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

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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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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