Environmental and Molecular Mutagenesis 21:144–153 (1993)

Effects of Methyl Methanesulfonate on Mouse Sperm Chromatin Structure and Testicular Cell Kinetics

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Effects of methyl methanesulfonate (MMS) on mouse testicular cell kinetics and sperm chromatin structure were determined flow cytometrically. Mice were exposed to a single ip injection of saline containing 0 or 150 mg/kg MMS. Relative ratios of 1N, 2N and 4N testicular cells were not affected until 22 days postexposure. Ratios of 1N cell types were altered from 13 to 22 days and were near normal by 25 days. This study revealed an MMS induced alteration of chromatin structure in testicular, elongated spermatids by the sperm chromatin structure assay (SCSA), a flow cytometric measure of the susceptibility of acridine orange stained sperm DNA to denaturation in situ. The SCSA also detected alterations in cauda sperm chromatin structure at 3 days, which was 8 days prior to alterations in sperm head morphology, indicating the increased sensitivity of the SCSA. SCSA data were practically similar whether measuring either fresh or frozen/thawed sperm, or whether measured by two different types of flow cytometers: a) laser driven, orthogonal optical axis; or b) low cost mercury arc lamp system with epiillumination. The data support the model of Sega and Owens [*Mutat Res* 111:227–244:1983] that MMS alkylates cysteine –SH groups in sperm protamines, thereby destabilizing sperm chromatin structure and leading to broken chromosomes and mutations.

Key words: sperm chromatin structure assay, alkylation, protamine sulfhydryl groups, elongated spermatid chromatin structure, flow cytometer, acridine orange

INTRODUCTION

Spermatogenesis

Chromatin structure of meiotic round spermatids undergoes dramatic changes during spermiogenesis, including replacement of nuclear histones by transition proteins [Grimes et al., 1977] and finally by low molecular weight, arginine rich protamines [Bellve et al., 1975; Balhorn et al., 1977]. These protamines are also relatively rich in cysteine [Bedford and Calvin, 1974; Bellve et al., 1975] which contain–SH groups that participate in the formation of S-S crosslinks within and between protamine molecules [Balhorn, 1982]. This crosslinking provides a unique resistance of sperm chromatin to sonication and strong detergents, but may be decondensed following incubation with sulfhydryl-reducing agents [Mahi and Yanagimachi, 1975; Evenson et al., 1980b]. These unique alterations contribute to the high degree of chromatin condensation which is characterized by a restricted accessibility to binding of DNA dyes [Gledhill et al., 1966; Evenson et al., 1986b], and a reduced stainability of free –SH groups [Pellicciari et al., 1983; Evenson et al., 1989a].

MMS as Clastogenic Agent

Methyl methanesulfonate (MMS) is a potent electrophilic, monofunctional alkylating agent which induces clastogenic damage including dominant lethal mutations [Ehling et al., 1968]. Dominant lethal mutation in males is measured by frequency of live and dead F_1 embryos derived from matings of mutagen treated males with untreated females at various times after treatment. Dominant lethals are clearly a manifestation of chromosome breakage [Sega and Owens, 1978] resulting in a loss of that broken segment at anaphase and giving rise to monosomic embryos which die in utero.

The temporal patterns of dominant lethal frequency induction by MMS [Sega and Owens, 1983], or a related chemical, ethyl methanesulfonate (EMS) [Sega and Owens, 1978] are closely paralleled by the temporal pattern of methylation/ethylation of mouse sperm. In mice, sperm heads reached a maximum methylation/ethylation about 8–11 days after treatment. Increased alkylation during this period does not represent accumulated alkylation of sperm cells since MMS and EMS are very reactive in biological systems [Cumming and Walton, 1970], probably disappear-

Received May 12, 1992; revised and accepted September 10, 1992.

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ing within several hours after injection [Sega and Owens 1978]. Sega and Owens [1983] also showed that DNA alkylation in mice did not show a temporal pattern related to fetal death rate; the greatest number of methylations/ethylations occurred by 4 hr after MMS/EMS treatment and was very slowly reduced over the next 2–3 week period. This pattern of alkylation likely reflects different levels of interaction between MMS/EMS and DNA in various germ cell stages, or it could be a result of loss of alkylated bases by hydrolysis from DNA [Verly, 1974]. In contrast, Sega and Owens [1983] and Teaf et al. [1985] showed that alkylation of cysteine, likely the strongest nucleophile present in mammalian protamine [Ross, 1958], was temporally correlated. Hydrolyzed samples of protamine from ^{3}H-MMS-exposed mice showed that about 80% of labeled material eluted with S-methyl-L-cysteine [Sega and Owens, 1983]. The mechanism of action of both MMS and EMS on the developing germ cells appears to be similar, although for equimolar exposures, MMS alkylates the germ cells 5–7 times more than does EMS [Sega and Owens, 1983].

The observation that maximum methylation and ethylation of protamine occurred in the germ-cell stages most sensitive to MMS and EMS-induced incidence of dominant lethal mutations led Sega and Owens [1983] to propose the following model of how dominant lethals are induced in germ cells by these chemicals: alkylation of cysteine sulfhydryl groups contained in sperm protamine blocks normal disulfide bond formation preventing proper chromatin condensation in the sperm nucleus. Subsequent stresses produced in the chromatin structure eventually lead to chromosome breakage, with resultant dominant lethality.

SCSA as a Measure of Chromatin Structure

It was hypothesized in this study that MMS induced alterations of chromatin structure could be measured by the sperm chromatin structure assay (SCSA) which is a measure of the susceptibility of sperm DNA to heat or acid induced denaturation in situ [Evenson et al., 1980a, 1985; Evenson, 1989]. Whole sperm cells or isolated nuclei are treated with a low pH buffer and then stained with the metachromatic dye, acridine orange (AO) [Darzynkiewicz et al., 1976]. AO intercalated into native DNA fluoresces green while that associated with denatured, single-stranded DNA fluoresces red. The extent of DNA denaturation can be determined for each of thousands of cells in a population by flow cytometric measurement of the metachromatic shift of AO fluorescence from green (double-stranded DNA) to red (single-stranded DNA) wavelengths. DNA in sperm with normal chromatin structure does not denature while DNA in sperm obtained from infertile animals [Evenson et al., 1980a; Ballachey et al., 1987, 1988] or animals treated with reproductive toxic chemicals [Evenson et al., 1985, 1986a, 1989b] demonstrates various degrees of denaturation.

MATERIALS AND METHODS

Mice

Seven- to eight-week-old male F1 mice (C57BL/6J × C3H/HeJ; The Jackson Laboratory, Bar Harbor, ME) were obtained about 5–6 weeks prior to chemical exposure. Animals were housed in transparent polycarbonate cages with rust-proof stainless steel wire lids; pine wood shavings were used as bedding. The mice were allowed free access to Purina Certified Rodent Laboratory Chow #5002 (Ralston Purina Co., St. Louis, MO) and deionized water. Lighting was maintained on a 12-hr light:dark cycle and room temperature kept at 21 ± 2°C. Mice were killed by cervical dislocation.

Chemical Exposure

Mice were randomly allocated to dosage and/or time groups as detailed below. Animals were exposed to fresh preparations of methyl-methanesulfonate (MMS, Polysciences, Inc., Warrington, PA) by i.p. injections with 0.5 ml Hank's balanced salt solution (HBSS, vehicle) or HBSS plus MMS. Two control mice were sampled at every sampling period.

In our first study, groups of 5 mice were injected with 75 mg/kg MMS and killed at 9, 16, 23, 30, 37, 51, and 59 days after exposure. We noted the greatest response in altered chromatin structure occurred at 9 days with recovery over the remaining time points. These data are not shown in this paper since the experiment reported here demonstrated that the most relevant alterations in chromatin structure occurred prior to 9 days. A pilot study was also conducted on the effects of a single exposure of 0, 100, and 150 mg/kg MMS at 5 and 11 days after exposure. These pilot experiments supported all data obtained in the experiment reported here but are not shown due to their redundancy.

This study examined effects on sperm and testicular cells exposed to 0 or 150 mg/kg MMS. Two mice in each dosage group were measured every other day from 1 to 15, and at 19, 22, and 25 days.

Testicular Germ Cells

Body and both testicular weights were recorded immediately after cervical dislocation. One testis from each mouse was minced with curved scissors into a cellular suspension in a 60-mm petri dish containing two ml HBSS at 4°C and then transferred to 12 × 75 mm polystyrene tubes. After settling of tissue fragments, the supernatants were filtered through 53 μm nylon mesh (Tetko, Inc., New York, NY) into 12 × 75 mm tubes and kept on crushed ice (4°C) until measured by flow cytometry (FCM).

Epididymal Sperm

Cauda epididymi were surgically removed and placed in a 60 mm Petri dish containing 2.0 ml TNE buffer (0.15 M

NaCl, 0.01 M Tris-HCl, 0.001 EDTA, pH 7.4) at 4°C and then minced with curved scissors. The sperm suspensions were gently aspirated and expelled several times through Pasteur pipettes, then filtered through 153 μm nylon mesh into 12 × 75 mm tubes and kept on crushed ice until measured by FCM.

An aliquot from each sperm sample was placed in a 2.0 ml Corning cryogenic vial, admixed with glycerol to a final concentration of 10% (v/v) and frozen at −20°C for 2 hr, and then transferred to a −100°C freezer for long term storage until all samples in a study were collected. All frozen samples of sperm were individually thawed and measured again by the SCSA, as a set, on both the ICP22A and Cytofluorograf flow cytometers.

Sperm Head Morphology

Two drops of each filtered epididymal sperm suspension 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 allowed to air dry. The slides were 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 350 sperm heads per sample 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)

Aliquots (0.20 ml) of testicular or epididymal sperm samples at a concentration of 1–2 × 10^6/ml 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 acridine orange (AO, Polysciences, Inc., Warrington, PA) per ml of AO buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0) [Darzynkiewicz et al., 1976; 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), AO intercalated into native, double-stranded DNA fluoresces green (F_{530}); AO associated with single-stranded nucleic acid (DNA or RNA) emits red fluorescence ($F\geqslant_{630}$). The fluorescence observed in AO stained testicular cells reflects the relative content of DNA (green fluorescence) and RNA (red fluorescence) [Darzynkiewicz, 1979; Evenson and Melamed, 1983; Evenson et al., 1986b]. Since normal, mature sperm cells contain virtually no RNA [Monesi, 1965], red fluorescence is minimal [Evenson and Melamed, 1983]. Normal isolated sperm nuclei and/or whole cells treated with RNAse demonstrate the same fluorescence pattern as whole cells indicating that the fluorescence is due to DNA staining and not nuclear or cytoplasmic RNA [Evenson et al., 1985; Ballachey et al., 1987].

Sperm Chromatin Structure Assay

To distinguish it from other AO staining protocols, the staining procedure described above has been termed the Sperm Chromatin Structure Assay (SCSA) when utilized with sperm [Evenson, 1986, 1990]. Although this low pH treatment does not cause denaturation of histone-complexed DNA, it apparently causes partial DNA denaturation in sperm with altered chromatin structure [Evenson et al., 1980a, 1985]. Abnormal chromatin structure, defined as an increased susceptibility to induced denaturation, is determined by FCM measurements 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 total (red + green) fluorescence. SCSA variables studied include mean ($X\alpha_t$), standard deviation ($SD\alpha_t$), cells outside the main population ($COMP\alpha_t$) of alpha t and mean red and green fluorescence.

Flow Cytometry Measurements

Fresh sperm and testicular cells were stained with AO and measured with a Cytofluorograf II flow cytometer. Frozen aliquots of sperm cells were thawed at 37°C, stained and measured with both the Cytofluorograf and ICP22A flow cytometers (Ortho Diagnostics, Inc., Westwood, MA). The Cytofluorograf II, with orthogonal axes of light illumination (488 nm), sample flow and ultrasense collection optics was equipped with a Lexel 100 mW argon ion laser operated at 35 mW. The ICP22A flow cytometer was equipped with a mercury arc lamp as the excitation source and epiillumination optics. Both instruments allow for dual parameter measurements to determine amounts of a) DNA vs RNA in testicular cells, and b) double-stranded vs. single-stranded DNA in sperm cells. By use of dichroic mirrors and filters, fluorescent signals were separated into green (515–530 nm) and red (>630 nm) components. Computer protocols were used to calculate α_t values for each cell as well as α_t distribution values for each sample population. The stained samples were stable over a period of time; however, for uniformity, recorded measurements of 5 × 10^3 cells for each sample were begun 3 min after staining. Measurement rate was about 250 cells/sec.

Days of time stated in the text indicate days of time following exposure to MMS on which measurements were made.

Data Analysis

Correlations and analysis of variance (GLM) were run on PC-SAS version 6.03 [SAS, 1988].

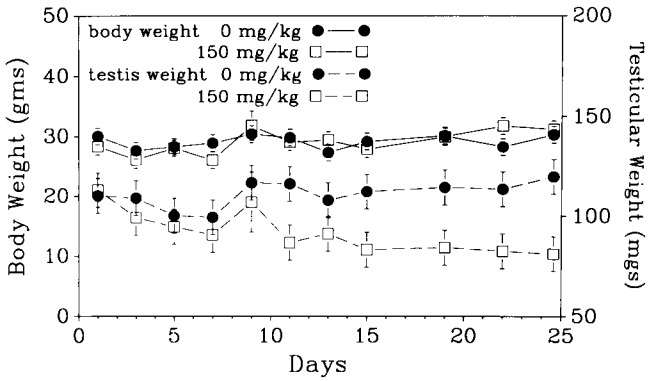

Fig. 1. Body and testis weights of mice treated with 0 or 150 mg/kg MMS and killed at 1, 3, 5, 7, 9, 11, 13, 15, 19, 22, and 25 days.

RESULTS

Body and Testis Weights

Analysis of variance revealed that mouse body weights were unaffected by MMS exposure to 150 mg/kg over the time frame of the experiment as seen in Figure 1.

Although testicular weights generally decreased following exposure to 150 mg/kg MMS, the decrease was statistically significant ($P < .05$) only at days 11, 15, 19, 22, and 25 as shown in Figure 1.

Testicular Cell Kinetics

Figure 2 shows dual parameter green (native DNA) vs. red (single-stranded RNA or DNA) fluorescence flow cytometry measurements of acridine orange (AO) stained mouse testicular cells. These distributions easily resolve cell populations with tetraploid, diploid and haploid DNA content. Two subpopulations of both tetraploid and diploid cells, differing in red fluorescence (RNA), and three subpopulations of haploid cells, differing in red (RNA) and green (DNA) fluorescence, were discernible although all diploids and tetraploids were separately grouped for analysis.

The percentage of each different cell type identified by discrete clusters was determined by computer software protocol. As exemplified in Figure 3 the kinetics are such that if the percentage of one population increases or decreases, the relative percentages of the others will also be affected

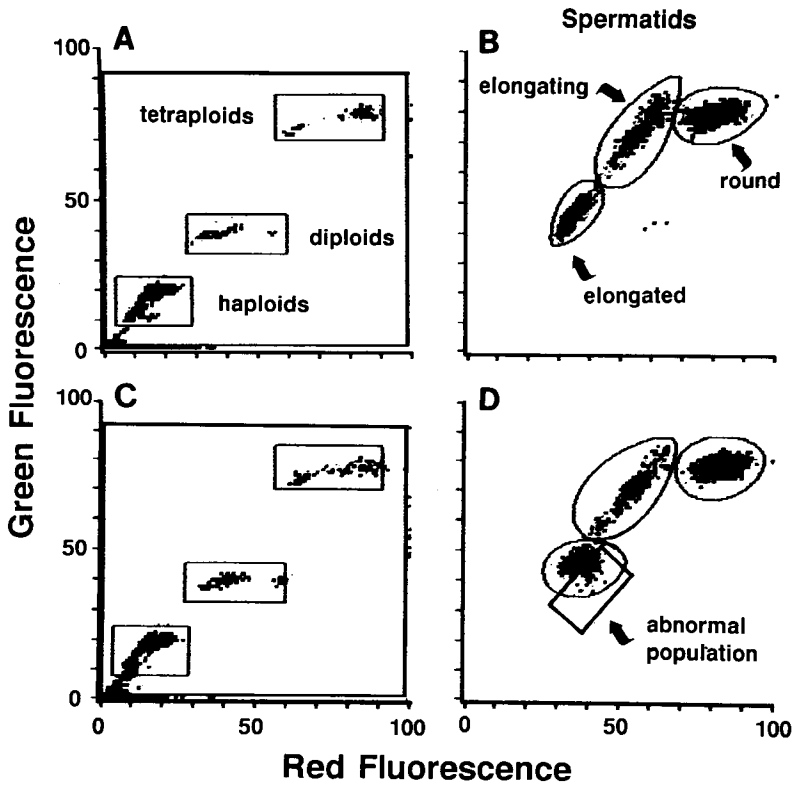

Fig. 2. Typical green vs. red fluorescence cytograms showing testicular populations for mice treated with 0 **(A,B)** and 150 mg/kg MMS **(C,D)** and killed at 5 days. Cytograms B and D were generated from cytograms A and C, respectively, by a software protocol that expands the haploid cell population values thereby providing better resolution between the three populations of round, elongating, and elongated spermatids. The box in cytogram D represents the subpopulation of elongated spermatids with denatured DNA (discussed below).

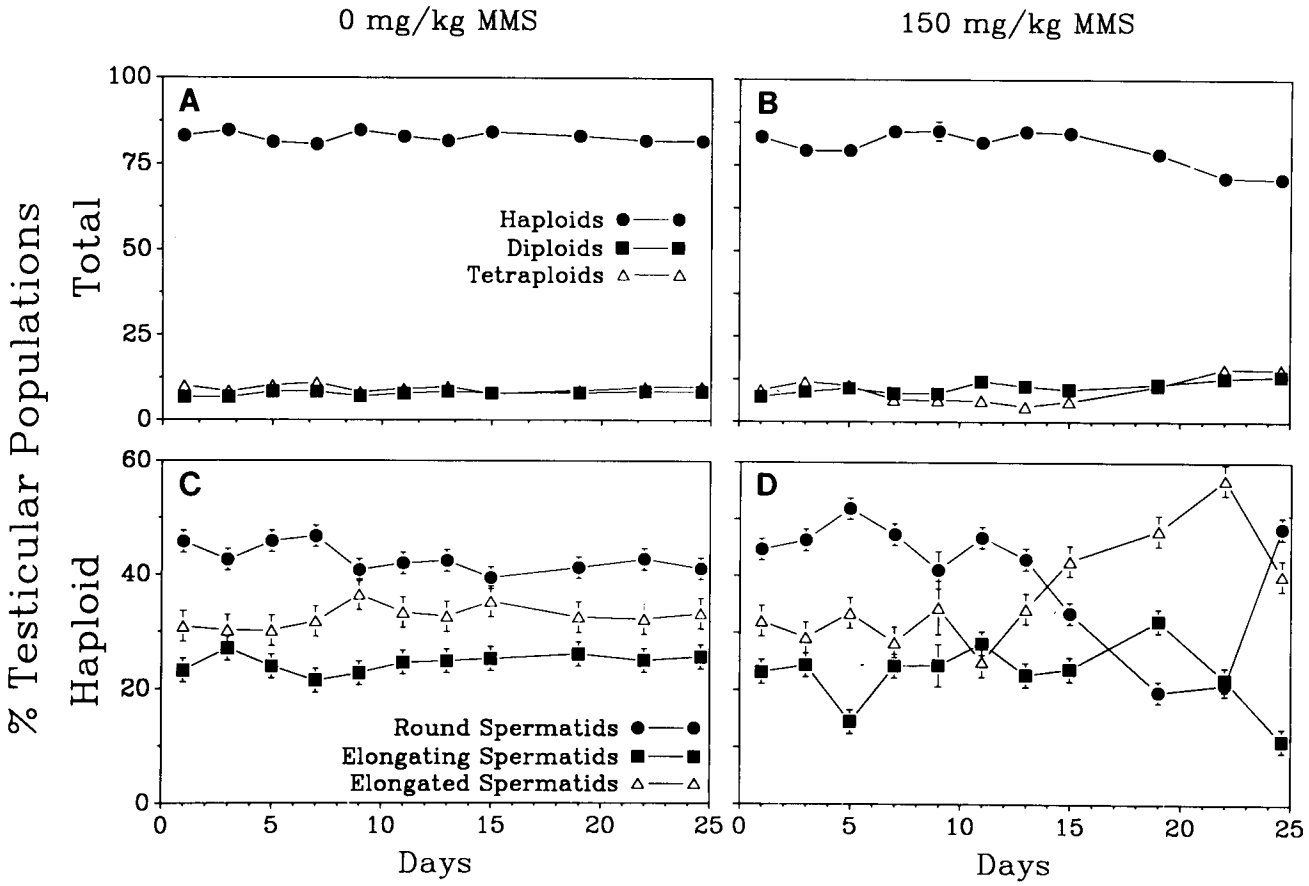

Fig. 3. Relative percentages of testicular cell populations, both total and haploid subpopulations for 0 and 150 mg/kg MMS mice measured at 1, 3, 5, 7, 9, 11, 13, 15, 19, 22, and 25 days.

whether the absolute numbers are affected or not. The only significant alteration in ratios of total haploid, diploid, and tetraploid testicular cell populations occurred at days 22 and 25 with an approximate 12% decrease ($P < .01$) in haploid cell types and a 60% increase (9.7% to 15.5%; $P < .01$) in tetraploid cell types relative to the control values.

All three haploid cell sub-populations consisting of round, elongating and elongated spermatids were affected by the exposure to MMS over time (elongating, $P < .05$; rounds and elongated, $P < .01$) (Fig. 3D). The relative percentage of round spermatids initially increased up to day 5 (52%) before dropping to a low on day 19 (20%) and then back to control values by day 25 (48%). Most significant differences occurred on days 15, 19, and 22. The relative percent of elongating spermatids fluctuated up and down over the study with lowest values on days 5 and 25 (15 and 11%, respectively). The *relative* elongated spermatid population increased steadily from day 11 (25%) to day 22 (57%) and dropped back to control values again on day 25 (40%).

Effects of MMS on Sperm Chromatin Structure and Head Morphology

The effect of MMS on susceptibility of sperm nuclear DNA to denaturation in situ was determined by the SCSA. Figure 4A,C shows examples of two parameter cytograms (green vs. red fluorescence) and α_t frequency histograms (Fig. 4B,D) produced from Cytogluorograf flow cytometer measurements of caudal sperm obtained from mice exposed to 0 or 150 mg/kg MMS at 13 days. Measurement of normal sperm produces a very narrow α_t distribution, while that of sperm with denatured DNA is broader and has a larger percent of *c*ells *o*utside the *m*ain *p*opulation of α_t ($COMP\alpha_t$). Mean α_t ($X\alpha_t$) and standard deviation of α_t ($SD\alpha_t$) describe the extent of chromatin structure abnormality within a population. Figure 4E–H shows examples of the same parameters derived by measuring an aliquot of the samples represented in A–D with an ICP22A flow cytometer. The elongated green fluorescence (DNA) stainability signal (Fig. 4A) from the Cytofluorograf (orthogonal light axes) is due to an optical artifact described by Gledhill et al. [1979] that has no significant effect on α_t values (Fig. 4B). Epiillumination used in the ICP22A flow cytometer avoids this artifact; consequently, the shape of the cytogram distribution is more rounded (Fig. 4E). Note the similarity of the α_t profiles generated by the two instruments (Figs. 4B,F).

Figure 5 shows the effects of 0 and 150 mg/kg MMS on sperm chromatin structure as measured by the SCSA on both types of flow cytometers. Results obtained from the Cyto-

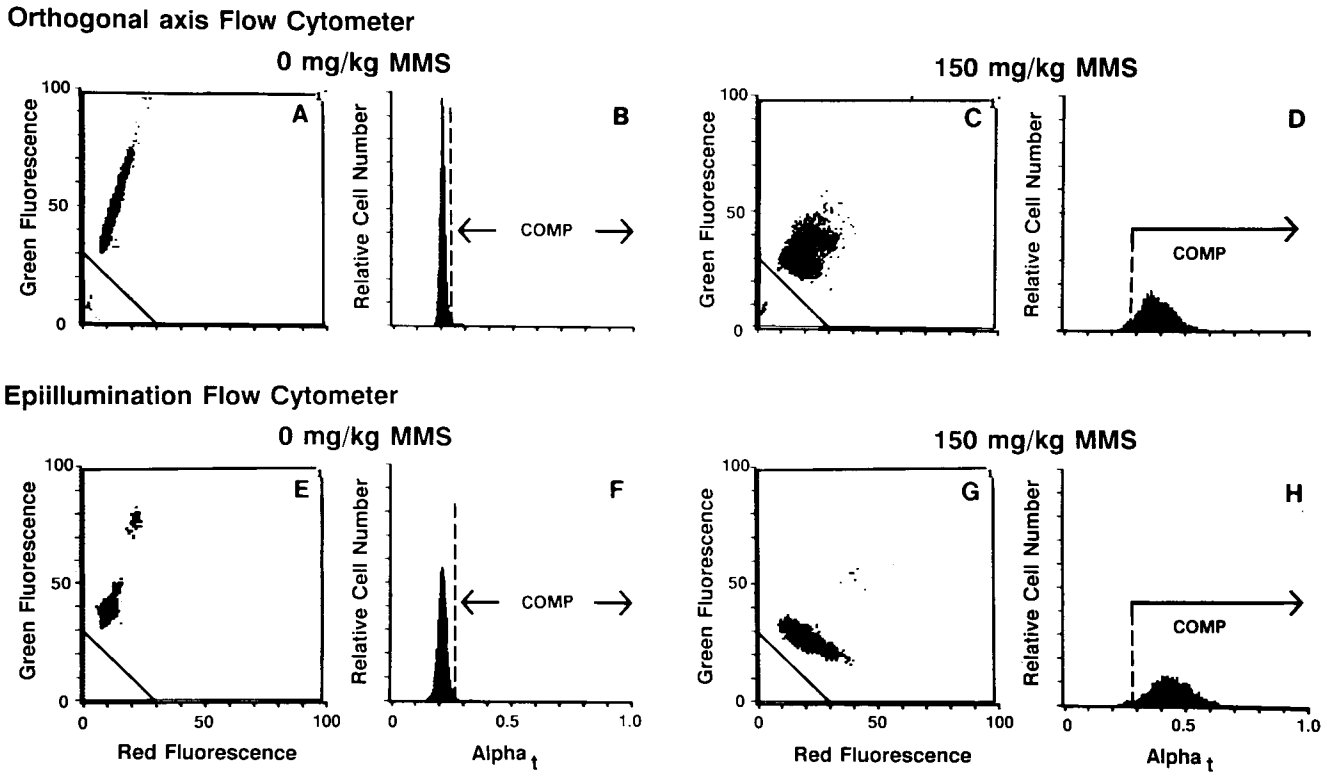

Fig. 4. Green vs. red fluorescence cytograms of SCSA data obtained by measuring caudal mouse sperm with a Cytofluorograf **(A,C)** or ICP22A **(E,G)** flow cytometer. Sperm were obtained from mice treated with 0 or 150 mg/kg MMS and killed at 13 days. Panels **B,D,F,** and **H** are α_t frequency histograms; cells to the right of the vertical lines are cells with denatured DNA ($COMP\alpha_t$).

fluorograf are not meaningfully different from the ICP22A for $X\alpha_t$, $SD\alpha_t$, and $COMP\alpha_t$. Correlations between SCSA data from the two instruments were .96, .99 and .98 (all $P < .001$) for $X\alpha_t$, $SD\alpha_t$, and $COMP\alpha_t$, respectively. Red and green mean fluorescence values had differences as expected due to differences in the nature of raw data and instruments settings. Thus, absolute values for the treated mouse group between instruments differed slightly but interpretation of data remains the same. The greatest response for the SCSA variables was for $COMP\alpha_t$ which shifted to 100% by day 3 (day 2 not measured) and remained at that level until day 11. For the other variables, the greatest response occurred on day(s) 7 ($X\alpha_t$, red and green fluorescence) and 15 ($SD\alpha_t$). The MMS exposed mice differed from control for $X\alpha_t$ and $COMP\alpha_t$ except on days 1, 19, 22, and 25 days. Increase of $SD\alpha_t$ responded to the 150 mg/kg MMS treatment in a delayed manner, related to decrease in $X\alpha_t$, and differed from controls on days 7, 11–25.

In order to assess the reliability of recalibrating the flow cytometer over each of a number of days and also to determine whether freezing, thawing and sonication of the sperm had any effect on α_t parameters, data from flow cytometric measurements made on frozen samples during a single measuring period were compared with measurements made on fresh samples over time, both on the Cytofluorograf. As seen in Figure 6, data from control mice (0 mg/kg MMS) on any given day in the experiment shows that no differences existed for any SCSA variables between any fresh and frozen sperm (Fig. 6). Minimal variations seen between fresh and frozen sample sets from 150 mg/kg treated mice are not considered biologically significant. Correlations between fresh vs frozen/thawed derived data were .93, .95, and .98 (all $P < .001$) for $X\alpha_t$, $SD\alpha_t$, and $COMP\alpha_t$, respectively.

As seen in Figure 7, percentage of abnormal morphological sperm obtained from MMS exposed mice started rising at day 11 (25.3 ± 10.5%) and plateaued at the maximum value (47%) at 13–22 days before starting to fall at 25 days. Note that this increase occurred 8 days after the dramatic increase in α_t values, particularly $COMP\alpha_t$ values as shown in Figures 5 and 6.

Effect of MMS on Chromatin Structure of Elongated Spermatids

A unique response of the elongated spermatid population, not described previously, is illustrated in Figure 2D. Note the subpopulation of the elongated spermatids, boxed off in the figure that has increased red fluorescence. Since the round and elongating populations are not shifted toward increased red fluorescence relative to the controls, this phe-

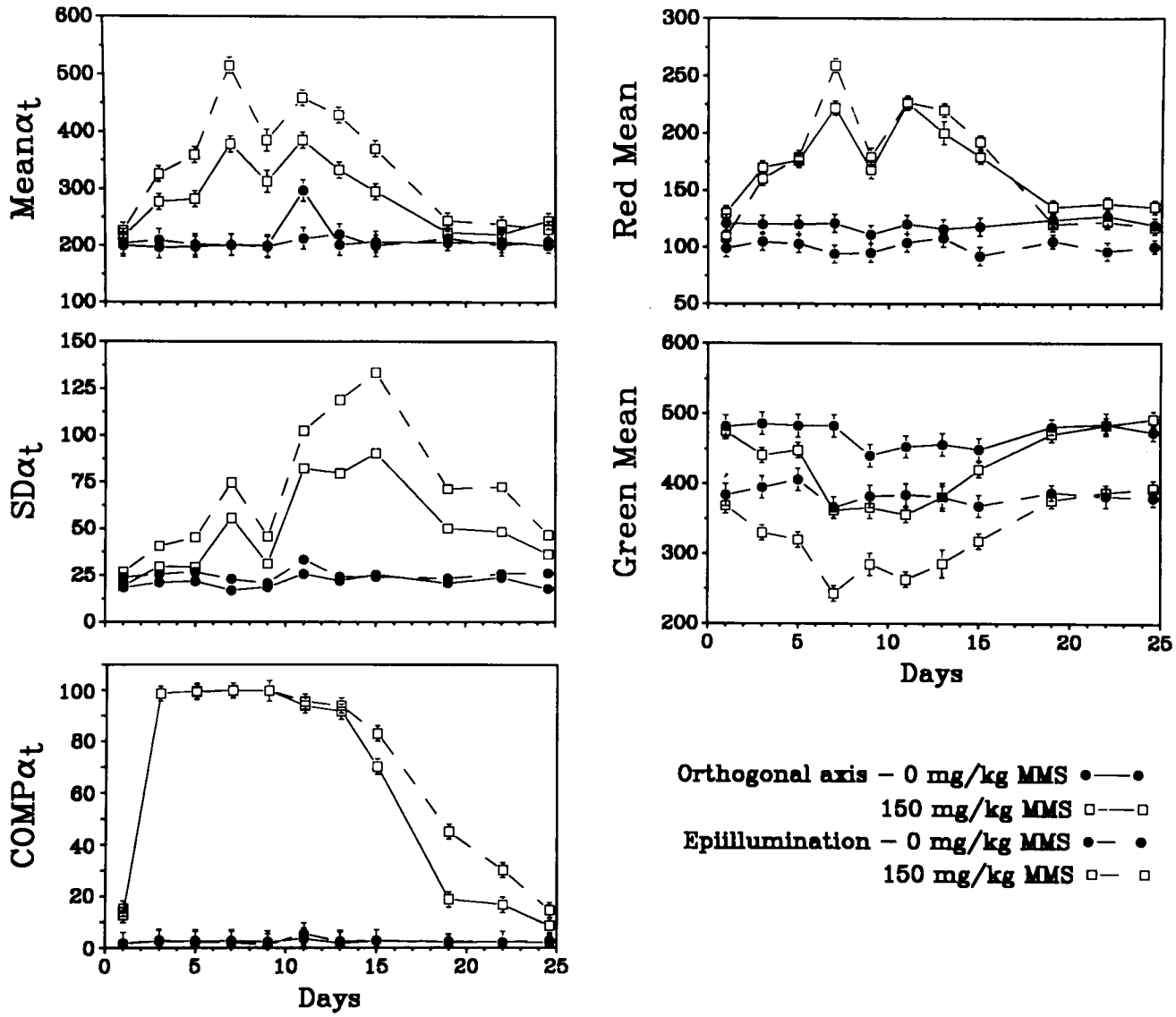

Fig. 5. Comparison of SCSA data derived by measurements made with a Cytofluorograf (orthogonal light axes) and ICP22A (epiillumination) flow cytometer on frozen/thawed and sonicated sperm. SCSA data include Meanα_t (Xα_t), SDα_t, COMPα_t, and mean red and green fluorescence. Sperm samples were obtained at 1, 3, 5, 7, 9, 11, 13, 15, 19, 22, and 25 days. Sperm from two mice were used for each treatment per time point.

nomenon cannot be due to an increased residual content of RNA. Rather, it is concluded that this population represents DNA that has denatured in the same fashion as described above in this study for epididymal sperm. Figure 8 shows that SDα_t and COMPα_t values of these elongated spermatids calculated in the same manner as for epididymal sperm cells described above. These data indicate that elongated spermatids demonstrate MMS induced damage by 3 days (25% COMPα_t) with recovery occurring by 5 and 7 days.

DISCUSSION

Data obtained in this study support the view of Sega and Owens [1983] that MMS destabilizes sperm chromatin structure, likely due to alkylation of cysteine –SH groups. Evidence is shown here for the first time that testicular elongated spermatids are susceptible to MMS induced chromatin structural changes; this also fits with the model that MMS targets the cysteine rich protamines that are replacing histones and transition proteins at that stage.

Other studies on ethyl nitrosourea [Evenson et al., 1985], triethylenemelamine [Evenson et al., 1989b] and thiotepa [Evenson et al., 1986a] have shown that the SCSA has about the same, or somewhat higher, sensitivity to detect damaging effects of alkylating agents as the sperm head morphology assay [Wyrobek and Bruce, 1975]. In sharp contrast, the SCSA was a much more sensitive test than sperm head morphology to detect MMS induced alterations as shown in

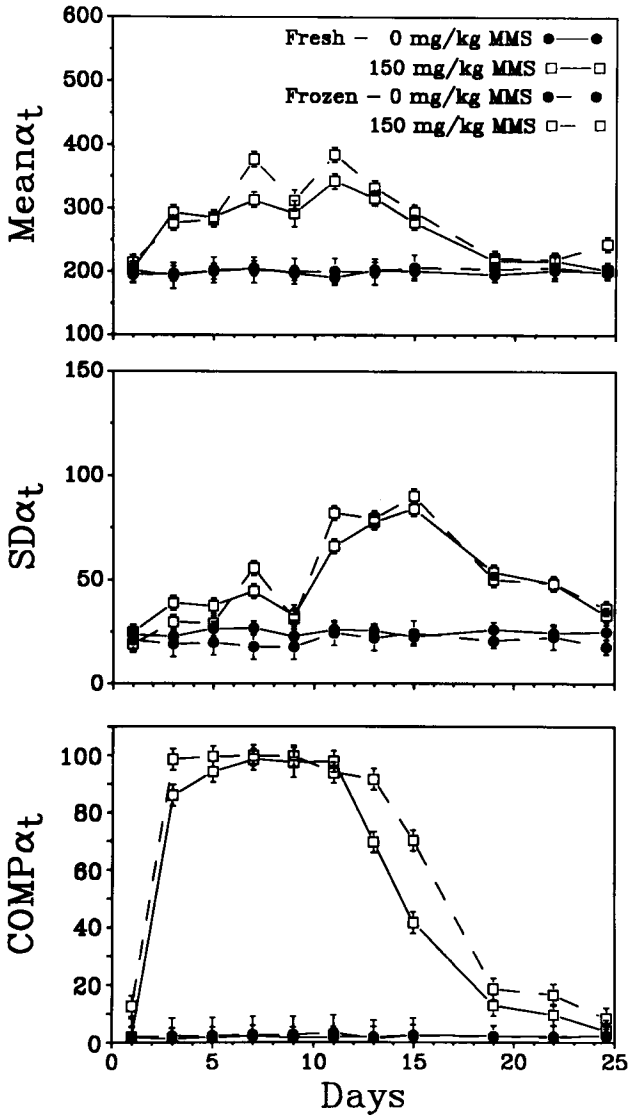

Fig. 6. Effects of 0 or 150 mg/kg MMS exposure on SCSA parameters measured on the Cytofluorograf as both fresh whole sperm (solid lines) and frozen/thawed sonicated sperm (dotted lines). SCSA measures include Meanα_t (Xα_t), SDα_t, and COMPα_t.

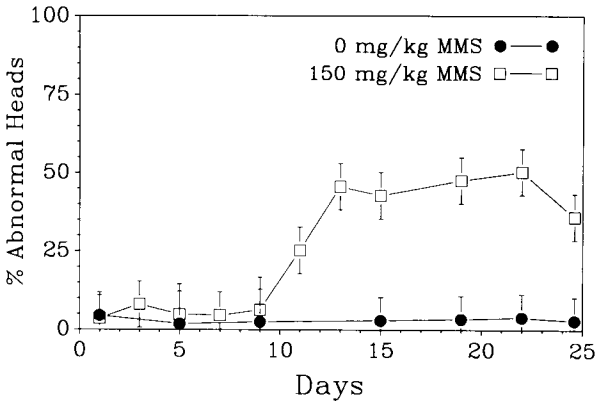

Fig. 7. Relationship between sperm head morphology abnormalities and days post exposure. Sperm samples were obtained at 1, 3, 5, 7, 9, 11, 13, 15, 19, 22, and 25 days.

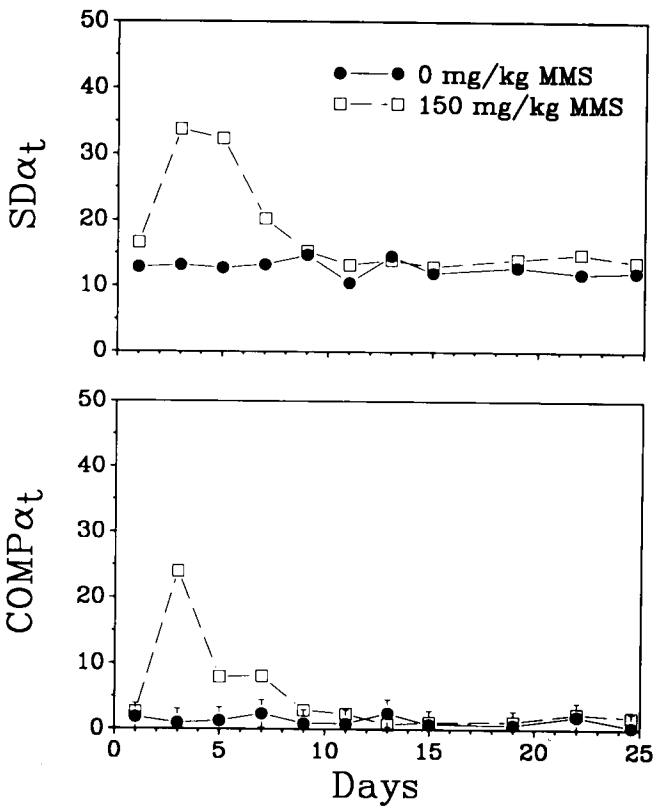

Fig. 8. COMPα_t and SDα_t of testicular elongated spermatids from mice exposed to 0 or 150 mg/kg MMS. Data were calculated from cytograms as illustrated in Figure 2D.

this study. Day 1 was not different from controls; however, COMPα_t was nearly 100% by day 3 indicating that the SCSA was detecting chromatin damage in all cauda sperm by day 3. Since developing mouse sperm reside for 3 days each in the cauda and caput epididymis and 5.8 days as elongated spermatids [Oakberg, 1956], the most sensitive cells ranged from elongated spermatids to cauda sperm. This argument is strengthen by the observation that elongated spermatids, but not round and elongating spermatids, had DNA susceptible to acid induced denaturation following exposure to MMS.

Sega et al. [1986] using alkaline elution of vas mouse sperm DNA obtained from MMS exposed mice, showed that increases in amounts of single-strand DNA breaks began by day 1 and steadily rose to a maximum by day 8. The level plateaued until about day 12 at which time it decreased. This decrease was temporally related to sperm which at the time of treatment were in germ-cell stages capable of DNA repair. Thus, the SCSA detects by day 3 100% of sperm with increased susceptibility to DNA denaturation, whereas alkaline elution detects an increase in total single-strand breaks on a population mean basis but not on a

cell by cell basis. The maximum values of $X\alpha_t$ temporally corresponded to the maximum single-strand breaks and dominant lethal mutations suggesting that the SCSA is measuring abnormal chromatin structure related to mutations in at least some cases.

Alkylation of cysteine residues likely caused instability of the developing spermatid chromatin. A model of sperm chromatin structure shows protamine molecules lying in the minor groove of DNA with the protruding protamine fitting into the major groove of a neighboring DNA strand in a fashion that permits DNA strands to be packaged side by side in a linear array [Balhorn, 1982; see review by Ward and Coffey, 1991] Such linear arrays have been seen by electron microscopy [Koehler, 1970; Koehler et al., 1983; Evenson and Darzynkiewicz, 1990]. Electron micrographs of AO precipitated mouse testicular sperm DNA showed that elongated spermatids had regional differences in susceptibility to denaturation likely due to maturation occurring from anterior to posterior regions [Evenson and Darzynkiewicz, 1990]. Testicular cell kinetics shown in Figure 1D would suggest that elongated spermatids may be partially blocked at this stage since at 22 days about 55% of the total haploid cells are elongated spermatids.

The $X\alpha_t$ generally paralleled $COMP\alpha_t$, which is expected. Recovery to near normal occurred rather quickly beginning at about 12 days. $SD\alpha_t$ response was slower; however, this would be expected since its calculation is CV/X and thus its rise coincides with the reduction of $X\alpha_t$. In previous studies, e.g., reproductive toxicity of ethyl nitrosourea [Evenson et al., 1985], less of a shift has been seen for the whole population. In such cases, $SD_{\alpha t}$ is likely a more sensitive indicator of chromatin damage to the sperm population than $X\alpha_t$.

In this study, as in previous studies [Evenson et al., 1989b; Evenson and Thompson, 1991], frozen/thawed sperm had the same SCSA values as fresh sperm. Thus, at least one freezing/thawing procedure does not alter the chromatin structure as measured by the SCSA. This provides a significant advantage to experimentation including the possibility to include more animals in each dosage/time group for better statistics. Thus, samples can be stored and measured at a convenient time, or sent on dry ice by overnight delivery service to a flow cytometry laboratory for measurement. Data in Figure 5 show that a flow cytometer can be calibrated on a daily basis over many days to be essentially as accurate as those on frozen sperm made in the time span of a day or two. Current use of frozen sperm aliquots to serve as the calibration agent has improved our capacity to do this as compared to the previous use of fluorescently stained polystyrene beads.

Sperm measured by the SCSA protocol on two different flow cytometers have very different green vs. red fluorescence cytograms as seen in Figure 4A,B. The elongated profile of AO stained sperm obtained on the Cytofluorograf flow cytometer is due to an optical artifact described previously by Gledhill et al. [1979]. In brief, the broad distribution on the green fluorescence axis is due to a light piping effect caused by the high refractive index of sperm and their asymmetrical shape. While this uncorrected artifact prohibits an accurate measurement of sperm DNA content, it has no significant effect on the α_t profile as α_t is a ratio of red to total fluorescence. This optical artifact can be eliminated if the sperm are measured in a flow cytometer that utilizes epiillumination [Gledhill et al., 1979]. This optical configuration does not produce a light piping effect and the raw signals are tightly grouped. The important observation made here is that the α_t profiles obtained on either instrument on sperm from control or MMS treated mice are very similar (Fig. 4). Furthermore, the α_t curves, derived from the two kinds of flow cytometers, plotted over time are very similar with very high correlations. The slight differences in absolute values are mostly due to absolute differences in green fluorescent values; this is expected since the ICP22A values are lower due to the more coherent distribution of DNA staining values. Of more importance, the biological interpretation of the data would be the same for data generated by either instrument. Comparative instrument measurements have been made on sperm from a number of other species and there is a very high degree of correlation between the α_t parameters [Evenson et al., in preparation]. Thus, the low cost epifluorescent flow cytometer is a viable alternative to the high cost laser driven flow cytometers with orthogonal axes of sample flow, laser beam, and optical lens collection.

ACKNOWLEDGMENTS

This work was supported in part by March of Dimes Grant No. FY91-0528, USDA Competitive Research Grant Number 90-37240-5717, and NSF Grant Number EHR-9108773. This is South Dakota Agricultural Experiment Station Publication No. 2648 of the journal series.

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Accepted by—
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