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• *Original Contribution*

GLUTATHIONE DEPLETION POTENTIATES ETHYL METHANESULFONATE-INDUCED DAMAGE TO SPERM CHROMATIN STRUCTURE

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Abstract — Male rats were treated with phorone at dosages previously shown to reduce glutathione in rodent reproductive tracts, followed by a single challenge with ethyl methanesulfonate, a known mutagenic and clastogenic agent. Epididymal sperm collected 8 and 15 days after exposure from phorone pretreated animals had a significantly greater alteration of sperm chromatin structure, defined as an increased susceptibility to DNA denaturation in situ, relative to sperm obtained from animals injected with saline alone or saline + EMS (50, 100, 150, or 200 mg/kg bw). These data support the hypothesis that ethyl methanesulfonate-induced alkylation of developing sperm chromatin protamines causes a significant stress on chromatin structure leading to increased DNA damage. This is the first report showing that glutathione depletion potentiates EMS-induced chromatin structural alterations that are likely related to dominant lethal mutations.

Key Words: phorone-depleted glutathione; ethyl methanesulfonate alkylation; rat spermatozoa; protamine; sperm chromatin structure assay; DNA denaturation.

INTRODUCTION

Glutathione (GSH) is a tripeptide containing a cysteine residue with a free sulfhydryl group that is present in mammalian cell types in a 0.5 to 10 mM concentration (1). GSH functions directly or indirectly in many important biologic events and plays an important role in drug metabolism and toxicity (2). GSH is the predominant intracellular nucleophilic scavenger and is usually the most abundant sulfhydryl compound present in animal tissues. The high specific activity of GSH-dependent enzymes in testis and epididymis of a variety of species (3,4) suggests the significance of GSH to biochemical processes of male reproductive function. However, the relative importance of the GSH system in protecting developing germ cells from chemically induced injury is not fully understood. Compromise of testicular or epididymal GSH-dependent protective mechanisms may lower the threshold for germ cell mutagenicity.

Cells in the seminiferous epithelium are protected by the blood–testis barrier. However, major portions of the male reproductive tract, such as the rete testis and epididymis, fall outside the functional blood–testis barrier. Because of higher accessibility in these regions of the reproductive tract, germ cells in these areas may be more severely affected by xenobiotics, thereby accentuating the potential importance of testicular and epididymal GSH in protecting against chemically induced insult. An important function of GSH addressed in this study is its role in detoxification of electrophilic agents, and a determination of whether depletion of GSH results in potentiation of clastogenic action.

Ethyl methanesulfonate (EMS) is a potent, electrophilic, monofunctional alkylating agent that induces clastogenic damage, including dominant lethal mutations (5,6) and heritable translocations (7,8). Dominant lethal mutation in males is measured by determining the frequency of live versus dead (that is, resorbed) fetuses occurring after mating mutagen-treated males with untreated females at various times after treatment. Dominant lethals are a manifestation of chromosomal breakage resulting in loss of that broken segment at anaphase and giving rise to monosomic embryos that die in utero. Sega and

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Owens (9) showed that the temporal pattern of dominant lethal mutations induced by EMS was closely correlated with the temporal pattern of alkylation of mouse sperm, and they proposed that the mechanism of EMS-induced dominant lethal mutations was by ethylation of protamines of the late spermatids or early spermatozoa. The germ cells become increasingly sensitive in mid-to-late spermatid stages as chromosomal histones are replaced by arginine-rich basic protamines (10,11). These protamines are not only rich in arginine, but also relatively rich in cysteine (10), which is a target of alkylation by EMS (12). Evenson and colleagues (13) showed methyl methanesulfonate (MMS)-induced damage to chromatin structure in testicular elongated spermatids. The germ cell stage apparently most sensitive to EMS alkylation occurs as the cells pass through the rete testis into the caput epididymis prior to the extensive disulfide bonding that occurs during passage through the epididymis (14,15). This sensitive stage occurs about two weeks prior to the time that mature sperm are ready for ejaculation (16,17).

EMS is an electrophile that readily conjugates with GSH. Therefore, any perturbation of testicular or epididymal GSH may influence the extent of EMS-induced dominant lethal mutations. Previous studies have demonstrated that pretreatment with compounds that lower tissue GSH levels prior to EMS administration to male rats potentiate the number of dominant lethal resorptions in females mated to these treated males (18–20).

We have employed flow cytometry analysis of caudal spermatozoa from Sprague–Dawley rats to determine the dose-dependent and temporal pattern of chromosomal damage following EMS treatment. In addition, this study addresses the question of whether phorone (diisopropylidene acetone), an alpha, beta-unsaturated carbonyl compound, and a known depleter of GSH from animal tissues, (21–23) potentiates EMS-induced chromatin structure abnormality. Phorone treatment has been found to significantly lower GSH in the reproductive tract (20,23). The working hypothesis was that pretreatment with phorone, a reproductive tract glutathione depleter, will enhance the known clastogenic action of EMS, as observed in previous studies (9,17) and this will be manifested as an increased susceptibility of DNA to denaturation in situ (24,25).

MATERIALS AND METHODS

Animals

Sexually mature male Sprague–Dawley rats (300 to 400 g) were used in all experiments. The rats were obtained as weanlings (25 days old) from the vivarium of the National Center for Toxicological Research (Jefferson, AR). They were housed on Beta Chips (Northeastern Products Corp., Warrenburg, NY), two per cage, and maintained on a 12/12 light dark cycle with food and water provided ad libitum. Rats were killed by CO_2 asphyxiation.

Chemicals and treatments

The dose-dependent and temporal pattern of EMS-induced alterations of sperm chromatin structure were assessed by flow cytometric analysis. For dose-dependent experiments, EMS (Sigma Chemical Co., St Louis, MO) was administered i.p. to groups of rats (n = 6) at either 50, 100, 150, or 200 mg/kg mixed in 0.9% saline. These doses were based on previous studies investigating the doses necessary to induce dominant lethal mutations (16,17). Control rats received 0.9% saline at 2 mL/kg. The animals were killed at 15 days (posttreatment) by CO_2 asphyxiation, and caudal spermatozoa were isolated. For time-dependent studies, groups of animals (n = 6) were administered EMS ip at 200 mg/kg, and killed at either 8, 15, or 22 days. These time periods were chosen to allow germ cells of various stages of development to mature and move into the epididymis where they were collected for flow cytometry measurements. Significant alteration of epididymal sperm chromatin structure after specific times posttreatment will indicate the germ cell stage of development that is sensitive to EMS-induced effects. Rats exposed to EMS 2 to 3 weeks prior to mating produced dominant lethal mutations (16,17).

Phorone (Sigmal Chemical Co., St. Louis, MO) was used to deplete glutathione levels. Previous experiments in our laboratory have demonstrated that significant lowering in hepatic and reproductive tract glutathione levels was achieved by phorone injection ip at 250 mg/kg. Maximum time of depletion was between 2 and 4 h after treatment. During this time period, testicular GSH was decreased to 65% of control values, and epididymal GSH was decreased to 20% of control values (20,23). Therefore, phorone was administered to 6 or 7 rats/group as a pretreatment (250 mg/kg ip, in corn oil, 125 mg/mL) 2 h prior to EMS challenge. Control animals received an ip injection of the corresponding vehicle.

Isolation of caudal sperm samples

On an appropriate day after EMS administration, rats were killed by CO_2 asphyxiation and both cauda epididymides from each animal were excised and pooled. The tissues were then minced with fine scissors in TNE buffer (0.01 M Tris, 0.15 M NaCl,

0.001 M EDTA, pH 7.5) with 10% glycerol and immediately frozen at −80 °C. Aliquots of each sperm sample were counted in a hemocytometer to determine the number of cells per mL. Samples were shipped on dry ice by overnight mail to South Dakota State University for flow cytometric analysis.

Sperm chromatin structure assay (SCSA)

Frozen caudal sperm samples were thawed in a 37 °C water bath and diluted with TNE buffer to 1 to 2 × 10^6 cells/mL. One-half-mL aliquots were immediately sonicated for 30 s (Branson Sonifier, Model 450, 70% duty cycle, power level 3) and measured by the SCSA. Aliquots (0.20 mL) of the sonicate were diluted to a concentration of 1 to 2 × 10^6 nuclei/mL and 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 s, the cells were stained by adding 1.2 mL of solution containing 6.0 μg chromatographically purified acidine orange (AO; Polysciences, Inc., Warrington, PA) per mL of AO buffer (370 mL 0.1 M citric acid and 630 mL 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0) (26–28). Flow cytometric measurements were begun 3 min after initiation of this procedure.

Acridine orange stained cells were passed at a rate of about 200 cells/s through the quartz flow cell in a Cytofluorograf II flow cytometer (Becton Dickinson Immunocytometry Systems, Westwood, MA) equipped with ultrasense optics and a Lexel 100 mW argon ion laser operated at 35 mW (488 nm) and interfaced with a 2150 data handler. Total fluorescence from each AO-stained nucleus in the sample population was separated into green (515 to 530 nm, native DNA) and red (>630 nm, denatured DNA) components (29).

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 (28,30). Abnormal chromatin structure, defined here as an increased susceptibility to acid-induced denaturation, is determined by flow cytometric measurements of the shift from green (native DNA) to red (denatured, single-stranded DNA) fluorescence. This shift is expressed as alpha t (α_t) and is the ratio of red to total (red + green) fluorescence (31). By definition, α_t values range from 0 to 1.0 (26); however, for convenience of working with whole numbers, they are expressed as ranging from 0 to 1000. Data were based on 5 × 10^3 nuclei per sample.

In the SCSA, α_t is calculated for each sperm, and results are expressed for each sample as the mean ($X\alpha_t$), standard deviation ($SD\alpha_t$), and percentage of cells outside the main population of α_t ($COMP\alpha_t$). Measurement of normal sperm produces a very narrow α_t distribution, but that of sperm with denatured DNA has a broader distribution with larger $SD\alpha_t$ and $COMP\alpha_t$ and often increased $X\alpha_t$. (24).

Statistics

The General Linear Model of the Statistical Analysis System (32) was used to calculate least square means and significant treatment differences for SCSA data.

RESULTS

Effect of EMS exposure on chromatin structure

Figure 1 shows raw flow cytometric data and computer-processed frequency histograms of α_t values of sperm obtained from animals treated with a single exposure of 0, 100, or 200 mg EMS/kg body weight at 15 days (post exposure). Each dot in the cytograms represents a single cell the position of which is relative to the amount of native DNA (green fluorescence) versus denatured DNA (red fluorescence). Note that the profile of fluorescence signal from EMS-treated animals has lower green and higher red values. Thus, the α_t values as seen in panel C are increased (both $P < 0.01$) following exposure to 100 or 200 mg/kg EMS.

Dose–response relationship of EMS on sperm chromatin structure

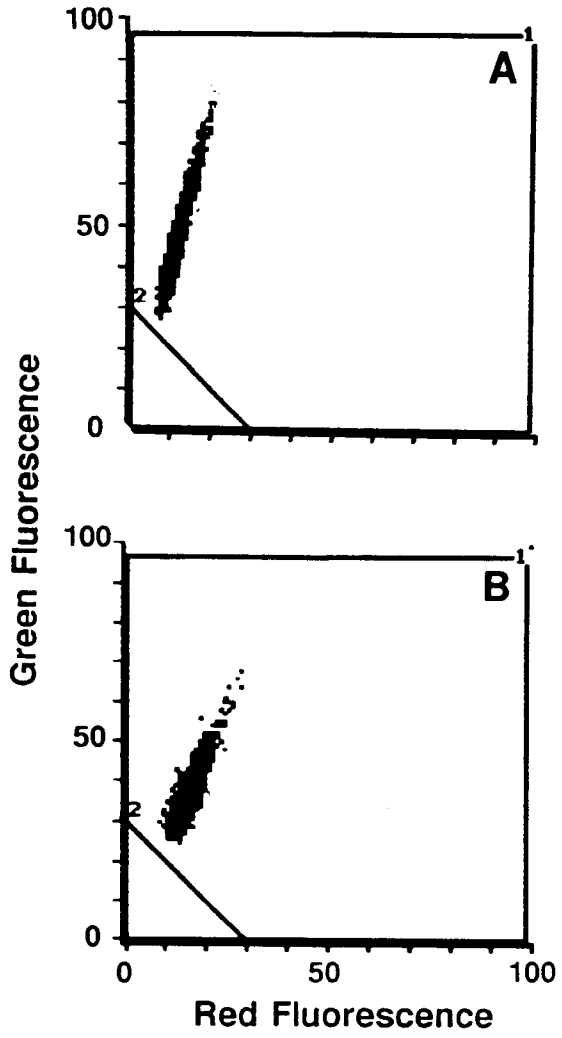
Dose–response effects of EMS at 15 days on altering sperm chromatin structure are shown in Figure 2. Increasing dosage levels from 0, 100, 150, and 200 mg/kg EMS caused a corresponding increase in $X\alpha_t$ and $COMP\alpha_t$. All three dosages caused a significant ($P < 0.001$) increase in $COMP\alpha_t$, whereas only the two highest dosages caused a significant ($P < 0.05$) increase in $X\alpha_t$.

Temporal pattern of EMS-induced sperm damage

The greatest effect on all SCSA values measured occurred with 200 mg/kg, which was used to determine the temporal pattern of EMS-induced damage. Rats were injected with 200 mg/kg EMS, and cauda sperm were isolated at 8, 15, and 22 days. All values for $X\alpha_t$ and $COMP\alpha_t$ were significantly increased from controls ($P < 0.001$) with the greatest differences being seen on days 8 and 15 and then a drop in values at day 22 as seen in Figure 3.

Effect of phorone pretreatment on potentiating EMS damage to sperm chromatin

Comparative SCSA data derived from animals either preteated with phorone or not and subse-



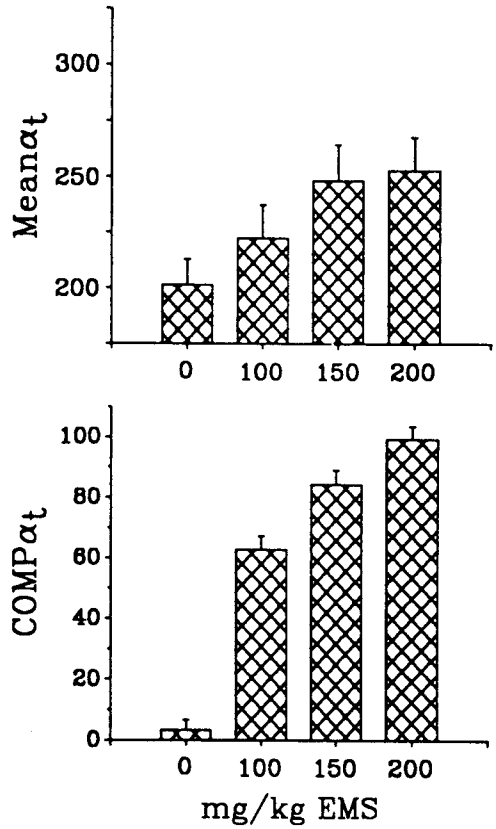
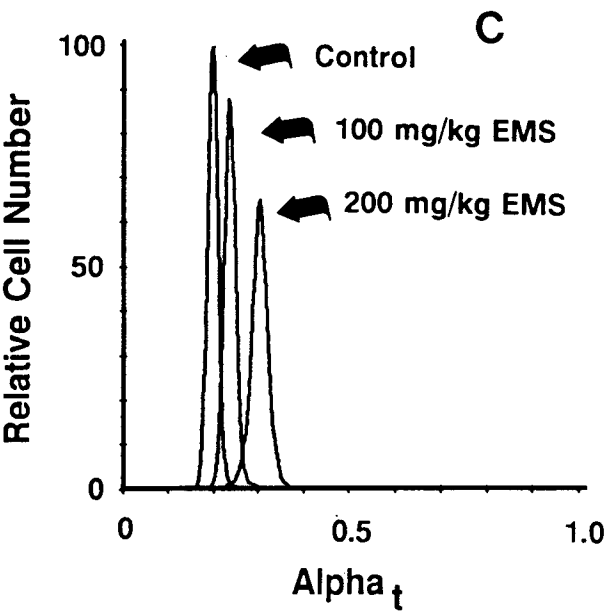


Fig. 2. Each bar represents the average ± SEM value for the effects of 0, 100, 150, or 200 mg/kg EMS on caudal sperm $X\alpha_t$ and COMPα_t at 15 days. 150 and 200 mg/kg EMS $X\alpha_t$ values were significantly different from 0 ($P < 0.05$). All treatments had COMPα_t significantly different from 0 ($P < 0.001$). Duplicate measurements were made on all samples from 6 rats/dosage (n = 12/dosage).



quently exposed to 0, 50, or 100 mg/kg EMS and killed 15 days later (Figure 4) or exposed to 0, 50, 100, or 200 mg/kg EMS and killed 8 days later (Figure 5) demonstrated that phorone pretreatment potentiated EMS action on chromatin structure. At the lowest dose (50 mg/kg), EMS + phorone had a significantly higher $X\alpha_t$ ($P < 0.05$) than did the same dose of EMS alone (Figure 4). Likewise 100 mg/kg EMS + phorone (Figures 4 and 5) or 200 mg/kg EMS + phorone (Figure 5) had a significantly higher

Fig. 1. Flow cytometry derived dual parameter green fluorescence (native DNA) versus red fluorescence (denatured DNA) relative intensity cytograms of caudal sperm obtained at 15 days from a rat exposed to 0 (panel A) or 200 (panel B) mg/kg EMS. Panel C shows α_t frequency histograms of SCSA-measured caudal sperm obtained from rats exposed to 0, 100, or 200 mg/kg EMS. Duplicate measurements were made on all samples from 6 rats/dosage (n = 12/dosage).

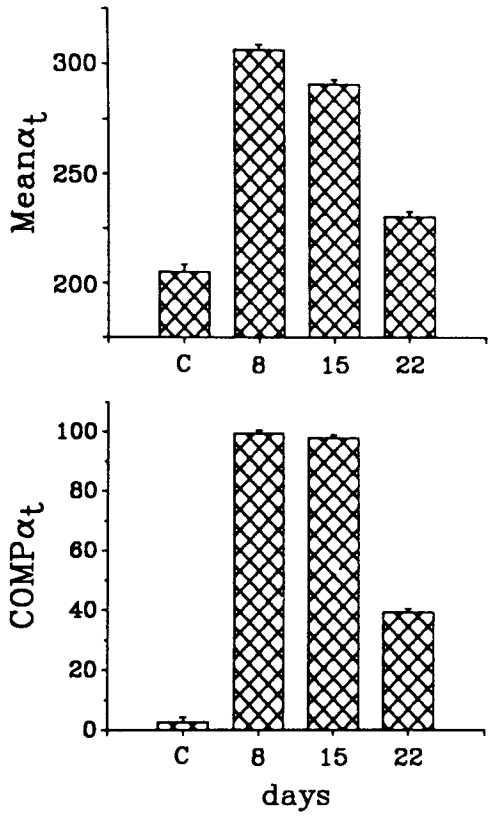


Fig. 3. Each bar represents the average ± SEM value for the effects of 200 mg/kg EMS on caudal sperm $X\alpha_t$ and COMPα_t at 8, 15, and 22 days. All values were significantly different from control ($P < 0.001$) for both $X\alpha_t$ and COMPα_t. Duplicate measurements were made on all samples from 6 rats/dosage (n = 12/dosage).

$X\alpha_t$ and COMPα_t ($P < 0.01$) than the equivalent dose of EMS alone (Figure 4). In a separate and independent experiment, 100 and 200 mg/kg EMS + phorone increased $X\alpha_t$ ($P < 0.01$) and COMPα_t ($P < 0.001$) values over that of EMS alone.

DISCUSSION

Sega and colleagues (9,12,33) have proposed that the mechanism for EMS-induced dominant lethal mutations is through ethylation of cysteine sulfhydryl groups of germ cell protamines, thereby blocking normal disulfide-bond formation and leading to a weakening of the quaternary structure of chromatin. This likely results in stress-induced single or double DNA strand breaks, eventually leading to chromosome breakage and dominant lethality. Our SCSA analysis of sperm from EMS-treated rats supports this hypothesis.

EMS induced significant damage to sperm chromatin structure in situ, as measured by the SCSA; the data are similar to those seen on measurements of sperm from mice exposed to methyl methanesulfonate (13). EMS treatment resulted in significant clastogenic effects in a dose- and time-dependent manner. The greatest damage was measured at 8 and 15 days. This temporal pattern of damage assessed by the SCSA is essentially the same as that observed by Teaf and colleagues (16,17) when assessing EMS-induced dominant lethal mutations in the rat. They demonstrated that the temporal pattern of EMS-induced dominant lethal resorptions was elevated at mating periods 8 and 15 days, followed by a return to control levels at 22 days. Therefore, the temporal pattern of EMS-induced damage measured by the SCSA is maximal at the same times as in dominant lethal assay. This time of maximal

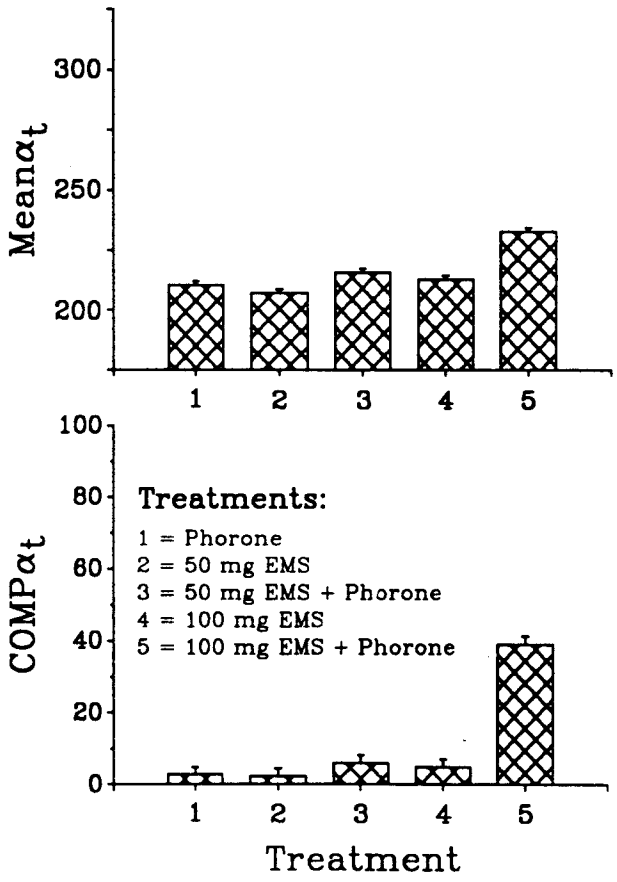


Fig. 4. Each bar represents the average ± SEM value of the effects of 5 different EMS and EMS + phorone treatments on caudal sperm $X\alpha_t$ and COMPα_t at 15 days. The most important significant differences are between the pairs of EMS and EMS + phorone at each treatment level. For $X\alpha_t$, both the 50 and 100 mg/kg pair differed ($P < 0.01$) while only the 100 mg/kg pair differed for COMPα_t ($P < 0.01$). Duplicate measurements were made on all samples from 6 rats/dosage (n = 12/dosage).

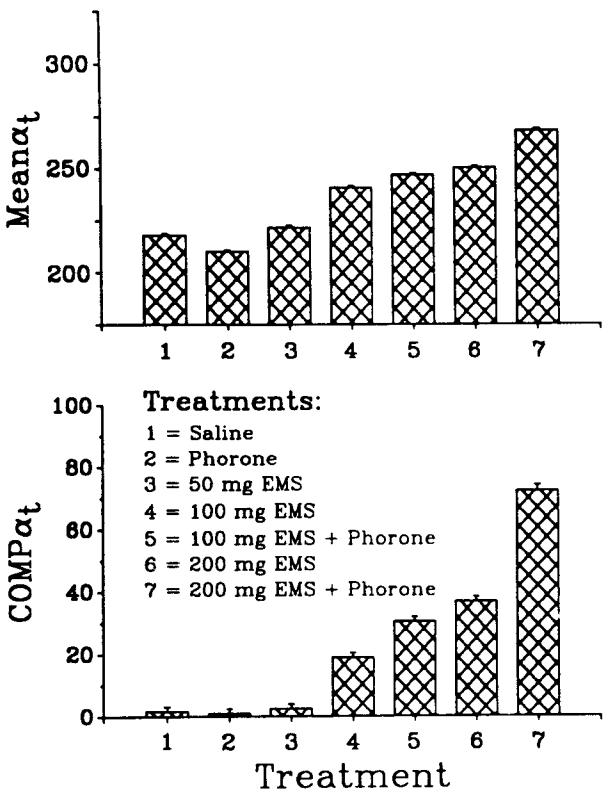


Fig. 5. Each bar represents the average ± SEM value of the effects of 7 different EMS and EMS + phorone treatments on caudal sperm $X\alpha_t$ and COMPα_t at 8 days. The most important significant differences are between the pairs of EMS and EMS + phorone at each treatment level. For $X\alpha_t$, every value is significantly different ($P < 0.01$) from every other value, while all values for 100 and 200 mg/kg EMS levels differed from each other and all lower treatments for COMPα_t ($P < 0.01$). Duplicate measurements were made on all samples from 7 rats/dosage (n = 14/dosage).

damage 8 to 15 days prior to recovery of caudal sperm indicates effects on those germ cells that were in late testicular stages or were caput epididymal sperm at the time of EMS treatment. It is in these mid to late spermatid stages that free −SH groups on protamines provide a maximal target for EMS alkylation, which is consistent with the hypothesis of Sega and Owens (9,12), discussed above. Based on the results of this study, we suggest that the first step in altered chromatin structure resulting from EMS alkylation and leading to increased susceptibility to DNA denaturation is the loss of quaternary structure, which is the same first step leading to genetic aberrations.

Figure 3 shows that COMPα_t is 40% at day 22, although the $X\alpha_t$ is approaching control levels. Thus, the SCSA is detecting an abnormality of chromatin structure manifested as an increased susceptibility of DNA to denaturation in situ at a time when dominant lethal effects have returned to near normal (17). According to Teaf and colleagues (17), cauda sperm are alkylated by EMS at a rate of approximately 5.2×10^6 ethylations per sperm head one day after EMS treatment, which is about 1/4 of the 20×10^6 ethylations at 8 and 15 days. Ethylations per sperm head then dropped to 2.3×10^6 at 22 days. Thus, alkylation likely occurs following EMS exposure at days 1 and 22, but the extent of binding is apparently not great enough to cause early embryo death. It should be noted that the mating intervals used by Teaf and colleagues were days 15 to 19 and days 22 to 26. Thus, effects that may be present at exactly day 22 are likely greater than the average of days 22 to 26. A recent study on effects of MMS on mouse sperm chromatin structure (13) showed that MMS exposure produced a COMPα_t of about 10% for cauda sperm on day 1, but nearly 100% of sperm had increased susceptibility to DNA denaturation by days 3 to 13 and a return to a 10% level by day 19.

Considering both our observations and those of Teaf and colleagues (17), we conclude that damage to chromatin structure seen at day 22 is due mostly to alkylation-induced damage to protamines resulting in changes and damage to quaternary and perhaps tertiary chromatin structure leading to an increased susceptibility to DNA denaturation. At the higher dosages of EMS exposure and/or optimal susceptible time periods, both protein alterations and DNA strand breaks could occur, with both events contributing to the increased susceptibility to DNA denaturation. Agents other than those that produce DNA strand breaks have been shown to cause increased susceptibility to DNA denaturation; these agents include methyl-benzimidazol-2-yl carbamate (inhibits tubulin polymerization and microtubule formation) (34), zeranol (a resorcylic acid lactone used as a growth stimulant) (35), and zinc-poor diets (36). These agents, or lack thereof, likely cause an abnormality of quaternary and tertiary structure of chromatin that leads to an increased susceptibility to DNA denaturation. Likewise, low levels of ethylation of sperm chromatin protamines may cause an abnormality leading to increased susceptibility to DNA denaturation, but the chromatin is not damaged sufficiently to cause dominant lethal mutations. It is also reasonable to speculate that alkylation-induced chromatin structure abnormalities without significant DNA damage could subsequently lead to DNA damage during sperm chromatin remodeling prerequisite to pronuclear formation in the oocyte (see 37 for review). The SCSA thus appears to be a more sensitive test for EMS-induced damage to

sperm nuclear chromatin than the dominant lethal mutation assay.

An additional objective of this study was to investigate the possible role of reproductive tract GSH in protecting against EMS-induced germ cell clastogenesis. A previous study (23) found that phorone (250 mg/kg) reduced testicular and epididymal GSH to 65% and 20% of control levels, respectively, with maximal reduction occurring after 2 to 4 h. Therefore, this study addressed the question of whether phorone-induced depletion of reproductive tract glutathione potentiates the chromatin structure abnormality induced by EMS, manifested as an increased susceptibility to DNA denaturation, in situ. The hypothesis that glutathione depletion would potentiate EMS damage was confirmed. Both EMS and phorone–EMS combination produced changes in chromatin structure in a dose- and time-dependent fashion. Depleting GSH prior to EMS treatment only increased the sensitivity of these stage-specific germ cells.

It is proposed that GSH functions as a local nucleophilic scavenger in reproductive tissues, which is similar to its role in other organs. Depression of GSH by phorone and other chemical depleters compromises this protective function; this reduction coupled with an increase in free EMS may be at least partially responsible for the effects observed in this study and in studies that detect increased dominant lethal mutations (17). During week 2 (day 8) and 3 (day 15), but not during week 1 after exposure to EMS, susceptible sperm are in the caput epididymis and rete testis. By the time sperm have reached the cauda epididymis, most or all the sites that might be ethylated are unavailable due to the normal formation of disulfide crosslinking and decrease in free $-SH$ sites during epididymal sperm maturation (15,17,19).

An unresolved question relative to the current experimental design regards the relative protective role of hepatic GSH versus reproductive tract GSH. The dose of phorone that lowered GSH at the site of EMS-induced clastogenesis (that is, rete testis and caput epididymis) also altered hepatic levels of GSH. Therefore, it is not possible to determine the relative contribution of liver metabolism in preventing distribution of EMS to the reproductive tract versus the role of target site GSH in protecting the developing germ cells. Experiments are in progress to address this issue.

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REFERENCES

1. Meister A, Anderson ME. Glutathione. Annu Rev Biochem. 1983;52:711–60.
2. Chasseaud LF. The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. In: Klein G, Weinhouse S. eds. Advances in cancer research; vol 29. New York: Academic Press; 1979:175–274.
3. Calvin HI, Turner SI. High levels of glutathione attained during postnatal development of rat testes. J Exp Zool 1982;219:389–93.
4. Hales BF, Hachey C, Robaire B. The presence and longitudinal distribution of the glutathione-*S*-transferases in rat epididymis and vas deferens. Biochem J. 1980;189:135–42.
5. Ehling UH, Cumming RB, Malling HV. Induction of dominant lethal mutations by alkylating agents in male mice. Mutat Res. 1968;5:417–28.
6. Soares ER, Crenshaw JW. Dominant lethal mutations induced in mice by orally administered ethyl methanesulfonate. Mutat Res. 1974;26:385–9.
7. Bishop JB, Kodell RL. The heritable translocation assay: its relationship to assessment of greater risk for future generations. Teratogenesis Carcinog Mutagen. 1980;1:305–22.
8. Anderson D, Hodge MCE, Palmer S, Purchase IFH. Comparison of dominant lethal and heritable translocation methodologies. Mutat Res. 1981;85:417–29.
9. Sega GA, Owens JG. Ethylation of DNA and protamine by ethyl methanesulfonate in the germ cells of male mice and the relevancy of these molecular targets to the induction of dominant lethals. Mutat Res. 1978;52:87–106.
10. Bellve AR, Anderson E, Hanley-Bowdoin L. Synthesis and amino acid composition of basic proteins in mammalian sperm nuclei. Dev Biol. 1975;47:349–65.
11. Balhorn R. A model for the structure of chromatin in mammalian sperm. Cell Biol. 1982;93:298–305.
12. Sega GA, Owens JG. Methylation of DNA and protamine by methyl methanesulfonate in the germ cells of male mice. Mutat Res. 1983;111:227–44.
13. Evenson DP, Jost LK, Baer RK. Effects of methyl methanesulfonate on mouse sperm chromatin structure and testicular cell kinetics. Environ Molec Mutagen. 1993;21:144–53.
14. Calvin HI, Yu CC, Bedford JM. Effects of epididymal maturation, zinc (II) and copper (II) on the reactive sulfhydryl content of structural elements in rat spermatozoa. Exp Cell Res. 1973;81:333–41.
15. Evenson DP, Baer RK, Jost LK. Flow cytometric analysis of rodent epididymal spermatozoal chromatin condensation and loss of free sulfhydryl groups. Mol Reprod Devel. 1989;1:283–8.
16. Teaf CM, Bishop JB, Harbison RD. Germ cell mutagenesis and GSH depression in reproductive tissue of the F-344 rat induced by ethyl methane-sulfonate. Mutat Res. 1985;144: 93–8.
17. Teaf CM, Bishop JB, Harbison RD. Depression of glutathione in male reproductive tissues and potentiation of EMS-induced germ cell mutagenesis by L-buthionine sulfoximine. Teratogenesis Carcinog Mutagen. 1987;7:497–513.
18. Teaf CM, Bishop JB, Harbison RD. Potentiation of ethyl methanesulfonate-induced germ cell mutagenesis and depression of glutathione in male reproductive tissues by 1,2-dibromoethane. Teratogenesis Carcinog Mutagen. 1990;10: 427–38.
19. Gandy J, Teaf CM, Adatsi FA, James RC, Harbison RD. The dependence of male reproductive toxicity on germ cell stage. In: Fujji T, Adams PM, eds. Functional teratogenesis: functional effects on the offspring after parental drug exposure. Tokyo: Teikyo University Press; 1987:133–43.

20. Gandy J, Bates HK, Conder LA, Harbison RD. Effects of glutathione enhancement and depletion on ethyl methanesulfonate-induced dominant lethal mutations in Sprague–Dawley rats. Teratogenesis Carcinog Mutagen. 1992;12:61–70.
21. Van Doorn R, Leijdekkers CM, Henderson PT. Synergistic effects of phorone on the hepatotoxicity of bromobenzene and paracetamol in mice. Toxicology. 1978;11:225–33.
22. Younes M, Sharma SC, Siegers CP. Glutathione depletion by phorone: organ specificity and effect on hepatic microsomal mix-function oxidase system. Drug Chem Toxicol. 1986;9:67–73.
23. Gandy J, Millner GC, Hudson KB, Casciano DA, Harbison RD. Effects of selected chemicals on the glutathione status in the male reproductive system of rats. J Toxicol Environ Health. 1990;29:45–57.
24. Evenson DP. Flow cytometry evaluation of male germ cells. In: Yen A, ed. Flow cytometry: advanced research and clinical applications; vol 1. Boca Raton, Florida: CRC Press; 1989:217–46.
25. Evenson DP. Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science. 1980;240:1131–3.
26. Darzynkiewicz Z, Traganos F, Sharpless T, Melamed M. Lymphocyte stimulation: a rapid multiparameter analysis. Proc Natl Acad Sci USA. 1976;73:2881–4.
27. Evenson DP. Flow cytometric analysis of male germ cell quality. In: Darzynkiewicz Z, Crissman H, eds. Methods in cell biology; vol 33: Flow cytometry. New York: Academic Press. 1990:401–10.
28. Evenson DP, Higgins PH, Grueneberg D, Ballachey BE. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry. 1985;6: 238–53.
29. Darzynkiewicz Z, Kapuscinski J. Acridine orange: a versatile probe of nucleic acids and other cell constituents. In: Melamed MR, Lindno T, Mendelsohn ML, eds. Flow cytometry and sorting, 2nd ed. New York: Wiley–Liss; 1990: 291–314.
30. Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol Reprod. 1987;36:915–25.
31. Darzynkiewicz Z, Traganos F, Sharpless T, Melamed M. Thermal denaturation of DNA in situ as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res. 1975;90:411–28.
32. SAS. SAS procedures guide, release 6.03 edition. Cary, North Carolina: SAS Institute; 1988.
33. Sega GA. Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulfonate. Proc Nat Acad Sci. 1974;71: 4955–9.
34. Evenson DP, Janca FC, Jost LK. Effects of the fungicide methyl-benzimidazol-2yl carbamate (MBC) on mouse germ cells as determined by flow cytometry. J Toxicol Environ Health. 1987;20:387–99.
35. Ballachey BE, Miller HL, Jost LK, Evenson DP. Flow cytometry evaluation of testicular and sperm cells obtained from bulls implanted with zeranol. J Anim Sci. 1986;63: 995–1004.
36. Evenson DP, Emerick RJ, Jost LK, Kayongo-Male H, Stewart SR. Zinc–silicon interactions influencing sperm chromatin integrity and testicular cell development in the rat as measured by flow cytometry. J Anim Sci. 1993;71: 955–62.
37. Perreault SD. Chromatin remodeling in mammalian zygotes. Mutat Res. 1992;296:43–55.