ABSTRACT. The purpose of this study was to determine whether apoptotic cell death of mouse testicular germ cells varies with increased age, or with exposure to an acute systemic oxidative stress. Results show that the percent of seminiferous tubules with apoptotic cells, and the number of apoptotic cells/tubule cross section were not significantly altered with age. However, there were significantly more apoptotic metaphase spermatoocytes at tubule stage XIV in 24-month-old mice than in 6-month-old mice. Oxidative stress significantly increased apoptotic metaphase spermatoocytes in young mice, and severely reduced testicular apoptosis in old mice. Our results have potential clinical relevance to changes with increased age in human sperm aneuploidies.

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INTRODUCTION

Recent reports have demonstrated an age-related increase in human sperm aneuploidy, resulting in a significant association between paternal age and increased risk of fathering aneuploid offspring with specific physical and mental abnormalities characteristic of chromosomal mutations (1). The mechanism of this age-related increase in sperm aneuploidy is not known. It has been recently shown that apoptosis, a form of programmed cell death designed to remove individual superfluous, flawed or damaged cells from an organism, is the primary mechanism of elimination of germ cell in rodent testis, both under spontaneous conditions and following chemical injury (2-6). However, there is little published information on age-related changes in testicular germ cell apoptosis. The present study was designed to determine whether apoptotic germ cell death in mouse testis varies with increased age and/or in response to an acute systemic oxidative stress.

MATERIALS AND METHODS

Six-month and 24-month-old male B6C3F1 mice (N=25 per age group) were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.) two weeks before the study; upon receipt, their mean (± SEM) weight was 30.2±1.6 and 37.7±4.3 g, respectively. All experimental procedures described below were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio (San Antonio, TX, USA).

Dietary components for the AIN-76A formula diet were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, USA). Iron dextran and antioxidant free menhaden fish oil were purchased from Sigma (St. Louis, MO, USA). The Apoptag in situ apoptosis labeling kit was purchased from Oncor (Gaithersberg, MD, USA). All other materials were analytical reagents of the highest grade available, and were used without further purification.

Upon receipt, all mice were fed a standard AIN-76A diet containing 5% corn oil (CO diet). After two weeks of acclimatization, 5 mice per age group continued on the CO diet, and the remaining mice (N=20 per age group) were switched to a modified AIN-76A diet which contained 19% menhaden fish oil plus 1% corn oil (FO diet) for an additional two weeks. Diets were balanced for caloric content, and made fresh weekly as

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Correspondence: C.J. Barnes, Ph.D., Department of G.I. Oncology, Box 78, U.T. M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030-4095, U.S.A.

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described previously (7), stored in single cage portions at -20°C, and replaced in each cage daily. We have previously shown that adding a source of highly polyunsaturated fat such as fish oil to the standard AIN-76A diet significantly increased the degree of unsaturation (and thus susceptibility to lipid peroxidation) of cell membrane fatty acids (8). After 2 weeks on the different diets, young (i.e., 6-month-old) and old (i.e., 24-month-old) control CO and FO diet mice (N=5 per age and diet) received an intraperitoneal injection of normal saline, and were sacrificed 1 hour later. The remaining young and old mice consuming the FO diet (N=15 per age group) were challenged with a systemic oxidative stress by intraperitoneal injection of 125 mg iron/kg body weight as iron dextran (8, 9). Five mice from each age group were sacrificed by cervical dislocation 1, 5, and 24 hours post injection. We have previously shown that increasing cellular membrane polyunsaturated fatty acid composition through the dietary modification described above followed by acute iron overload (as a catalyst for lipid peroxidation) was an effective means of inducing an acute systemic oxidative stress, and that livers of young and old mice demonstrated significantly different responses to the induced stress (8).

Immediately following sacrifice, testes were removed, and the capsules were gently pierced with a 27 gauge needle prior to immersion fixation in 10% buffered formalin for 12 hours. Four μm-thick cross sections of formalin-fixed, paraffin-embedded tissue were mounted on coated glass slides, and stained for apoptotic cells using the Apoptag in situ apoptosis detection kit according to the manufacturer’s instructions, followed by counter-staining with methyl green. This technique labels the many fragmented DNA ends that normally occur following endonuclease activity during apoptotic cell death (10). In the testis, a number of investigators have demonstrated that this in situ labeling method is representative of testicular germ cell apoptosis, and the DNA end labeling technique has been validated against other apoptosis identification techniques, including DNA laddering and apoptotic cell morphology (2, 4, 5, 11-13). One or more morphological characteristics of apoptosis were almost always present in Apoptag-positive cells in the mouse testis (Fig. 1). Also, we conducted a preliminary study comparing apoptosis identification by morphological criteria on H&E-stained sections with the DNA end labeling (Apoptag) technique, and the results demonstrated a significant linear correlation between the two methods ($r^2=0.975$). In situ labeling of fragmented DNA, therefore, was considered indicative, but not proof, of a cell undergoing apoptosis.

Positive cells were quantified using a light microscope with a 40× objective, and an ocular grid. For each mouse, approximately 100-150 tubules in each testicular cross section were examined for the number of tubules which contained apoptotic cells. The criteria established by Leblond and Clermont (14, 15) for identifying the specific stage (I - XIV) of the seminiferous epithelial cycle, and the specific cell types undergoing apoptosis were utilized for determination of these parameters in each apoptosis positive tubule cross section.

Data are expressed as the mean±SEM. Differences between age groups in the percent distribution of apoptotic cells among seminiferous tubule cross sections at different stages were analyzed by $\chi^2$ analysis. Differences in the percent apoptotic tubules, and in the percent apoptotic cells of different cell types between the different age groups and treatment groups were established by two-way analysis of variance followed by one-way analysis of variance and Student-Neuman-Keuls a posteriori tests. All analyses were performed using PRISM statistical software (GraphPad Software, San Diego, CA). Significance was accepted if $p<0.05$. 

![Figure 1 - Photomicrographs of stratified spermatogenic epithelium with Apoptag-labeled apoptotic mouse testicular germ cells (arrowheads). Asterisks mark the wall of the seminiferous tubule and late stage spermatids are located at the top of the photomicrograph. The chromatin in the germ cells indicated by arrowheads showed a dark brown color indicative of positive Apoptag labeling. Characteristic morphological features of apoptosis are also apparent in these cells, including nuclear marginalization, condensation and fragmentation of chromatin, cell shrinkage and membrane blebbing. Figure 1B illustrates an Apoptag-positive primary spermatocyte at the metaphase stage of meiotic division (arrowhead). Methyl green counter stain, magnification 1000x.](image-url)