Published-Ahead-of-Print October 4, 2006, DOI:10.2164/jandrol.106.001362
Journal of Andrology, Vol. 28, No. 2, March/April 2007

DOI: 10.2164/jandrol.106.001362

Spermatozoa Have Decreased Antioxidant Enzymatic Capacity and Increased Reactive Oxygen Species Production During Aging in the Brown Norway Rat

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Received for publication August 3, 2006; accepted for publication September 25, 2006.

Abstract

As the proportion of aged males attempting to reproduce continues to rise, so does the concern regarding the quality of spermatozoa from aged men. An imbalance between the generation of reactive oxygen species (ROS) and cellular antioxidant defenses, as occurs in aging, ultimately leads to decreased protein, lipid, and DNA quality. Spermatozoa are highly susceptible to oxidative damage, and thus an age-related shift in redox status may have serious implications for fertility. Therefore, we examined the effect of age on antioxidant enzymatic activity, ROS production, and extent of lipid peroxidation in both caput and cauda epididymal spermatozoa from young (4-month-old) and old (21-month-old) Brown Norway rats. Glutathione peroxidase (Gpx1, Gpx4) and superoxide dismutase (SOD) enzymes had decreased activity in aging spermatozoa. Immunofluorescence studies indicated that Gpx4 expression was decreased in both the head and midpiece regions of spermatozoa in aged animals. The decrease in nuclear Gpx4 points to a novel potential mechanism that may explain the previously noted decreased levels of protamine disulfide bonds in aged sperm nuclei. Further, hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet -}$) production were increased significantly in aging spermatozoa. Finally, lipid peroxidation was found to be drastically increased in aged spermatozoa. Taken together, these results suggest a decreased capacity for aged spermatozoa

to handle oxidative stress and provide a potential basis for understanding the underlying cause of decreased quality of spermatozoa during aging.

Key words: Oxidative stress, epididymis, glutathione peroxidase, superoxide dismutase, reproduction, sperm

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Oxidative stress is defined as an imbalance between the cellular antioxidant defense systems and the production of reactive oxygen species (ROS), ultimately leading to oxidative damage of cellular macromolecules (reviewed in Harman, 1981). There is a well-established association between aging and increased oxidative stress (Cadenas and Davies, 2000; Finkel and Holbrook, 2000). Since the free radical theory of aging proposed by Harman in 1956 (Harman, 1956), it has been demonstrated by several investigators that as an organism ages, it exhibits a higher propensity for the imbalance leading to oxidative damage (Golden et al, 2002; Sohal et al, 2002; Wei et al, 2002). Higher levels of ROS are found in many aged tissues (Schoneich, 1999) and can irreparably damage proteins, lipids, and DNA (Lucas and Szweda, 1998; Inal et al, 2001; van der Loo et al, 2003).

Spermatozoa are highly susceptible to ROS-induced damage due to the high percentage of polyunsaturated fatty acids (PUFA) in the membrane (Mack et al, 1986; Alvarez and Storey, 1995). Oxidation of these PUFA can lead to decreased fluidity and flexibility of the sperm membrane and can therefore compromise the fertilization potential of the sperm (de Lamirande and Gagnon, 1992, 1995, 1995). In addition to lipid membrane damage, increased ROS in aged spermatozoa has detrimental effects on chromatin quality (Zubkova et al, 2005), as well as on motility (Kidd et al, 2001; Jung et al, 2002) and morphology (Plas et al, 2000) parameters.

In spite of the major consequences oxidative stress may have on male fertility, very few studies have investigated how sperm quality is affected by age. Notably, with the mean age of fathers significantly increasing in recent years, there have been increased concerns about decreased sperm quality with advancing age (Kidd et al, 2001). There is now a significant proportion of men who father children while in their 50s (Plas et al, 2000); men in this age group have a higher incidence of subfertility, and the time to pregnancy is increased (Rolf et al, 1996).

Spermatozoa have long been known as efficient producers of ROS (Tosic and Walton, 1950; Holland and Storey, 1981; Aitken and Clarkson, 1987). ROS such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl ($HO^{\bullet}$) radical are formed as byproducts of aerobic cellular processes. While under normal conditions ROS are essential for various sperm-specific physiological processes (de Lamirande et al, 1997), when produced in larger than normal quantities ROS are associated with male infertility (Aitken et al, 1996; Griveau and Le Lannou, 1997). The mechanism for this is likely due to detrimental ROS effects on the motility of spermatozoa, peroxidation of membrane lipids leading to compromised sperm-oocyte fusion, and decreased chromatin quality (Aitken, 1999; Christova et al, 2004; Zubkova and Robaire, 2004).

From the time they are made in the testes through to ejaculation and travel in the female reproductive tract, spermatozoa are constantly exposed to oxidizing environments. Perhaps most critically, the epididymis can be seen as a highly pro-oxidizing environment for the sperm (Vernet et al, 2004). Spermatozoa entering the caput epididymidis are functionally immature; during their maturation they are under constant attack from intracellular and extracellular production of ROS (Vernet et al, 2004).

It is thus crucial that spermatozoa be protected from oxidative damage. To this end, they contain

enzymatic antioxidant defense systems available to deal with excess ROS production. Glutathione reductase (GR), glutathione peroxidases (Gpx), and superoxide dismutase (SOD) have been shown to exist in epididymal spermatozoa (Tramer, 1998), while the presence of catalase (CAT) remains controversial (Holland and Storey, 1981; Alvarez et al, 1987; Bilodeau et al, 2000). The glutathione peroxidases are involved in repairing oxidative damage to cellular macromolecules and scavenging H_2O_2 while utilizing glutathione (GSH) as a reducing equivalent. Their continued activity depends on the ability of GR to recycle oxidized GSH back to the reduced form.

The family of GSH peroxidases currently contains 5 characterized isoforms. Gpx4 is involved in forming a structural part of the midpiece (Ursini et al, 1999), is a protamine thiol peroxidase in the sperm nucleus (Conrad et al, 2005), and is also involved in reduction of oxidized phospholipids in the membrane of the spermatozoa (reviewed in Ursini et al, 1997). The importance of Gpx4 in the maturation process of spermatozoa during their transit through the epididymis has been demonstrated previously (Conrad et al, 2005). In its nuclear form, Gpx4 is responsible for the continual process of protamine thiol oxidation in the sperm nucleus from the testes, to the caput, and the cauda epididymidis (Conrad et al, 2005); this process is critical for proper chromatin condensation and packaging. A schematic representation of spermatozoa, ROS, and the enzymatic antioxidant defense system is illustrated in Figure 1.

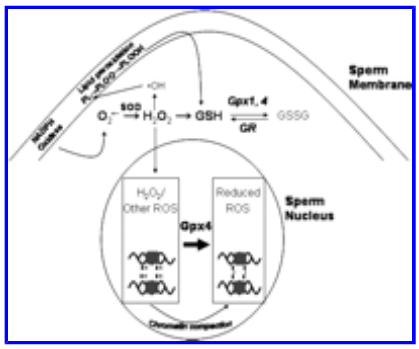

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Figure 1. ROS production, enzymatic antioxidant defence, and the dual role of ROS in the spermatozoon. Superoxide radical (O2•–) is produced by the activity of NADPH oxidase in the sperm lipid membrane. Superoxide is dismutated to hydrogen peroxide (H_2O_2) via the action of superoxide dismutase (SOD). H_2O_2 can initiate lipid peroxidation via production of the hydroxyl radical (•OH) in both the sperm membrane and the sperm nuclear membrane (PL indicates phospholipid). H_2O_2 and lipid radicals (PLOO•, PLOOH) are detoxified by the glutathione (GSH)-dependent antioxidant system. The gluthatione peroxidases (Gpx) utilize GSH as a reducing equivalent and catalyze the reduction of radical oxygen species. GSSG is formed as a result of the oxidation of GSH, and GSH is then recycled via the action of glutathione reductase (GR). H_2O_2 and other radicals such as the phospholipid radical are also indirectly involved in the chromatin compaction process in the sperm nucleus. Gpx4 utilizes the protamine thiol groups (SH) of the sperm chromatin as reducing equivalents to detoxify the radicals. In the process, disulfide bridges are created (SS-SS) between the protamines, ensuring the necessary compact state of the chromatin. While ROS are required for this process, too much ROS can lead to DNA damage. Conversely, too little ROS or impaired Gpx4 activity can lead to improper chromatin compaction, leaving the DNA vulnerable to oxidative attack.

The objective of this study is to examine the enzymatic antioxidant activity and ROS production of maturing spermatozoa during aging and to assess the extent of lipid peroxidation in aging sperm. Our hypothesis is that aged rat epididymal spermatozoa are less efficient than young rats in handling increased oxidative stress and that this loss of efficiency is due to a decreased antioxidant capacity coupled with increased ROS production, leading to increased damage to the sperm lipid membrane.

Materials and Methods

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Reagents

All chemicals were of the highest reagent grade and purchased from Sigma Chemical Co (St Louis, Mo) with the following exceptions: deferoxamine mesylate salt (Spectrum Chemical Corp, Gardena, Calif), the lipid peroxidation kit (Cayman Chemical Co, Ann Arbor, Mich), A23187 (AG Scientific, San Diego, Calif), and the Gpx4 polyclonal antibody (Abcam Inc, Cambridge, Mass).

Animals

Male Brown Norway rats (aged 4 months and 21 months) were obtained from the National Institute of Aging (Bethesda, Md) and housed on a 14L:10D cycle. Food and water were provided ad libitum. All animal studies were conducted in accordance with the guidelines outlined in *A Guide to the Care and Use of Experimental Animals* prepared by the Canadian Council on Animal Care (McGill protocol 206).

Sperm Extraction and Purification

Animals were euthanized by CO_2 asphyxiation, and testes were checked for regression in the 21-month-old animals. Epididymides with corresponding regressed testes were not used for sperm extraction. In total, 6 young (4 months) and 5 old (21 months) animals were used for the study. Caput and cauda epididymides were rapidly removed and placed into buffer on ice, and sperm were collected as previously described (Seligman et al, 1991). Spermatozoa were released into either 1x PBS (for lipid peroxidation and ROS measurements) or the antiperoxidant media BSPDR (for enzymatic analyses, 0.01 mol Tris-Cl (pH 7.5), 0.12 mol NaCl, 1 mmol phenylmethylsulfonyl fluoride (PMSF), 0.1 mmol deferoxamine mesylate, and 0.1 mmol resveratrol) on ice and filtered. Purification and fractionation was carried out essentially as described in Tramer et al (1998). Final pellets were suspended in 1 mL of either 1x PBS or BSPDR and were aliquoted, counted, and stored at −80 °C for subsequent analysis.

Enzymatic Analyses

Prior to analysis, purified epididymal sperm (3 x 10^6/mL) were sonicated (Vibra-Cell; Sonica and Materials Inc, Danbury, Conn) on ice 3 x 20 seconds and then incubated with 0.2% peroxide-free Triton-X at room temperature for 5 minutes. Activities were measured on a Beckman DU-7 spectrophotometer (Beckman Coulter, Fullerton, Calif) using the kinetic function. Final reaction volumes in all enzymatic assays were 1 mL. All enzyme activities were measured within the linear portion of the absorbance/time curve. Assays were run in triplicate.

Gpx1 activity was determined using t-butyl hydrogen peroxide as the substrate by following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm in the presence of an excess of GR (EC 1.6.4.2; Sigma), as previously described (Flohe and Gunzler, 1984). Kinetic analysis was done over 10 minutes recording the rate of decrease in absorption at 340 nm. Final results were expressed in nmol NADPH oxidized/min/10^6 cells.

GR activity was determined by following the oxidation of NADPH at 340 nm via addition of oxidized GSH (GSSG), as previously described (Goldberg and Spooner 1983). Final results were expressed in nmol NADPH oxidized/min/10^6 cells.

Total SOD activity was determined at 550 nm by measuring the inhibition of xanthine/xanthine oxidase–mediated reduction of 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tertrazolium-5-

carboxanilide (XTT), as previously described (Zubkova and Robaire, 2004). One unit of superoxide dismutase activity is defined as the amount needed to inhibit 50% of XTT reduction. The assay was run simultaneously with varying known concentrations of SOD to generate the standard curve. Results are expressed in Units of SOD activity/10^6 cells.

Gpx4 activity was determined by following the oxidation of NADPH at 340 nm with the addition of an excess of GR. Activity was calculated in assay conditions exactly as for Gpx1, except that phosphatidylcholine hydroperoxide (PCOOH) was used as the substrate (Maiorino et al, 1990). PCOOH was isolated via the reaction of phosphatidylcholine (Type IV/S; Sigma) with soybean lipoxygenase (EC 1.13.11.12, Type V; Sigma) in a C-18 Sep Pak cartridge (Waters Corp, Milford, Mass), as previously described (Maiorino et al, 1990). PCOOH was extracted from the solid phase using methanol. The final substrate concentration was quantified spectrophotometrically at 234 nm. Results were expressed in nmol NADPH oxidized/min/10^6 cells.

Gpx4 Immunofluorescence and Quantification

For the determination of Gpx4 immunofluorescence, epididymal spermatozoa were treated with either 40 mmol GSH or 40 mmol dithiothreitol (DTT) for 40 minutes at room temperature before being spun onto glass slides using the StatSpin Cytofuge (Iris Sample Processing, Westwood, Mass). Treatment with GSH facilitates head staining, while DTT treatment is specific for midpiece Gpx4 staining (Haraguchi et al, 2003). Cells were fixed in −20 °C methanol for 10 minutes, blocked, and incubated with a polyclonal anti-Gpx4 primary antibody (1:50). Secondary antibody was FITC-conjugated (1:200); imaging was done using standard fluorescence microscopy.

Quantification of immunofluorescence was done with the microcomputer imaging software (MCID, version 7.0; Imaging Research, St Catherine's, Canada), using the relative optical density (ROD). GSH-treated spermatozoa were used to assess relative head fluorescence intensity, and DTT-treated sperm were used to assess relative midpiece fluorescence. ROD values were converted to gray levels by the formula [$(1/10^{ROD})$ x 256]. A minimum of 100 sperm were counted per group (n = 3).

ROS Measurements

H_2O_2 Steady State Measurements— Determination of H_2O_2 steady state concentration in epididymal sperm suspensions after 10 minutes incubation was done by fluorometric assay using the horseradish peroxidase (HRP)—p-hydroxyphenylacetic acid (pHPA) system, as previously described (Panus et al, 1993). Spermatozoa were suspended in 1x PBS, 0.05 mg/mL A23187 in 1x PBS, or 200 U/mL CAT in PBS and incubated for 10 minutes at 37 °C. Suspensions were then diluted (1:2.5) in prewarmed (37 °C) 100-mmol phosphate buffer (pH 7.4) containing 2.8 U/mL HRP and 40 µmol p-HPA. Final reaction volume was 1.25 mL. Fluorescence of the samples was measured at 37 °C on a SpectraMax Gemini fluorescence spectrophotometer (Molecular Devices, Sunnyvale, Calif) at 317 nm excitation-414 nm emission wavelengths. H_2O_2 concentration was determined by subtracting the value of the sample treated with 200 U/mL CAT from the untreated samples (buffer alone or buffer + A23187). A standard curve was run in parallel with known concentrations of H_2O_2. H_2O_2 concentration after 10-minute incubation was then calculated using the following formula: $[H_2O_2]$ = fluorescence intensity x V_{final} /slope x $V_{aliquote}$.

Superoxide Production— Superoxide production was determined by chemiluminescence using a computer-driven BMG LUMIStar Galaxy chemiluminometer (BMG Labtech Inc, Durham, NC), as previously described (de Lamirande and Gagnon, 1995, de Lamirande and Gagnon, 1995) at 5-minute intervals for 70 minutes, in the integration mode (output summed for 10 seconds). Spermatozoa were suspended in

either 1x PBS, 0.05 mg/mL A23187 in 1x PBS, or 200 U/mL SOD in PBS (negative control). Epididymal spermatozoa, at a final concentration of 1 x 10^6/mL, were diluted 1:2 in 100 mmol phosphate buffer (pH 7.8) and incubated at 37 °C. Chemiluminescent measurements were started immediately after addition of the probe, 2-methyl-6-(p-methoxyphenyl)-3, 7-dihidroimidazo [1,2-a] pyrazin-3-one (MCLA), at a concentration of 20 µmol, from a prepared 10-mmol stock in DMSO stored in the dark at −80 °C. Due to the potential for autoxidation of MCLA in the assay, every sample was run in parallel with the same sample supplemented with SOD. The SOD signal was then subtracted from the sample data to determine the SOD-inhibitable signal. Because the medium itself can generate superoxide, it was also tested by itself, with or without SOD.

Lipid Peroxidation

Lipids were extracted and lipid peroxide products quantified directly using the LPO kit from Cayman Chemical Co. Briefly, lipids were extracted from epididymal spermatozoa into deoxygenated chloroform. Lipid peroxide products were then quantified directly using a SpectraMax Plus (Molecular Devices) spectrophotometer via the chromogenic detection of the reaction of ferrous ions with lipid peroxides (Milhaljevic et al, 1996). Known concentrations of lipid peroxide were run simultaneously to generate the standard curve, and appropriate blanks were run. The chromogen-peroxide complex was measured at 500 nm. Results were expressed in µmol lipid peroxides/10^7 cells.

Statistical Analysis

Data were analyzed by SigmaStat 2.03 software (SPSS Inc, Chicago, Ill), using 2-way ANOVA and the Holm-Sidak post hoc test, unless otherwise indicated. Error bars represent SEM. Differences were considered significant at *P* less than .05.

Results

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Antioxidant Enzymes

Figure 2 shows the effect of age on the antioxidant enzymatic activities (GR, Gpx1, Gpx4, and SOD) of spermatozoa from the caput and cauda epididymidis. The activity of GR was not significantly affected by age in spermatozoa from either epididymal segment (Figure 2a). Gpx1 activity was significantly decreased in sperm from aging rats taken from the caput epididymidis but not from the cauda region (Figure 2b). Aging also significantly decreased the thiol-oxidizing activity of the chromatin and membrane-associated thiol-dependent Gpx4 in spermatozoa from both segments of the epididymis, but most notably in sperm from the caput epididymidis, in which the activity of sperm from old animals was less then half that of the young (Figure 2c). Further, the activity of total SOD was significantly decreased in the aged cauda epididymal spermatozoa but not in the caput epididymal sperm (Figure 2d).

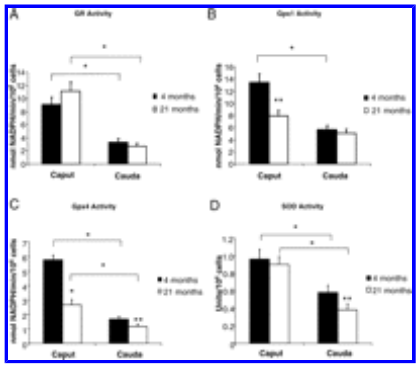

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Figure 2. **(A)** Glutathione reductase (GR), **(B)** glutathione peroxidase-1 (Gpx1), **(C)** glutathione peroxidase-4 (Gpx4), and **(D)** superoxide dismutase (SOD total) activity in caput and cauda epididymal spermatozoa of young (4 months, n = 6) and old (21 months, n = 5) rats. Activities were measured on a spectrophotometer and units of activity standardized per 10^6 cells. Data were analyzed using 2-way ANOVA and Holm-Sidak post hoc test; **P* < .001, ***P* < .05. There was no significant effect of age on GR activity. Aging significantly decreased Gpx1 activity in caput epididymal sperm and significantly decreased SOD activity in cauda epididymal sperm. Further, aging significantly decreased Gpx4 activity in spermatozoa from both segments. In addition, caput epididymal spermatozoa exhibited significantly higher activities then sperm from the cauda in all but the Gpx1 activity of 21-month-old rats.

The results demonstrate a highly significant segment-specific epididymal effect on GSH-dependent GR and Gpx1 antioxidant enzymes (Figure 2a and b), on the thiol-dependent Gpx4 (Figure 2c), and on SOD (Figure 2d). For all 4 enzymes, activities in spermatozoa from the caput were significantly higher than those in the cauda epididymidis, with the exception of the Gpx1 activity of 21-month-old rats.

Gpx4 Immunofluorescence

Spermatozoa were treated in either 40 mmol GSH (Figure 3a) or 40 mmol DTT (Figure 3b) in order to allow the Gpx4 antibody to gain access to the head or midpiece, respectively. DAPI counterstaining was done for comparison purposes. Figure 3a shows that in both GSH-treated caput and cauda epididymal spermatozoa, aging significantly decreased the intensity of fluorescence for Gpx4 in the head of spermatozoa; these levels are quantified in Figure 3c. In both caput and cauda epididymal sperm, Gpx4 staining was twice as intense in the young compared to the old. GSH treatment also appeared to facilitate midpiece staining in the young animals

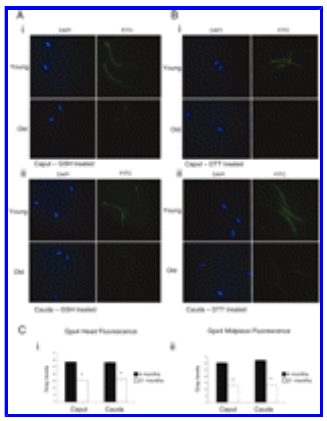

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Figure 3. **(A)** Gpx4 immunofluorescence, GSH treated spermatozoa. GSH treatment facilitates head staining of Gpx4 in spermatozoa. Young (4 months) and old (21 months) caput (i) and cauda (ii) epididymal spermatozoa were treated in 40 mmol GSH for 45 minutes prior to fixation and counterstained with DAPI. 21-month-old rats exhibited marked decrease in intensity of head staining compared to the 4-month-old rats. Magnification was at 400x. **(B)** Gpx4 immunofluorescence, DTT treated spermatozoa. Young (4 months) and old (21 months) caput (i) and cauda (ii) epididymal spermatozoa were treated in 40 mM DTT for 45 min. prior to fixation and counterstained with DAPI. DTT treatment revealed Gpx4 midpiece staining only. In both caput and cauda spermatozoa, fluorescence intensity of the midpiece was drastically decreased in the 21-month-old rats. Magnification was at 400x. **(C)** Gpx4 staining intensity was quantified on the MCID computer program. In both GSH (i) and DTT (ii) treated slides, relative intensity was significantly higher (*P* < .001) in the young samples compared to the old, in sperm from both the caput and cauda. There was no significant difference between the spermatozoa from the 2 epididymal segments (*P* > .05).

DTT treatment facilitated midpiece Gpx4 staining in caput and cauda epididymal spermatozoa (Figure 3b). The aged animals exhibited a marked decrease in midpiece fluorescence intensity in both caput and cauda epididymal spermatozoa. Quantification of Gpx4 staining in the DTT-treated spermatozoa

again indicated a highly significant effect of age (Figure 3c). In both caput and cauda epididymal spermatozoa, staining intensity was over twofold higher in spermatozoa from young rats.

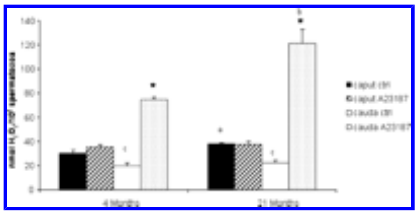

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Figure 4. Steady-state levels of H_2O_2 production at 10 minutes postincubation in caput and cauda epididymal spermatozoa of young (4 months, n = 6) and old (21 months, n = 6) rats. Spermatozoa were incubated with buffer alone, buffer + 200 U/mL CAT, or buffer + 0.05 mg/mL A23187 (calcium ionophore). H_2O_2 levels were assessed by fluorometric assay at 317 excitation-414 nm emission wavelengths after a 10-minute incubation period. Results are expressed in nmol H_2O_2 per 10^6 spermatozoa. There was no significant effect of A23187 in caput epididymal sperm ($P > .05$). The results demonstrate a highly significant increase in H_2O_2 levels (*, $P < .001$) in the cauda epididymal spermatozoa of 4-month-old and 21-month-old rats incubated in A23187. Further, aging significantly increased H_2O_2 levels in cauda epididymal spermatozoa, but only in the samples incubated in A23187 (b, $P < .001$). Aging also increased H_2O_2 levels in caput epididymal spermatozoa, but only in the control spermatozoa (a, $P < .05$). In addition, there was also a significant effect of segment on H_2O_2 levels. In the 4-month-old and 21-month-old animals, control spermatozoa from the cauda epididymidis exhibited significantly lower H_2O_2 levels relative to caput (c, $P < .05$). This scenario was reversed (that is, the caput epididymal spermatozoa exhibited significantly lower H_2O_2 levels then the cauda) in the spermatozoa incubated in A23187. Data were analyzed using a 3-way ANOVA; bars represent mean ± SEM.

ROS Production: H_2O_2

Figure 4 shows the steady state H_2O_2 concentration after 10 minutes of incubation of spermatozoa from the caput and cauda epididymides of young and old rats, as monitored by the p-HPA/HRP system, utilizing sperm that were incubated with a calcium ionophore (A23187) or in control buffer. Samples containing CAT were run in parallel so that only the CAT-inhibitable signal was recorded. Caput epididymal sperm H_2O_2 generation was unaffected after incubation with A23187 relative to control in both the 4-month-old and 21-month-old animals. In the cauda epididymal spermatozoa, A23187 induced a highly significant ($P < .001$) burst in H_2O_2 production (greater than sixfold that of control) in the 21-month-old animals; A23187 induced H_2O_2 levels greater than fourfold higher in the 4-month-old cauda epididymal spermatozoa relative to control. Further, aging significantly increased H_2O_2 levels in the cauda epididymal spermatozoa, but only under capacitating (ie, incubation with A23187) conditions ($P < .001$), while in control sperm (ie, no A23187) the increase was nonsignificant. Spermatozoa from 21-month-old caput epididymidis also showed a significant increase in H_2O_2 levels relative to 4-month-old caput epididymal sperm, but this was only significant in the control group ($P < .05$). In addition, H_2O_2 levels were significantly lower in control spermatozoa from the cauda epididymidis ($P < .05$) when compared to caput spermatozoa from both the 4-month-old and 21-month-old animals. Yet H_2O_2 levels were significantly higher in A23187-incubated cauda epididymal spermatozoa relative to sperm from the caput epididymidis in both 4-month-old and 21-month-old animals.

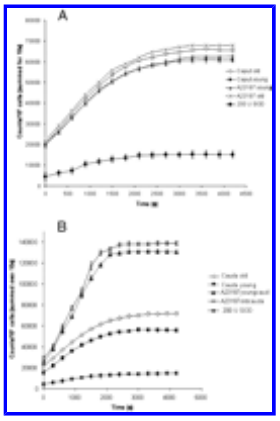

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Figure 5. Superoxide generation in caput **(A)** and cauda **(B)** epididymal spermatozoa of young and old rats. Spermatozoa were suspended in 1x PBS (control), A23187 in 1x PBS, or 200 U SOD in 1x PBS (negative control). Superoxide production was measured on a chemiluminometer immediately after the addition of 20 µmol MCLA. Counts represent the single output from one well summed for 10 seconds, taken at 5 minute intervals. A23187 had no effect on caput epididymal sperm $O_2^{\bullet -}$ production in either young or old rats (a). A23187 induced a highly significant increase in $O_2^{\bullet -}$ production in cauda epididymal spermatozoa in both old and young rats relative to respective controls (b, $P < .001$). Aging significantly increased $O_2^{\bullet -}$ production in the control cauda epididymal spermatozoa at all time points studied (b), and also had a significant effect on caput epididymal spermatozoa, but only at time points 1200 seconds and after (a). Further, in the 4-month-old animals, caput epididymal spermatozoa exhibited a significantly higher level of $O_2^{\bullet -}$ production ($P < .05$) at time point 1500 seconds and after relative to cauda. In the 21-month-old animals, however, cauda spermatozoa had a significantly higher level of $O_2^{\bullet -}$ production relative to caput after 1500 seconds ($P < .05$). Data were analyzed using the Student's *t* test with Bonferroni correction. Error bars represent ± SEM.

$O_2^{\bullet -}$ Production

Figure 5 shows the generation of $O_2^{\bullet -}$ by caput and cauda epididymal spermatozoa from young and old rats. There was no effect of A23187 on the caput epididymal spermatozoa (Figure 5a). However, A23187 induced a highly significant increase in signal in spermatozoa from both the young and old cauda epididymidis relative to control (Figure 5b). Further, spermatozoa from the cauda epididymidis of 21-month-old rats showed significant increases in $O_2^{\bullet -}$ production at all time points studied ($P < .05$) compared to the samples from 4-month-old rats, while in those from the caput epididymidis, this difference of age was only significant at the 1200-second time point and thereafter. In the 4-month-old animals, caput epididymal spermatozoa had a significantly higher level of $O_2^{\bullet -}$ production ($P < .05$) at time point 1500 seconds and after, relative to those from the cauda epididymidis. In the 21-month-old animals, however, spermatozoa from the cauda region had a significantly higher level of $O_2^{\bullet -}$ production than those from the caput epididymidis after 1500 seconds ($P < .05$).

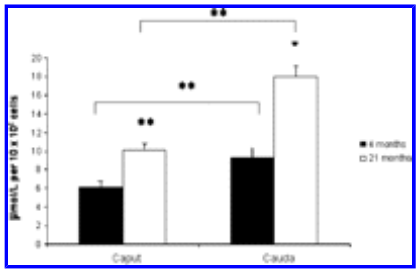

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Figure 6. Extent of lipid peroxidation in caput and cauda epididymal spermatozoa of young (4 months, n = 6) and old (21 months, n = 5) rats. Lipid peroxides were quantified directly using a spectrophotometer following the chromogenic detection of the reaction of lipid peroxide products with ferrous ions. Results are expressed as µmol/L lipid peroxides per 10 x 10^6 cells. Data were analyzed by 2-way ANOVA using the Holm-Sidak post hoc test; *$P < .001$, **$P < .05$. Bars represent mean ± SEM. Lipid peroxides increased significantly with age in both caput and cauda epididymal spermatozoa. Further, cauda epididymal spermatozoa exhibited significantly higher levels of lipid peroxides compared to caput epididymal sperm.

Lipid Peroxidation

Figure 6 shows the extent to which lipid peroxidation was significantly altered with age in

epididymal spermatozoa. Total lipid peroxides from extracted lipids were significantly increased in aged spermatozoa as compared to young, by twofold. Further, spermatozoa from the cauda epididymidis showed significant increases in total lipid peroxides when compared to spermatozoa from the caput region in both young and old animals.

Discussion

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Antioxidant defense is crucial in decreasing oxidative damage to sperm lipids and DNA (Alvarez and Storey, 1989; Muratori et al, 2003). Data from our laboratory have shown decreased expression of key antioxidant enzymes, Gpx4 and SOD, in the aged epididymis (Jervis and Robaire, 2002), and a significant decrease in Gpx1 activity in the cauda epididymidis of aged rats (Zubkova and Robaire, 2004). Here we demonstrate that the epididymal spermatozoa enzymatic antioxidant defense is impaired in aging. Interestingly, recent evidence suggests that these decreases in activity are not restricted to spermatozoa in the male genital tract, having also been detected in Leydig cells (Luo et al, 2006). Hence it is likely that the age-related decline of the reproductive system via oxidative stress is a complex issue, affecting more than one component of the system.

The extent to which the decrease in the thiol-dependent antioxidant enzymatic activity could potentially be explained by a decrease in the availability of substrates has not been fully explored. The degree to which Gpx1, GR, and Gpx4 (all thiol-dependent enzymes) utilize GSH as a substrate is unclear due to the very low levels of GSH in rat epididymal spermatozoa (Tramer, 1998). If GSH does exist at appreciable levels, there is evidence to suggest that these levels decrease in aging (Maher, 2005); in particular, GSH concentrations have been shown to decrease in the testis in the mouse model (Rebrin et al, 2003). In light of these observations, it is possible that the sustained levels of GR activity in aged rat spermatozoa observed in the current study represent a cellular compensatory mechanism for maintaining the levels of GSH. Another candidate as a substrate for the thiol-dependent antioxidant enzymes is cysteine, which has also been shown to decline in aging (Droge, 2005). In fact, under conditions in which the substrate concentrations are low, these enzymes are more prone to oxidative damage and degradation (Miller, 1987).

Of particular interest is the effect of age on Gpx4 activity. Gpx4 expression is drastically decreased in infertile men (Imai et al, 2001); combined with other lines of evidence (Garrido et al, 2004), these data point to a direct correlation between decreased Gpx4 activity and abnormal sperm morphology. Here we show that Gpx4 activity is significantly lower in aging rat spermatozoa and that Gpx4 expression is significantly lower in the head and midpiece of spermatozoa from aged animals. Previous data from our lab have demonstrated that aging decreases the number of disulfide bonds that exist between the protamines (as assessed using the mBBr technique, there were 25% fewer disulfide bonds in aged cauda spermatozoa) (Zubkova et al, 2005), a phenomenon directly associated with chromatin condensation (Conrad et al, 2005). Fewer disulfide bonds indicate a more open structure, and hence a DNA more vulnerable to oxidative damage. In light of the proven role of Gpx4 and protamine thiol oxidation in the sperm nucleus, the current study provides a novel potential mechanism for this decrease in disulfide bond formation. Further, the decreased expression of Gpx4 in the midpiece may indicate a compromised structure, potentially impacting motility. Previous data from our lab in the same model have shown that aging does affect certain sperm motility parameters in the rat (Zubkova and Robaire, 2004).

We have demonstrated that spermatozoa from the caput epididymidis have significantly higher antioxidant enzymatic activity then spermatozoa from the cauda region. This is in agreement with a

previous report by Tramer et al (1998), although this study noted no difference in GR activity between segments. An explanation for the phenomenon of higher antioxidant enzymatic activity in the immature caput spermatozoa may reflect the greater need for control of ROS-related maturational events at this stage of spermatozoa maturation. That is, the sperm in the cauda epididymidis are already mature and capable of carrying out their biological function. Hence this loss in antioxidant activity could simply be interpreted as a diminished need for the mediation of ROS-controlled sperm maturational events.

In order to gain a more comprehensive insight into the extent of oxidative stress in aged spermatozoa, we examined H_2O_2 and $O_2^{\bullet -}$ production. Following previously noted age-associated increases in oxidant generation in other mammalian tissues such as heart (Sohal et al, 1994), brain (Gabbita et al, 1997), and testis (Chen et al, 2001), we noted that spermatozoa from the aged rat showed significant increases in H_2O_2 and $O_2^{\bullet -}$ production. Interestingly, the age-related increase in H_2O_2 production in cauda epididymal spermatozoa was only significant in the sperm incubated under conditions that induce the acrosome reaction, that is, with A23187. Functionally, this may mean that aged spermatozoa under capacitating conditions exhibit bursts of H_2O_2 that are much higher than required for capacitation and most probably are deleterious to the cell. Further, in both young and old animals, H_2O_2 production was twofold higher in caput epididymal spermatozoa than in spermatozoa from the cauda region. This trend was similar in the $O_2^{\bullet -}$ production of the 4-month-old animals, albeit at a lower level of significance. These results support previous findings that rat caput epididymal spermatozoa produce more ROS then spermatozoa from the cauda epididymidis (Vernet et al, 2001).

A23187 did not significantly induce either H_2O_2 or $O_2^{\bullet -}$ production in the spermatozoa from the caput epididymidis that were analyzed. This is expected, given that spermatozoa from the caput are less capable of undergoing the Ca^{2+}-mediated processes of the acrosome reaction and capacitation, and hence would be less affected by an initiator of this process. In fact, it has been shown previously that induction of these Ca^{2+}-initiated events is dependent on epididymal maturation (Sirivaidyapong et al, 2001), and that Ca^{2+} accumulation in sperm increases as the sperm transit through the epididymis (Okamura et al, 1992). Further, it has been demonstrated that there is a difference in the type of activated Ca^{2+} channels in spermatogenic cells compared to mature spermatozoa (Serrano et al, 1999). These findings suggest a difference in the response to Ca^{2+} between immature (caput) and mature (cauda) spermatozoa. Here we support these findings, as only spermatozoa from the cauda epididymidis were affected by A23187 with respect to ROS production.

Using a more direct method of assessing lipid peroxidation, we demonstrated that age had a highly significant effect on sperm membrane peroxidation in spermatozoa from both the caput and cauda epididymal regions. Peroxidation levels were drastically increased (~twofold) in aged spermatozoa. The increased extent of lipid peroxidation in sperm from aged animals indicates serious deficiencies in sperm membrane quality, potentially interfering with lipid membrane fluidity and flexibility, leading to compromised fusogenic potential (Aitken and Sawyer, 2003). Because of the demonstrated importance of various lipids in promoting sperm-egg fusion (Riffo et al, 1997; Weerachatyanukul et al, 2001), this increase in peroxidation of spermatozoa from old rats also provides a plausible explanation for the increase in infertility generally associated with aged men (Mathieu et al, 1995; Jung et al, 2002).

Taken together, these data suggest an overall decreased capacity for aged spermatozoa to manage excess oxidative challenge. A decreased antioxidant capacity coupled with an increased ROS

production is a hallmark of aging and, as demonstrated with the lipid peroxidation end point, can have significant effects on macromolecular integrity. In light of the aforementioned susceptibility of spermatozoa to ROS damage, it is likely that these processes contribute significantly to the decline in sperm quality parameters that have been observed in aging males.

Footnotes

Supported by a grant from the Canadian Institutes of Health Research. B.R. is a James McGill Professor of McGill University.

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