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Effects of X-Irradiation on Mouse Testicular Cells and Sperm Chromatin Structure

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The testicular regions of male mice were exposed to x-ray doses ranging from 0 to 400 rads. Forty days after exposure the mice were killed and the testes and cauda epididymal sperm removed surgically. Flow cytometric measurements of acridine orange stained testicular samples indicated a repopulation of testicular cell types following x-ray killing of stem cells. Cauda epididymal sperm were analyzed by the sperm chromatin structure assay (SCSA), a flow cytometric measurement of the susceptibility of the sperm nuclear DNA to in situ acid denaturation. The SCSA detected increased susceptibility to DNA denaturation in situ after 12.5 rads of x-ray exposure, with significant increases following 25 rads. Abnormal sperm head morphology was not significantly increased until the testes were exposed to 60 rads of x-rays. These data suggest that the SCSA is currently the most sensitive, noninvasive method of detecting x-ray damage to testicular stem spermatogonia.

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Key words: sperm, x-rays, testis, acridine orange, chromatin structure, abnormal head morphology

INTRODUCTION

Adverse effects of ionizing radiation on reproduction were first observed about a century ago [Albers-Schonberg, 1903]. Radiation, either scattered or directly applied to the testes, can produce temporary or permanent sterility in males [Meistrich, 1993]. The killing effect of radiation is likely due to DNA damage by free radicals generated by radiation ionization of water [Goldman and Johnson, 1993]. DNA damage from radiation consists of strand breaks and base damage. Radiation induced double strand DNA breaks are thought to contribute to lethality, while DNA base damage causes problems during DNA replication [van Loon et al., 1993]. Damage to DNA can lead to cell death or the induction of mutations [Grosovsky et al., 1988], even triggering apoptosis in certain cell types [Mori et al., 1992]. Radiation preferentially affects actively growing cells, such as those undergoing mitosis, meiosis, and early in the DNA synthesis (S) phase [Goldman and Johnson, 1993]. Radiation exposure can produce highly variable DNA content in mature spermatozoa, as measured by Pinkel et al. [1983] in mouse sperm using flow cytometry and the fluorescent DNA probes ethidium bromide-mithramycin (EBMI), 4,6-diamidino-2-phenylindole, and acriflavine feulgen. Hacker-Klom et al. [1989], using EBMI, have detected diploid spermatids in radiation exposed mice.

Various methods have been used to detect radiation damage to mammalian testes. In rodents, alkaline elution has been used [van Loon et al., 1993] to determine the degree of strand breaks in testicular cell DNA after in vitro exposure to ionizing radiation. In humans, these studies have been limited to routine semen analysis [Pryzant et al., 1993; Meistrich, 1993] due to ethical considerations. Evaluation of human sperm quality is primarily done via light microscope measurement of cell concentration, motility index, and morphology. These indicators, while useful, do not take into account more than a few parameters of the complex phenomenon of fertility. Flow cytometric methods have been developed to measure the traditional light microscopy parameters more rapidly, while determining other parameters which are unique to flow cytometry. Evenson et al. [1991] have shown that flow cytometric SCSA data are more consistent within an individual, compared to the classical semen analysis parameters. Flow cytometric methods allow for rapid determination of cell concentration, ratios of cell types present, chromatin structure, viability, and mitochondrial function [Graham et al., 1990].

Acridine orange (AO) has been used in conjunction with flow cytometry to monitor the effects of toxic insults on mouse testicular cells and cauda epididymal cells [Evenson et al., 1980, 1986a, 1989, 1993; Evenson and Jost, 1993]. AO intercalated into double stranded DNA fluoresces green, while AO bound to RNA or single stranded DNA fluoresces red. By utilizing the metachromatic properties of acridine orange [Darzynkiewicz, 1979], seven testicular cell popula-

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tions can be monitored [Evenson and Melamed 1983; Evenson et al., 1986a]. The SCSA uses AO to monitor the susceptibility of sperm chromatin DNA to acid induced denaturation in situ [Darzynkiewicz et al., 1976].

Prior experiments have shown that the SCSA is a sensitive measure of sperm chromatin structure as related to fertility [Ballachey et al., 1987] and reproductive toxicology. Exposure to triethylenemelamine (TEM) [Evenson et al., 1989], thiotepa [Evenson et al., 1986a], ethylnitrosourea (ENU) [Evenson et al., 1985], and hydroxyurea (HU) [Evenson and Jost, 1993] caused alterations in the relative ratios of testicular cell populations indicative of damage to precursor spermatogonial stem cells and/or spermatogonia. Exposure to methyl methanesulfonate (MMS) [Evenson et al., 1993] caused chromatin damage in elongated spermatids as well as in epididymal sperm due to alkylation of protamine sulphydryl groups [Sega and Owens, 1983]. Previous studies [Bruce et al., 1974; Wyrobek and Bruce, 1975] have shown radiation induces altered sperm head morphology. In the present work, flow cytometric measurements of AO stained testicular and cauda sperm cells were used to monitor mouse spermatogenesis and alterations in chromatin structure following x-ray exposure to the testes. Flow cytometric data were correlated with testicular weights and abnormal cauda epididymal sperm head morphology. The utility of the SCSA to detect changes in chromatin structure was evaluated to determine whether the SCSA was a more sensitive biomarker of radiation effects on spermatogenesis than sperm head morphology.

MATERIALS AND METHODS

Mice

For the experiment reported here, twenty-eight 7–8-week-old male B6C7F1/J mice (C57BL/6J♀ × C3H/HeJ♂), from Jackson Laboratories (Bar Harbor, ME), were randomly divided into seven treatment groups and allowed to acclimatize in our animal facilities 4 weeks prior to x-ray exposure. The mice were kept on a 07:00–19:00 hr light schedule, allowed free access to Purina Certified Rodent Laboratory Chow and deionized water, and housed four per cage in polycarbonate cages with wire mesh tops and pine shavings for bedding. Fifty mice of the same strain and handled in the same manner were utilized in two pilot studies. Data from the pilot studies utilizing x-ray dosages from 0 to 400 rads and measuring the results 35 to 40 days later were confirmed by the present experiment.

X-Ray Exposure

The mice were transported 50 miles by car in their cages to the Medical X-Ray Center (Sioux Falls, SD) at which point they were anesthetized by i.p. injection of 80 mg/kg of pentobarbital. Testicles were digitally manipulated into the scrotal area, and x-rays were administered with a Varian Clinac 6X linear accelerator to the scrotal region at doses of 0, 12.5, 25, 60, 125, 250, and 400 rads. X-rays were delivered with 6 MV photons at a source to axis distance of 80 cm. One centimeter of tissue equivalent bolus was placed over the testes and dose was prescribed to a depth of D/max. Dose rate was 200 rads per min with a field size of 2 × 2 cm. Dosages of x-rays selected were below those required to reduce sperm counts permanently to 50% of control levels in humans and mice [Meistrich, 1986]. The highest dose (400 rads) has been shown to reduce testicular weights to 40% of control values when applied as full body exposure to mice [Di Paola et al., 1980].

Sample Collection

Forty days after x-ray exposure, the mice were killed by cervical dislocation and weighed. Cells present in the reproductive tract at this time point would have been spermatogenic stem cells at the time of radiation exposure. The testes and epididymi were immediately removed surgically, and the testes' weights recorded. All tissues were kept on ice (4°C) from the time of dissection to either measurement of fresh samples by flow cytometry or freezing (−100°C) for later analysis.

Testes or epididymi from each mouse were placed into a 60 mm petri dish containing approximately 2 ml of Hanks' balanced salt solution (HBSS, Gibco, Grand Island, NY) or TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M disodium EDTA, pH 7.4), respectively. The tissue was minced for approximately 1 min with curved scissors. The cell suspension was pipetted into a 12 × 75 mm test tube and placed on ice for several minutes so the tissue fragments would settle out. The cell suspension was drawn off and gravity filtered through a 53 μm (testis) or 153 μm (epididymi) Nitex filter (Tetko, Inc., New York, NY). Filtered epididymal cell suspensions were split into two aliquots and stored in a −100°C freezer. A single freezing and thawing of epididymal sperm has no detrimental effect on AO stainability as described previously [Evenson et al., 1989, 1994].

Sperm Head Morphology

Two drops of fresh epididymal sperm suspension per animal were stained with two drops filtered 1% Eosin Y in water. After 30 min, the stained samples (2 per animal) were smeared onto glass slides and air dried. The slides were then dipped into methanol to remove excess stain, air dried, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). Using a 100× oil immersion differential interference contrast objective on a Nikon Optiphot light microscope, a minimum of 400 sperm heads per animal were scored as having either normal or abnormal morphology by the criteria of Wyrobek and Bruce [1975]. Head abnormalities were not subclassified; acrosome and tail abnormalities were not scored.

Cell Staining With Acridine Orange (AO)

Cellular suspensions of testicular cells or epididymal sperm were diluted to a concentration of 1–2 × 10^6/ml. Two hundred microliter aliquots of the cell suspensions were admixed with 0.40 ml of 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.4. After 30 sec, the cells were stained by adding 1.2 ml of a solution containing 6 μg chromatographically purified AO (Polysciences, Inc., Warrington, PA) per ml of AO buffer (370 mls of stock 0.1 M citric acid, 630 mls of stock 0.2 M Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, pH 6.0) [Darzynkiewicz et al., 1975; Evenson et al., 1985].

Triton X-100 is used in the first step of the staining procedure to permeabilize cell membranes thereby providing accessibility of DNA for AO staining. When excited by blue laser light (488 nm), the fluorescence observed in AO stained testicular cells reflects the relative content of native DNA (Green fluorescence; $F_{515-530}$) and RNA (red fluorescence; $F_{\geq 630}$) [Darzynkiewicz, 1979; Evenson and Melamed, 1983; Evenson et al., 1986b]. Since normal, mature epididymal sperm cells contain virtually no RNA [Monesi, 1965], red fluorescence is minimal [Evenson and Melamed, 1983]. Normal isolated sperm nuclei and/or whole sperm cells treated with RNAse demonstrate the same fluorescent pattern as untreated whole sperm cells, indicating that the red fluorescence is due to single strand DNA staining and not nuclear or cytoplasmic RNA [Evenson et al., 1985; Ballachey et al., 1987].

Sperm Chromatin Structure Assay

When used on epididymal, vas deferens, or ejaculated sperm, the procedure involving AO staining and subsequent flow cytometric measurements

has been termed the sperm chromatin structure assay (SCSA) [Evenson, 1989, 1990; Evenson and Jost, 1994]. Even though the low pH treatment does not cause denaturation of histone complexed DNA, low pH or heat treatment apparently does cause partial denaturation of protamine complexed DNA in sperm having altered chromatin structure [Evenson et al., 1985]. Abnormal chromatin structure, defined here as an increased susceptibility to acid induced denaturation in situ, is determined by flow cytometric measurement of the shift from green (native DNA) to red (denatured, single stranded DNA) fluorescence. The extent of this shift is expressed by alpha t (α_t) [Darzynkiewicz et al., 1975] and is the ratio of red to (red + green) fluorescence.

In the SCSA, α_t is calculated for each sperm, and results are expressed for each sample as the mean (mean α_t), standard deviation (SDα_t), and percentage of cells outside the main population of α_t (COMPα_t). Measurement of normal sperm produces a narrow α_t distribution. A sperm population with denatured DNA has a broader distribution, hence larger SDα_t and COMPα_t values.

Flow Cytometry

All flow cytometric measurements were made as the cells passed through the quartz flow channel of a Cytofluorograf 30 interfaced to a 2150 Data Handler (Ortho Diagnostics, Inc., Westwood, MA) and a Tektronix 4612 hard copier (Tektronix, Inc., Beaverton, OR). The flow cytometer was equipped with ultrasense optics and a Lexel 100 mW argon ion laser operated at 35 mW with an excitation wavelength of 488 nm.

After AO staining, 5,000 cells were measured by the flow cytometer at a rate of approximately 200 cells/sec. Recorded measurements were begun 3 min after staining, which allowed time for equilibration of the sample flow in the sheath flow. Red fluorescence ($F_{\geq 630}$) and green fluorescence ($F_{515-530}$) emitted from individual cells were separated optically and the digitized signals, processed in area mode (testicular cells) or peak mode (caudal sperm), were recorded as list mode data on the computer disk.

Data Analysis

List mode files were converted to histogram files using the 2150 Data Handler, and the histogram files were transferred by Multilink (Phoenix Flow Systems, San Diego, CA) to an IBM PC compatible computer for analysis. Multi2D Software (Phoenix Flow Systems) was used to analyze the histogram data. Descriptive statistics, correlations, and analysis of variance were carried out using The Student Edition of Minitab (MINITAB, Inc., State College, PA).

RESULTS

Figure 1 diagrams the relationship between radiation exposure and testicular weight (correlation = −0.936, $P < 0.05$) at 40 days post exposure. When compared to controls, animals receiving 250 or 400 rads of radiation exposure had significantly decreased testicular weight ($P = 0.001$, $P < 0.001$, respectively).

Figure 2 shows contour plots obtained from flow cytometric analysis of AO stained testicular samples. Seven regions, delineated by isocontour plots representing subpopulations of cells, can be classified (Fig. 2A), including two tetraploid (regions 1 and 2, Fig. 2A), two diploid (regions 3 and 4, Fig. 2A), and three haploid (regions 5–7, Fig. 2A,B) based on relative red (RNA content) vs. green (DNA stainability) fluorescence. The haploid population in Figure 2A was further enhanced and differentiated (Fig. 2B) into round (region 5), elongating (region 6), and elongated (region 7)

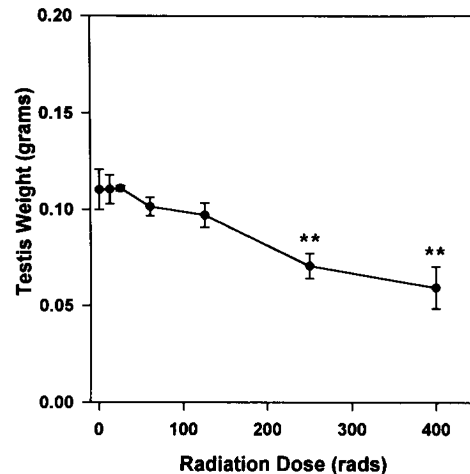

Fig. 1. Relationship between testicular weight and radiation dose. Forty days after x-ray exposure, mice were killed by cervical dislocation and both testes removed and weighed individually. A significant decrease in testicular weight was seen for mice exposed to 250 or 400 rads. $^{**}P < 0.001$.

spermatids. Cells in S-phase were present between the tetraploid (boxes 1 and 2) and diploid (boxes 3 and 4) populations (Fig. 2A).

Figure 3A graphically displays the changes in *relative* percent of testicular populations present 40 days post exposure in relation to increasing radiation dose. A statistically significant decrease in relative percent from control values is evident in the haploid population, beginning with 60 rads of radiation exposure through 400 rads of exposure. A corresponding relative increase in the diploid and tetraploid populations was observed beginning at 60 or 250 rads, respectively, and continuing through 400 rads of radiation exposure.

The relative percentages of AO stained haploid spermatids (round, elongated, and elongating) are shown in Figure 3B. A significant relative increase in the round spermatid population occurred between 60 rads through 250 rads, before returning to control values at 400 rads. Elongated spermatids showed a significant relative decrease at 60 rads, continuing to 400 rads. Elongating spermatids showed a significant relative increase at 125 rads, before returning to control levels.

AO stained normal cauda epididymal mouse sperm have a green vs. red fluorescence cytogram as shown in Figure 4A and α_t frequency histogram as shown in Figure 4C. A cytogram of AO stained cauda epididymal sperm from a mouse receiving 250 rads of radiation exposure is shown in Figure 4B, with the α_t frequency histogram shown in Figure 4D. Cells outside the main population (COMP α_t), showing increased DNA denaturation, are defined by the boxes in Figure 4A,B and the regions in Figure 4C,D. All SCSA variables (mean α_t, COMPα_t, and SDα_t; Figure 5A,B,C, respectively) showed an increase at 12.5 rads, which became significant from 25 rads to 400 rads.

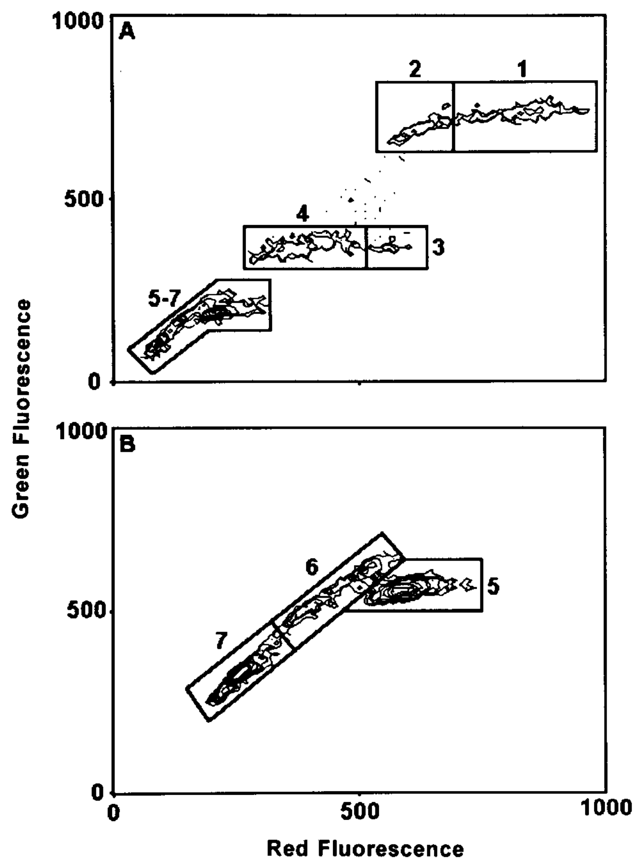

Fig. 2. Two parameter cytogram (A) of AO stained testicular sample. Red (RNA) vs. green (DNA) fluorescence for 5,000 cells showing tetraploid (boxes 1, 2), diploid (boxes 3, 4), and haploid (boxes 5–7) populations, and **B**) computer generated magnification of haploid population showing round (box 5), elongating (box 6), and elongated (box 7) spermatids. Cell populations are defined based on their relative red vs. green fluorescence. Contour plots were created using Multi2D (Phoenix Flow Systems, San Diego, CA) software using a threshold value of 1 (A) or 2 (B) for display purposes.

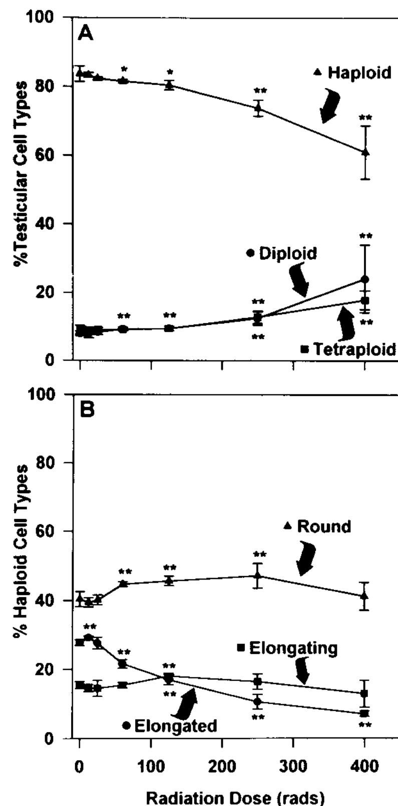

Fig. 3. Relative changes in testicular cell populations 40 days after x-ray exposure. **A:** Haploid, diploid, and tetraploid values were quantitated by Multi2D computer analysis of two parameter cytograms of AO stained testicular samples, as shown in Figure 2A. **B:** Relative changes in haploid cell populations 40 days after x-ray exposure quantitated by computer analysis of two parameter cytograms of AO stained testicular samples, as shown in Figure 2B. $^*P < 0.05$, $^{**}P < 0.001$.

Figure 5D shows the relationship between radiation exposure and percent of abnormal sperm heads (correlation = 0.773, $P < 0.001$) at 40 days post exposure. Percent of abnormal sperm heads (Fig. 5D) showed a significant increase at 60 rads, continuing through 400 rads. Regression analysis showed significant correlations between percent abnormal sperm heads and mean α_t (r = 0.782, $P < 0.001$), SDα_t (r = 0.816, $P < 0.001$), COMPα_t (r = 0.791, $P < 0.001$), and testicular weight (r = −0.825, $P < 0.001$).

DISCUSSION

Meistrich [1986] has shown that stem spermatogonia, as well as type A and B spermatogonia, are the most sensitive stages of spermatogenesis to radiation exposure. As spermatogonia progress to spermatocytes and spermatids, sensitivity to radiation damage decreases, especially in stage 12–16 mouse spermatids whose nuclei are sonication resistant. Fully differentiated spermatozoa have the lowest radiosensitivity. Irradiated cells usually progress through their first mitotic division but may die in subsequent cycles [Jung, 1982]. In stem cells, there usually is sufficient time for DNA repair [Hacker-Klom et al., 1984] before the cells enter the critical, mitotic phase of their cycle, at which time the damage is repaired or expressed as DNA strand breaks or base mismatches. Expression of radiation damage leads to a reduction in sperm count and persistent alterations in sperm head morphology [Meistrich, 1986]. Radiation does not alter the kinetics of spermatogenesis, only the cell types present during the various stages, leading to a minimum fertility in mice observed 5–6 wk after radiation treatment [Searle and Beechey, 1974; Ehling, 1977]. By using the timetable of mouse spermatogenesis proposed by Oakberg [1956a,b] and Sega [1974], cells present in the cauda epididymis 5–6 wk after radiation treatment were spermatogenic stem cells at the time of x-ray exposure. On this basis, developing cells present in the reproductive tract 40 days after radiation exposure were spermatogenic stem cells at the time of x-ray exposure.

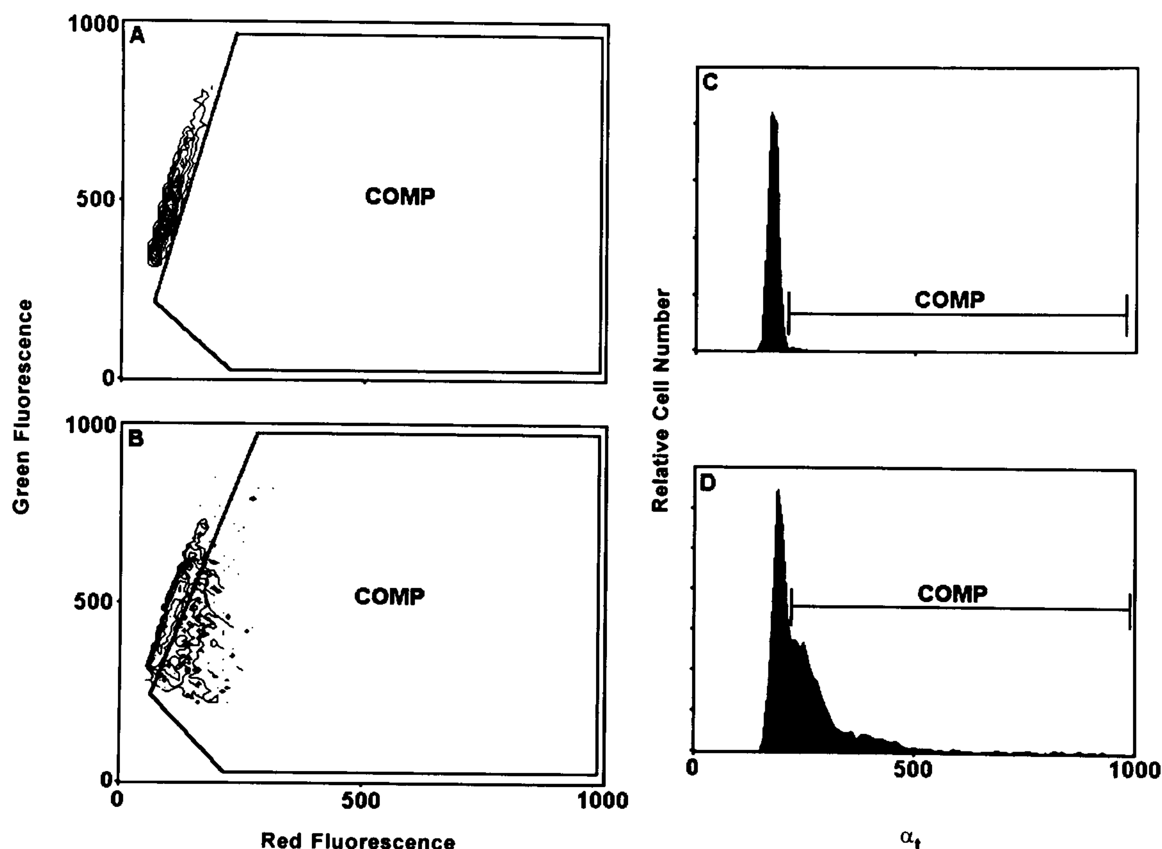

Fig. 4. Flow cytometric output from acid stressed AO stained cauda epididymal sperm. **A,B:** Green (double-stranded DNA) vs. red (single-stranded DNA) two parameter cytograms showing the definition of the cells outside the main population (COMP) cells. The large green fluorescence distribution is due to an optical artifact produced by the flat, oval shaped sperm nuclei passing through the orthogonal fluidics and optics of the flow cytometer [Gledhill et al., 1979] and has no bearing on α_t ratio calculations. Sample shown in A is from a control mouse, while B is from a mouse which received 250 rads of radiation exposure. **C,D:** Computer generated α_t histograms (ratio of red/red + green fluorescence) corresponding to samples presented in A and B. α_t is defined mathematically between 0 and 1, but numerical calculations are done on a 0 to 1,000 scale to facilitate working with whole numbers. COMP cells correspond to boxed areas in A and B, and designated regions in C and D. Contour plots and histograms were created using Multi2D software using a threshold value of 3 for display purposes (A,B) or 0 for calculation purposes (C,D).

Decreasing testicular weight (Fig. 1) with increasing x-ray dosage follows the pattern seen by Di Paola et al. [1980] following radiation exposure. Loss of testicular weight results from the killing of radiosensitive cell types, which are removed from the testes. Following the killing of radiosensitive cells, regeneration of stem cells occurs almost immediately [Meistrich, 1986], and repopulation of the tubules begins. Hacker-Klom et al. [1984] have seen a significant reduction in S-phase cells following radiation doses as low as 10 rads. The relative decrease in haploid cells and corresponding relative increase in diploid and tetraploid cell types (Fig. 3A) indicates regeneration and repopulation occurring after a portion of the stem cells have been killed. As the stem cells divide to replace those damaged by radiation, a relative drop in the later stage cell types (haploid) occurs, creating a relative increase in earlier stage cell types (diploid and tetraploid). The reduced percentage of elongated spermatids relative to the haploid cell populations as a whole (Fig. 3B) also indicates regeneration and repopulation, with later stage cell types not yet returning to their pre-radiation levels.

Forty days after exposure, doses of x-rays as low as 12.5 rads caused increased values for the SCSA variables mean α_t, SDα_t, and COMPα_t (Fig. 5A,B,C). These values were significantly increased over control values at 25 rads. As previously discussed [Evenson and Jost, 1993], damage leading to increased SDα_t may be indicative of chromatin abnormalities occurring within the population as a whole. At the lowest x-ray dose, the increase in SDα_t, even though non-significant, may be indicative of the damage affecting a large percentage of the population at higher x-ray dosages. The increases in mean α_t, SDα_t, and COMPα_t (Fig. 5A,B,C) indicate an increasing susceptibility of the sperm DNA to in situ denaturation. These measurements were made on caudal sperm which were spermatogenic stem cells at the time of x-ray exposure. This indicates that damage

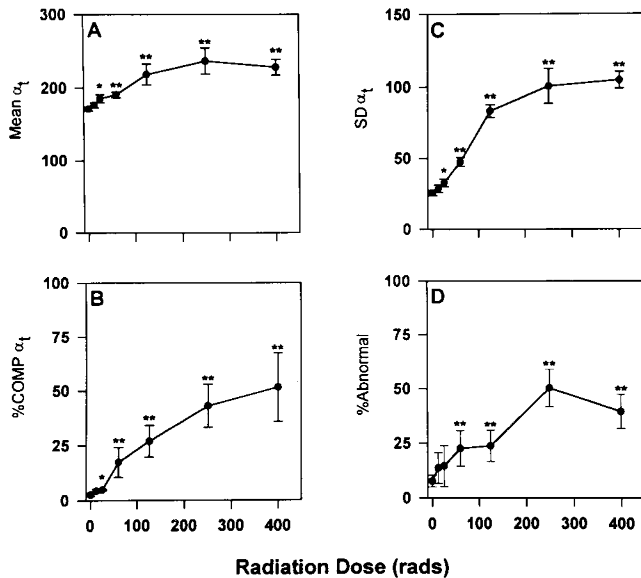

Fig. 5. Relationship between α_t variables, abnormal head morphology of caudal sperm obtained from mice 40 days after exposure to x-rays and radiation dose. **A,B,C:** Values obtained by Multi2D computer analysis of α_t histograms (Fig. 4B) of AO stained acid stressed cauda epididymal sperm. Mean α_t (A), SDα_t (B), and %COMPα_t (C) values increased at 12.5 rads, becoming significant at 25 rads. α_t values were highly correlated with both testicular weight (Fig. 1) (mean α_t r = − 0.807, $P < 0.001$; SDα_t r = −0.888, $P < 0.001$; COMPα_t r = −0.887, $P < 0.001$) and the percentage of abnormal sperm heads (D) (mean α_t r = 0.782, $P < 0.001$; SDα_t r = 0.816, $P < 0.001$; COMPα_t r = 0.791, $P < 0.001$). **D:** Cauda epididymal sperm head abnormalities did not show a significant increase until 60 rads of x-ray exposure. *$P < 0.05$, **$P < 0.001$.

caused to the spermatogonial stem cells was not completely repaired [Meistrich, 1986], but was expressed, or carried through the spermatogenic process. Increasing doses of radiation led to a higher degree of DNA denaturation susceptibility, indicating that the damage was relatively proportional to the radiation dose. Indirect damage to the mechanisms which package and protect the DNA may also lead to increased susceptibility to in situ DNA denaturation [Evenson et al., 1987].

Correlated with increased susceptibility to DNA denaturation was an increase in the number of sperm cells displaying abnormal head morphology (Fig. 5D). Percent of sperm cells with abnormal head morphology was increased over control values at 12.5 and 25 rads, with a significant increase beginning at 60 rads. The increase in cells with abnormal head morphology may be related to genetic changes in the genes responsible for spermatogenesis [Wyrobek and Bruce, 1975].

The disruption of spermatogenesis and killing of spermatogenic stem cells by radiation exposure follows the pattern seen in sperm and testicular populations after exposure to alkylating chemicals such as thiotepa [Evenson et al., 1986a], ENU [Evenson et al., 1985] and TEM [Evenson et al., 1989], and the metabolic inhibitor HU [Evenson and Jost, 1993] which preferentially affected cells in S-phase. The change in relative testicular populations follows the pattern seen after treatment with thiotepa, ENU, and TEM. A decrease in haploid spermatids with a corresponding relative increase in tetraploid and diploid cells was seen after radiation or chemical exposure. The increase in the percent of abnormal sperm heads indicates the possible presence of radiation or chemical induced mutations, resulting from improperly repaired DNA damage.

Previous reports [Bruce et al., 1974] have shown that head abnormalities increase measurably with doses of x-rays as low as 30 rads. The trend for increased head abnormalities was present at 12.5 and 25 rads, but due to the large range of values (3.5% to 26% and 5% to 29.5%, respectively) statistical significance could not be shown. SCSA values correlate well with those reported for abnormal head morphology [Bruce et al., 1974], showing a significant increase at 25 rads. Previously, researchers have examined testicular samples from mice following radiation exposure [Hacker-Klom et al., 1984], monitoring the induction of diploid elongated spermatids as a sign of radiation damage. A dose of 100 rads was necessary to induce a significant increase in the relative number of diploid elongated spermatids present in the testis [Hacker-Klom et al., 1984]. Monitoring the reduction in S-phase cells following radiation exposure is a more sensitive measure of testicular damage [Hacker-Klom et al., 1984], showing a significant reduction in S-phase cells 2 days following 10 rads of radiation exposure.

The disruption of spermatogenesis by radiation as measured by the SCSA likely occurs as 1) DNA damage in spermatogenic stem cells leading to cell death, or 2) DNA damage to spermatogenic stem cells expressed in mature spermatozoa. Cell death due to DNA strand breaks would result in the loss of testicular cells and a concomitant decrease in testicular weight. DNA damage or improperly repaired DNA strand breaks may lead to improper chromatin packaging in the mature sperm, increasing its susceptibility to in situ DNA denaturation. The SCSA is able to detect radiation induced damage occurring at lower doses in mature spermatozoa than traditional methods such as abnormal sperm morphology or by monitoring whole body dosimetry by counting chromosome aberrations in lymphocytes of the peripheral blood [Bender and Gooch, 1962]. Whole body dosimetry requires 1 day of chromosome aberration counting after high radiation exposure, and up to 10 days of counting after exposure to radiation doses below 30 rads [Hacker Klom et al., 1984] to count enough aberrations for the results to be statistically significant. The reduction of S-phase cells [Hacker-Klom et al., 1984] is more sensitive but requires testicular biopsy, an invasive procedure, to obtain samples. Another sensitive technique is monitoring the x-ray induction of meiotic micronuclei in early spermatids [Lahdetie & Parvinen, 1981]. Micronuclei are observed after 10 rads of radiation, with significant numbers present

after 50 rads, but this method also suffers from the need for testicular biopsy to obtain samples.

In contrast to other methods, the SCSA is non-invasive and objective, and allows for a sensitive analysis of 5×10^3 cells within 5 to 10 min. Overstreet [1984] has stated that tests must meet the following criteria to be useful for monitoring exposure to reproductive stresses: the test must be 1) objective, 2) technically sound, 3) biologically stable, 4) sensitive, and 5) feasible. The SCSA meets all of these criteria as concluded from studies of sperm obtained from toxin exposed mice [Evenson et al., 1985, 1986a, 1989, 1993; Evenson and Jost 1994], patients attending infertility and cancer treatment clinics [Evenson et al., 1984a,b], and results of the current study.

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