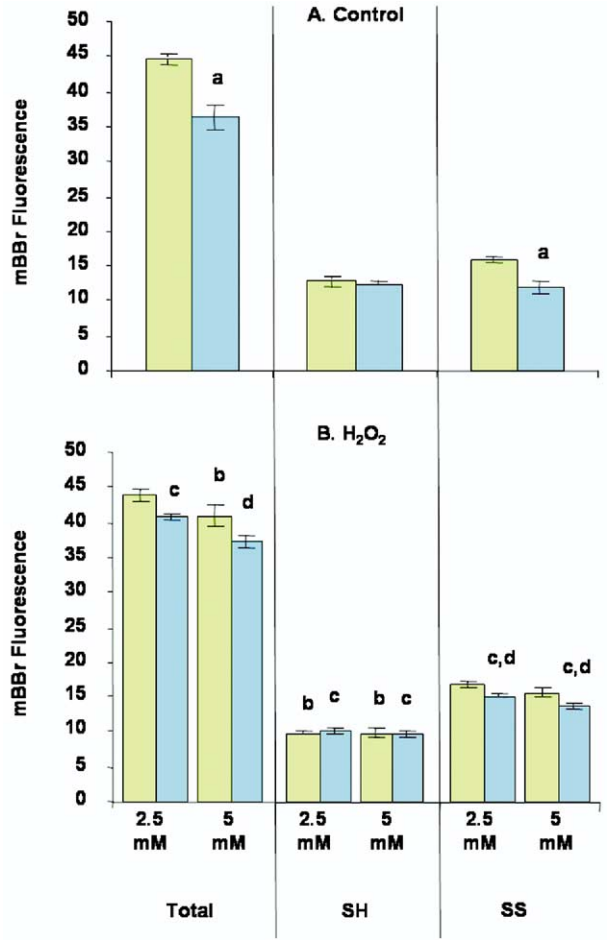
Monobromobimane Thiol Labeling

A significant overall decrease in thiol quantity was found in spermatozoa from older animals (Fig. 1A). When we looked at the free thiols and disulfides separately, we found that there was no age-related change in free thiol status. Disulfides, on the other hand, showed a significant decrease in spermatozoa from old rats, where the mean mBBr fluorescence intensity decreased by more than 25%.

After H_2O_2 treatment, total thiol levels continued to be lower in spermatozoa of aged animals, but only in the high H_2O_2 group (Fig. 1B). Free thiols significantly decreased after both low- and high-dose treatment; this decrease was not dose dependent and was a predictable result of treatment with an oxidant (Fig. 1B).

FIGURE 1

Disulfide bonds, free thiol, and total thiol quantification by mBBr staining. *Yellow bars* represent young and *blue bars* represent old. Statistics key: a = significant change between young control and old control; b = significant change between young control and young treated; c = significant change between old control and old treated; d = significant change between young treated and old treated. Mean ± SEM; n = 6.



Zubkova. Effect of age on spermatozoal chromatin. Fertil Steril 2005.

As a result of free thiols becoming oxidized by H_2O_2, disulfide levels increased in all groups. This increase was significant in spermatozoa from old rats at both doses, but not significant in spermatozoa from young rats.

Chromomycin A3 Labeling

We found a remarkable difference in CMA3 labeling in spermatozoa collected from young and old rats (Fig. 2). Spermatozoa from young rats had significantly more CMA3 incorporation when compared with spermatozoa from old animals, suggesting either increased protamination or decreased strength of protamine-DNA association or tighter chromatin structure with age. Incubation with H_2O_2 did not significantly alter CMA3 incorporation levels in any of the groups. Furthermore, the age-dependent difference was maintained, but it was statistically significant only in the control and low-dose groups.

Acridine Orange Assay for Sperm DNA Denaturation

Results from the sperm chromatin structure assay showed a clear dose-dependent increase in spermatozoa acid-induced DNA denaturation following incubation with H_2O_2 (Fig. 3).

Although there was no significant difference in the percentage of cells with abnormal DD between spermatozoa from young and old rats, we noticed a trend for increased chromatin denaturation in old rats when compared to young. Specifically, after the low-dose treatment, spermatozoa from old animals had a more than 5% greater percentage of abnormal DD, while at the high dose the increase in percentage of abnormal cells approached 10%. Furthermore, the *P* value for comparison between young and old decreased from 0.95 in controls to 0.35 in low dose to 0.11 in high dose, once again suggesting a trend for more dissociation with age.

Quantitative PCR

Before H_2O_2 treatment amplification of both DNA fragments remained constant regardless of the animals' age. After

FIGURE 2

Protamine association with sperm chromatin determined by CMA3 staining. *Yellow bars* represent young and *blue bars* represent old. Statistics key: a = significant change between young control and old control; b = significant change between young control and young treated; c = significant change between old control and old treated; d = significant change between young treated and old treated. Mean ± SEM; n = 6.

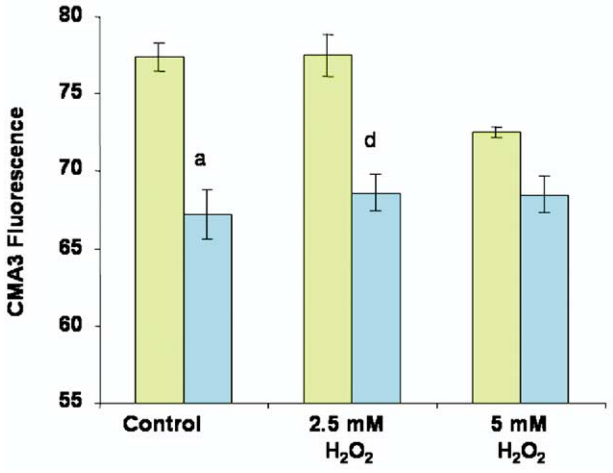


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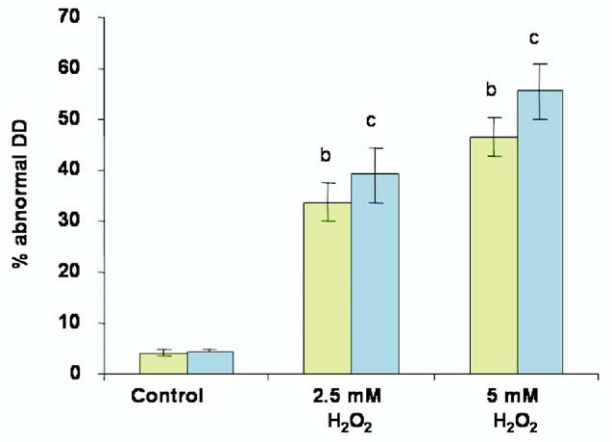
FIGURE 3

The proportion of sperm showing abnormal DNA denaturation (DD) as determined by the acridine orange assay. Values represent the mean proportion of sperm in each sample showing abnormally high levels of DD due to acid treatment (% DNA denaturation). *Yellow bars* represent young and *blue bars* represent old. Statistics key: a = significant change between young control and old control; b = significant change between young control and young treated; c = significant change between old control and old treated; d = significant change between young treated and old treated. Mean ± SEM; n = 6.



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H_2O_2, however, there was a clear age-dependant change in amplification.

Amplification of the clusterin gene from spermatozoa of young animals did not significantly change after treatment with H_2O_2 (Fig. 4A). In spermatozoa of old animals, however, there was a significant effect at the high H_2O_2 dose. Furthermore, DNA from old animals was significantly less amplified when compared to that from young rats after the high-dose H_2O_2 treatment ($P<.001$). A significant interaction between age and treatment was found.

Mitochondrial DNA, on the other hand, was affected by H_2O_2 treatment in both young and old, causing dose-dependent decreased amplification of the genome (Fig. 4B). Furthermore, similarly to what was seen with the chromatin fragment, DNA from aged animals was significantly less amplified than that extracted from young at the high H_2O_2 dose.

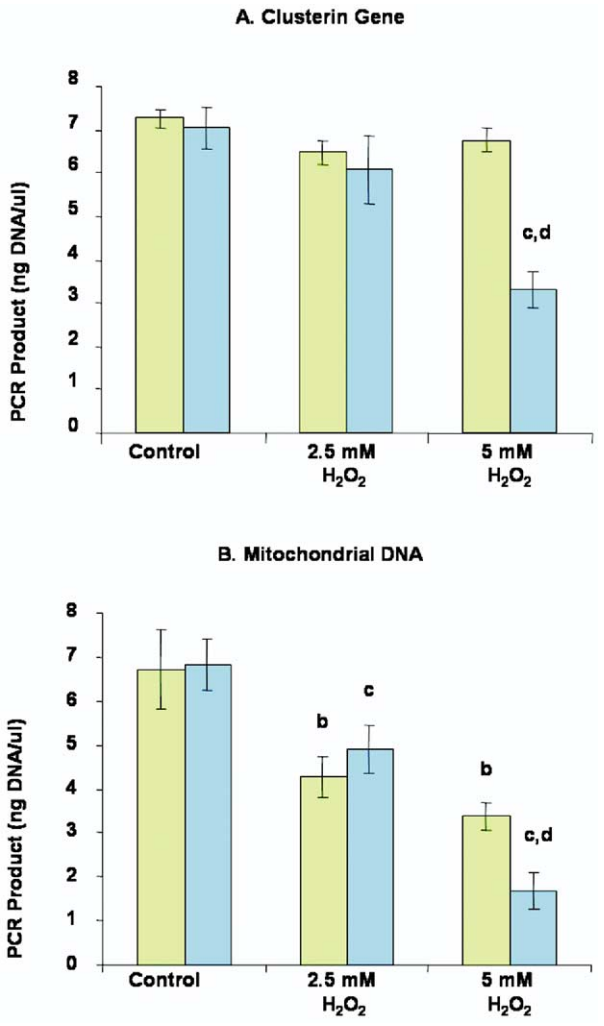
DISCUSSION

In this study, we used a complimentary set of assays to understand the effect that aging has on spermatozoa chromatin structure. By combining assays that test for physiologic processes (disulfide bond formation and chromatin protamination) with those that measure functional endpoints (DNA denaturation and damage), we found significant differences between spermatozoa from young and old rats.

The most dramatic change was a decrease of 25% in disulfide levels in old rats, implying a significant change in chromatin packaging. This change might be due to decreased intra- and interprotamine binding, thus potentially leaving spermatozoa of older rats less coiled and more vulnerable to

FIGURE 4

Detection of DNA breaks and oxidative modifications by PCR. *Yellow bars* represent young and *blue bars* represent old. Statistics key: a = significant change between young control and old control; b = significant change between young control and young treated; c = significant change between old control and old treated; d = significant change between young treated and old treated. Mean ± SEM; n = 6.



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oxidative insult. This observation may be of particular significance because Zini et al. (51) showed that spermatozoa of infertile men have a higher level of free thiols and are more susceptible to DNA denaturation than those from fertile controls.

Incubation of spermatozoa with H_2O_2, an oxidant, resulted in thiols being converted from the reduced to the oxidized form. However, the thiol groups responded similarly in spermatozoa from both young and old rats, indicating that sensitivity of thiols to H_2O_2 is not altered with age.

Having established that there is a change in overall thiol levels with age, we wanted to see if this change was associated with alterations in chromatin protamination. The CMA3 assay is becoming more extensively used for evaluating sperm quality as part of male fertility assessment (52, 53). It provides an indirect measure of assessing protamines in sperm (54, 55), where CMA3 competes with protamines for chromatin binding (47, 56).

We found more CMA3 labeling in spermatozoa from young than from old rats in both control and H_2O_2-exposed groups. This was surprising because we expected spermatozoa from young rats to have more extensive protamination due to the higher concentration of disulfides detected in the chromatin. However, increased CMA3 incorporation can also occur if protamines are more easily displaced by the staining reagent or if chromatin has a looser structure (57). Our results indicate that the relationship between thiols, protamines, and CMA3 incorporation in older males is not as straightforward as in younger males.

To investigate if the altered DNA packaging in spermatozoa of aged rats affects chromatin condensation, spermatozoal chromatin structure was assessed using the acridine orange assay. This assay utilizes the differential fluorescence of the nucleic acid–binding dye AO, which fluoresces red or green when bound to either denatured or condensed chromatin, respectively. The degree of condensation of sperm nuclei is related to fertilizing potential (58–62) and is a parameter indicative of toxicant-induced disruption of spermatogenesis (63).

When spermatozoa from young and old rats were compared, we saw no statistically significant difference in percentage of abnormal DD between the two age groups, either before or after H_2O_2 incubation. However, there was a clear trend toward greater decondensation in spermatozoa from old rats. A study done on rabbit spermatozoa supports this, as it also found that decondensation increased with age (64).

The increased chromatin dissociation after H_2O_2 treatment is most likely due to oxidant-induced DNA breaks. Owing to the condensed nature of the chromatin and the loss of most of their cytoplasm, mature spermatozoa lack appreciable levels of DNA repair (65–67). Knowing that $OH^{\bullet}$ damages DNA by inducing base modification and strand breaks (41), we used the quantitative PCR assay to examine if the slight difference in percentage of abnormal DD that we saw between young and old animals was indeed due to increased susceptibility to oxidative DNA modifications with age.

Quantitative amplification of the clusterin gene revealed that the level of DNA breaks was similar in young and old before H_2O_2 incubation. However, after oxidative stress, two very different responses emerged, where the chromatin from spermatozoa of aged rats showed a dramatic dose-dependent decrease in amplification. This supports our hypothesis that chromatin from spermatozoa of older rats is more sensitive to damaging agents than that of young ones. Furthermore, when these results were compared with those obtained from the mitochondrial genome of spermatozoa, DNA from both young and old rats showed a significant decrease in amplification after treatment, with a somewhat greater decrease at the higher H_2O_2 dose in the old rats. One possible explanation for the increased level of DNA damage in mitochondrial DNA is that it lacks protamines, proteins that act to protect spermatozoal DNA. This observation supports our findings regarding changes in chromatin packaging in the old rats.

Although only two studies have been published where the quantitative PCR technique was used in spermatozoa, our results correlate well with the previous report of significantly higher damage to the mitochondrial genome as opposed to the genomic DNA after H_2O_2 (68). It is of interest to note that in human spermatozoa, Sawyer et al. (69) showed much greater nuclear DNA damage than what was observed in our study, but this may be due to the fact that human spermatozoa are of lower overall quality than that found in other species (70). Additionally, human spermatozoa express both protamine 1 and protamine 2 (whereas rats have only protamine 1). It has been shown that the chromatin of spermatozoa from species expressing both protamines is more susceptible to decondensation (71); this property might also make it more susceptible to damage associated with oxidant exposure.

The changes in spermatozoal chromatin packaging observed with age could be due to a number of age-related changes in both testicular and epididymal function. Overall, the age-dependant accumulation of oxidative damage in an organism's DNA and proteins (72) can affect processes involved in the production of intact spermatozoa. In the testes, DNA mutations have been shown to accumulate in spermatozoa as a result of constant germ cell replication (73). A thickening of the basal membrane (74, 75) and a decrease in Sertoli cell numbers (76, 77) also occur in both humans and rats with age. Furthermore, we recently published our findings on changes in epididymal antioxidant capacity with age in rats (78)—these changes could also affect oxidative processes involved with chromatin compaction in spermatozoa.

In summary, we report here, for the first time, decreased CMA3 fluorescence and decreased disulfide formation in spermatozoa as animals age. These changes may be causing spermatozoa to become more vulnerable to oxidative damage with age and to develop more DNA breaks and oxidative

modifications. Therefore, although males remain fertile well into old age, the quality of their spermatozoal chromatin declines, which in turn may have an impact on the well-being of their offspring.

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