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# Overexpression of catalase in mice reduces age-related oxidative stress and maintains sperm production

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ABSTRACT

Advanced paternal age is associated with increased complications in pregnancy and genetic diseases in offspring. Oxidative stress is a major contributor to the damage accumulated in sperm during aging. Complex networks of antioxidants regulate reactive oxygen species (ROS) in the testis. While mounting evident shows that redox dysfunction compromises the quality of developing male germ cells, the mechanisms by which aging causes this remain unclear. Furthermore, therapies to successfully alleviate aging-associated loss in germ cell quality are limited. The antioxidant catalase (CAT) has been used in aging-associated pathologies to alleviate oxidative stress. We used mice overexpressing CAT (MCAT) to determine whether CAT overexpression alleviates the redox dysfunction observed with aging. We found that MCAT mice did not exhibit the age-dependent loss of spermatozoa, nor did they show aging associated loss in testicular germ and Sertoli cells seen in wild type (WT). Low overall ROS and reduced peroxynitrite levels were detected in spermatocytes from aged MCAT mice, following exposure to the pro-oxidant tert-butyl hydroperoxide. Germ cells from young MCATs showed elevated levels of DNA-damage repair markers, γ-H2AX and 53BP1, but this response was lost with aging. Finally, we found oxidative stress induced 8-oxodG lesions to increase in sperm with aging; these lesions were significantly reduced in aged MCAT and these mice showed no decrease in the age-dependent number of pups per litter. Thus we conclude that aged MCAT mice generate sperm at the same rate as young mice; these sperm are protected from oxidative stress associated damage.



## 1. Introduction

Epidemiological studies have linked advanced paternal age to a vast array of congenital abnormalities (Green et al., 2010) and diseases such as achondroplasia (Orioli et al., 1995), autism (Frans et al., 2013), schizophrenia (Hubert et al., 2011), and attention deficit hyperactivity disorder (ADHD) (D'Onofrio et al., 2014). Growing evidence suggests that the quality of developing germ cells deteriorates with aging (Serre and Robaire, 1998; Syntin and Robaire, 2001; Wyrobek et al., 2006) and that the principal cause of this cellular damage is oxidative stress (Harman, 1956). Increased levels of reactive oxygen species (ROS) and decreased antioxidant capacity define a state of oxidative stress and have been well documented in the male reproductive tract (Jervis and Robaire, 2004; Sikka, 2001), developing male germ cells (Selvaratnam et al., 2015), and mature spermatozoa (Weir and Robaire, 2007).

Spermatogenesis is a highly dynamic process during which germ cells generate copious amounts of ROS as a byproduct of cellular metabolism (Fisher and Aitken, 1997; Guerriero et al., 2014). Mitochondria are the main source of this ROS (Balaban et al., 2005), and excess/accumulated ROS can cause extensive damage to cellular components, including nucleic acids, lipids and proteins (Halliwell and Gutteridge, 1984). While some ROS are required for normal cellular functions (Ray et al., 2012), the extent of replication that naturally occurs in the germ cell epithelium makes it critical that the testes have defense mechanisms against ROS induced damage. Developing germ cells are protected from this damage by a complex antioxidant defense system (Aitken and Roman, 2008; Bauché et al., 1994; O'Flaherty, 2014); however, with aging the balance between ROS generation and antioxidants is disrupted (Aitken and Roman, 2008; Gershon and Gershon, 1970; Luo et al., 2006).

Antioxidants such as superoxide dismutase 1 (SOD1) break down superoxide ($O_2^{\bullet -}$) into hydrogen peroxide ($H_2O_2$), which can then be further neutralized into water by several antioxidants including: catalase (CAT) (Kirkman and Gaetani, 1984), peroxiredoxins (PRDXs), glutathione peroxidases (GPXs) and glutathione S-transferases (GSTs) (Aitken and Roman, 2008). Previous studies have shown that increased CAT provides resistance to ROS (Akman et al., 1989) and reduces oxidant-induced DNA damage (Libman et al., 2010). Moreover, mice overexpressing CAT (MCAT) in their mitochondria show reduced ROS

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toxicity and increased longevity (Schriner et al., 2005). However, the effect of CAT overexpression on developing male germ cells remains unknown, and the effects of such an overexpression on aging male germ cells have yet to be explored.

Aging affects both spermatogenesis and steroidogenesis in the testis (Chen et al., 2005; Luo et al., 2006); our studies focus on the effects of aging on spermatogenesis (Jervis and Robaire, 2004; Weir and Robaire, 2007). We examined how the composition of seminiferous tubule of MCAT testes was affected by aging, using a newly developed method involving automated immunofluorescent labeling. To determine the response of live spermatocytes extracted from the testes both young (3-mo) and aged (18-mo) MCAT mice were administered an oxidative insult, pro-oxidant tert-butyl hydroperoxide (TBHP). Finally we examined young and aged MCAT testes for DNA damage and repair markers, γ-H2AX and 53BP1, and MCAT spermatozoa for ROS induced 8-oxodG DNA damage lesions. We propose that MCAT mice display decreased ROS and that with aging MCAT animals show increased resilience to oxidative stress.

## 2. Materials and methods

### 2.1. Animals

The mice used for this study were bred in house, from wild-type (WT) and MCAT (B6.Cg-Tg(CAG-OTC/CAT)4033Prab/J) breeders purchased from The Jackson Laboratory (Bar Harbor, ME). MCAT mice have a CMV enhancer/chicken beta-actin promoter driving the expression of a human catalase gene in mitochondria (Schriner et al., 2005). All mice were on C57BL/6 background (n = 5–7) and housed at the McIntyre Animal Resources Centre, McGill University maintained under controlled temperature (22 °C) and lighting (12L:12D), with food and water provided ad libitum. WT and MCAT genotypes were determined by polymerase chain reaction (PCR) analysis of DNA extracted from tail biopsies using Qiagen DNeasy® Tissue Kits (Qiagen, CA). MCAT genotyping was done using a PCR protocol provided by The Jackson Laboratory. Mice were aged to 3-months and 18-months of age. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care (McGill Animal Care Committee protocol #206).

### 2.2. Hormone assays

Serum samples of 100 μl were used to measure testosterone using ELISA (IBL, Hamburg, Germany), and LH and FSH were assayed using the Milliplex mouse pituitary panel (Millipore, Billerica, MA, USA) by the Ligand Core Laboratory (University of Virginia, Charlottesville, VA). The intra- and interassay CV for testosterone was 4.3% and 7.4%, respectively. The intraassay CV for FSH/LH was 11.5%. Quantification of hormone levels was done in 4–6 serum samples per experimental group.

### 2.3. Administration of oxidative stress

Oxidative stress was induced with administration of organic peroxide, tert-Butyl hydroperoxide (TBHP) (Sigma Aldrich, St. Louis, MO). Mice of 3-mo or 18-mo were given a single intra-peritoneal injection of 30 mg/kg body weight 1-h prior to being euthanized. In control animals, TBHP was replaced with saline injections. After each treatment mice were euthanized using carbon dioxide followed by cervical dislocation. Tissues were removed, weighed, and fixed or flash-frozen and stored at −80 °C for later analyses.

### 2.4. High content screening

Immediately following euthanasia, testes were removed from both TBHP and saline (control) treated mice. The tunica albuginea was removed, and the testes were minced in phenol-red free media (Sigma Aldrich Canada, Oakville, ON) and put through a 56-μm nylon mesh. The mixed germ cells were incubated with nuclear stain Hoechst (2,5′-Bi-1H-benzimidazole, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl) (ThermoFisher, Burlington, ON) and fluorogenic probe CellROX® DeepRed Reagent (Invitrogen, Burlington, ON, Canada) for 30-min at 32 °C; following incubation, cells were washed in media and transferred to a 96-well Cell Carrier (PerkinElmer, Woodbridge, ON) plate with an optically clear bottom.

For detection of separate components of ROS, cells were incubated for 30-min with superoxide indicator MitoSOX™ red reagent and peroxynitrite sensor Aminophenyl fluorescein (APF) (ThermoFisher). Following incubation, cells were transferred to a 96-well plate with an optically clear bottom, centrifuged at 300 RCF at 4 °C for 5-min, and immediately scanned with the Operetta® (PerkinElmer) high content imaging system.

Images were exported to the Columbus® software (PerkinElmer, version 2.5.0) to create an algorithm to filter and select spermatocytes. This algorithm was previously confirmed to select >80% of spermatocytes identified as cell positive for synaptonemal complex protein 3 (SYCP3) (Fig. S1). We analyzed and quantified cytoplasmic ROS, superoxide, and peroxynitrite from images of live spermatocytes using this software.

### 2.5. Sperm counts

The number of testicular and epididymal sperm was determined as previously described (Robaire et al., 1977). Briefly, frozen tissues from mice of each group were weighed and then homogenized (Polytron PTA7, setting 5; Brinkman Instruments, Westbury, NY) for two 15-second periods separated by a 30 s interval in 2 ml of 0.9% NaCl (Millipore, Billerica, MA), 0.1% thimerosal (Sigma), and 0.5% Triton X-100 (BDH, Montreal, QC). Sperm heads were counted in aliquots of diluted homogenate using a hemocytometer. Testis and epididymal sperm counts were shown as the number of sperm per mg of tissue.

### 2.6. Testis fixation & histology

From each group: WT-3, WT-18, MCAT-3 and MCAT-18, mice were euthanized and their testes removed and immersion-fixed in modified Davidson's fixative (Latendresse et al., 2002) for 2 h, then the testes were sliced in half and further fixed for 6–8 h. Fixed tissues were then transferred to 70% ethanol overnight and were dehydrated and embedded in paraffin.

Each slide with several samples of testes paraffin sections (4 μm), was deparaffinized (Histoclear; Diamed Inc., Mississauga, Canada). A batch of 30 slides was loaded into the Tecan GenePaint™ (Tecan, Zurich, Switzerland) solvent delivery robot, which was programmed to pass them through a graded ethanol series to rehydrate the tissues and then perform Periodic Acid Schiff (PAS) (Sigma Aldrich) staining according to the manufacturer's protocol. The slides were mounted with Permount (ThermoFisher Scientific, Montreal, Canada) and coverslips and left to dry before observation with a light microscope. Tubule diameters and epithelium heights were measured using ImageJ (NIH, Bethesda, MD, USA) for 200 tubules per animal, with 5–6 animals per experimental group.

### 2.7. Immunofluorescence

Analysis of whole testis can lead to erroneous results because Leydig cells, as steroidogenic cells that produce large amounts of antioxidants to protect themselves from ROS, often mask differences in key markers in seminiferous tubule cells (Sertoli and germ cells) (Griswold et al., 1988). Therefore, we developed a new method of analysis that involved automated immunofluorescent labeling and the selection of tubules in specific stages of spermatogenesis (Stages I–IV; V–VII; VIII–IX; X–XII).