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Long-Term Effects of Triethylenemelamine Exposure on Mouse Testis Cells and Sperm Chromatin Structure Assayed by Flow Cytometry

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The toxic and potentially mutagenic actions of triethylenemelamine (TEM) on mouse body and testis weights, testicular cell kinetics, sperm production, sperm head morphology, and sperm chromatin structure were assessed in two experiments. The first experiment examined effects of four dose levels of TEM, assayed 1, 4, and 10 wk after toxic exposure. In the second study, effects from five dosage levels were measured at 1, 4, and 10 wk, and the highest dosage level was evaluated over 44 wk.

TEM produced an expected dose related loss of spermatogenic activity and subsequent recovery as determined by dual-parameter (DNA, RNA) flow cytometry (FCM) measurements of testicular cells. Both testicular weights and caudal sperm reserves remained generally below controls after 44 wk recovery following exposure to the highest (1.0 mg/kg daily × 5) dosage. Chromatin structure alterations, defined as increased susceptibility to DNA denaturation in situ, and sperm head morphology were highly correlated (.87–.93, $P<.001$) with dose and with each other. Data obtained from the sperm chromatin structure essay (SCSA) on fresh sperm was highly correlated with measurements of aliquots of the same sample collected over 44 wk, frozen, and then measured on the same day. Sperm head morphology and sperm chromatin structure remained abnormal at 44 wk for the 1.0 mg/kg TEM dosage, suggesting that the abnormalities, present long after the initial toxic response, may be a result of mutation. This study demonstrates that flow cytometry provides a unique, rapid, and efficient means to measure effects of reproductive toxins and potential mutagens.

Key words: spermatogenesis, sperm head morphology, DNA denaturation in situ, acridine orange

INTRODUCTION

Spermatogenesis is characterized by a very high rate of cell proliferation resulting in hundreds of millions of sperm produced daily within the extensive lengths of the testicular seminiferous tubules. In addition, mammalian spermatogenesis is characterized by unique events such as meiosis, exchange of somatic-like histones for sperm specific transition proteins [Grimes et al., 1977], and basic protamines [Balhorn, 1982], and shaping of species specific sperm head morphology. Thus, the mammalian testis can effectively serve as an environmental dosimeter for agents that may interfere with cell division and differentiation.

Several studies [Evenson et al., 1985, 1986a, 1989] have shown a correlation between chemical induction of mouse and rat sperm head morphology abnormalities and alteractions of sperm chromatin structure, defined as increased susceptibility to DNA denaturation in situ. Previous studies were limited to a recovery period of 4–10 wk. In this study mice have been exposed to triethylenemelamine (TEM), a potent alkylating agent, and recovery of spermatogenesis has been followed for 11 mo in an attempt to dissociate short-term from long-term effects including potential mutations.

TEM is a trifunctional alkylating agent with clastogenic effects in vivo and in vitro [Schmid, 1976]. Over three decades ago TEM was observed to induce dominant-lethal

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mutations [Cattanach and Edwards, 1958] and chromosome translocations in post-spermatogonial stages in the mouse [Cattanach, 1957] at doses that are low (1/100) relative to the approximate lethal dose [Vogel and Sobels, 1976]. The highest percentage of dominant lethals was produced from male mice exposed to TEM 11–14 days prior to mating with the developing sperm at the mid-spermatid stage [Hitotsumachi and Kikuchi, 1977]. Most of the resulting aberrations were chromosome types such as breaks and exchanges probably resulting from TEM-induced cross-links in the closely packed chromosomes of sperm. Although TEM is highly mutagenic to post-meiotic male germ cells, it has been reported to have relatively little mutational effect in spermatogonial stages [Cox and Lyon, 1975].

This study showed that exposure to TEM caused sperm alterations that persisted over the 44 weeks examined. Alterations included increased percentages of abnormally shaped sperm heads which were highly correlated with abnormal sperm chromatin structure measured by flow cytometry. Furthermore, this study showed a very high correlation of flow cytometry data obtained weekly from fresh samples versus frozen samples analyzed in a single day. The sperm chromatin structure assay was found to be reproducible for evaluating toxins of the male reproductive system.

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 4 wk 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 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 20, 24, 28, 32, 36, 40, and 44 wk after the last chemical exposure.

Chemical Exposure

Mice were randomly allocated to dosage groups of 0, 0.25, 0.50, 0.75, and 1.0 mg TEM/kg body weight for sampling at 1, 4, and 10 wk and 0 and 1.0 mg TEM/kg for the remaining weeks. Animals were exposed daily to fresh preparations of triethylenemelamine (TEM, Polysciences, Inc., Warrington, PA) for 5 consecutive days by i.p. injections with 0.5 ml Hanks' balanced salt solution (HBSS, vehicle) or HBSS plus TEM at the above listed concentrations. At every sampling period at least two control mice and three mice per dosage of TEM were sampled.

Testicular Germ Cells

Body weights were recorded immediately after cervical dislocation. Both testes were surgically removed, weighed, and 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 exactly 2.0 ml TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4) at 4°C. Each epididymis was sliced several times with a single edged razor blade 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, frozen at −20°C for 2 hr, −100°C overnight, and then stored in a liquid nitrogen tank until all samples were collected. All frozen samples of sperm were measured again by FCM at the same photomultiplier (PMT) settings on the same day.

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 using 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 $1–2 \times 10^6$/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 (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 permealize the cell membranes providing accessibility of DNA for AO staining. When excited by blue laser light, AO intercalated into native, double-stranded DNA fluoresces green (F_{530}); AO associated with single-stranded nucleic acid (DNA or RNA) emits red fluorescence ($F \geq_{600}$). The metachromatic 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., 1986a,b]. 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 [Evenson et al., 1985, 1989; Ballachey et al., 1987].

Sperm Chromatin Structure Assay

To distinguish it from other acridine orange staining protocols, the staining procedure described above has been termed the sperm chromatin structure assay (SCSA) when utilized with sperm. $SCSA_{acid}$ denotes the use of acid to denature DNA [Evenson, 1986, 1989]. 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., 1985]. Other studies utilizing heat ($SCSA_{heat}$) to denature sperm DNA produced results equivalent to acid denaturation [Evenson et al., 1985]. This study used the $SCSA_{acid}$ procedure only. Abnormal chromatin structure, defined as an increased susceptibility to acid- or heat-induced denaturation, is determined by FCM measurements of the shift from green (native DNA) to red (denatured, single-stranded DNA) fluorescence. This shift is expressed by alpha t (α_t) [Darzynkiewicz et al., 1975] and is the ratio of red to total (red + green) fluorescence. 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 cells outside the main population of α_t ($COMP\alpha_t$). Standard deviation of α_t describes the extent of chromatin structure abnormality within a population.

Flow Cytometric Measurements

Stained cells were measured in a Cytofluorograf II flow cytometer (Ortho Diagnostics, Inc., Westwood, MA) equipped with ultrasense optics and a Lexel 100 mW argon ion laser operated at 35 mW with an excitation wavelength of 488 nm. Dual parameter flow cytometry measurements were made to determine amounts of a) DNA vs. RNA in testicular cells, and b) double-stranded vs single-stranded DNA in sperm cells. Total fluorescence from each individual cell in the sample population was collected by an optical lens situated at right angles to the sample flow and laser beam. By use of dichroic mirrors and filters, fluorescent signals were separated into green (515–530 nm) and red (>600 nm) components. The computer calculated α_t values for each cell as well as α_t distribution values for each sample population. The stained samples are 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. The amplified and digitized signals were viewed live and brought back up from disk storage for analysis and displayed on the screen of the interfaced 2150 Data Handler computer (Ortho Diagnostic Systems, Inc.). Hard copies of the screen display were made with a Tektronics 4612 copier (Tektronics, Inc., Beaverton, Ore.).

Sperm Concentrations

Sperm concentrations were determined by both hemacytometer and FCM. To determine the sperm concentration by FCM, a known concentration (based on multiple hemacytometer counts) of fluorescent polystyrene beads (25% bright fluorospheres; Coulter Electronics, Hialeah, FL) was admixed with AO-stained sperm cells, and a cell:bead ratio obtained similar to that described by Stewart and Steinkamp [1982]. To ensure proper mixing, the bottle of beads was vigorously vortexed before an aliquot was removed for either hemacytometer or FCM counting methods. For hemacytometer counts, sperm cells were diluted in fixative consisting of 0.6 M $NaHCO_3$, 0.35% formalin, 0.85% NaCl.

Data Analysis

Correlation coefficients and means were computed using these procedures in the Statistical Analysis System [SAS, 1988]. Group means were tested using Duncan's Multiple Range Test within the ANOVA procedure of SAS.

RESULTS

Body and Testis Weights

1, 4, and 10 wk

At 1 wk post-treatment only the highest dose of 1.0 mg/kg TEM decreased the body weights significantly (17%; $P<.01$) (Fig. 1). Body weights were not affected at 4 wk and 10 wk. All TEM levels decreased ($P<.01$) testis weights 22–28% at 1 wk. Testis weights at 4 wk were significantly ($P<.01$) different from control. At 10 wk, .5 ($P<.05$), .75 ($P<.01$), and 1.0 ($P<.01$) mg TEM/kg decreased testis weights. Correlation coefficients of dose and testis weight were −.79, −.84, and −.92 (all $P<.001$) for 1, 4, and 10 wk, respectively.

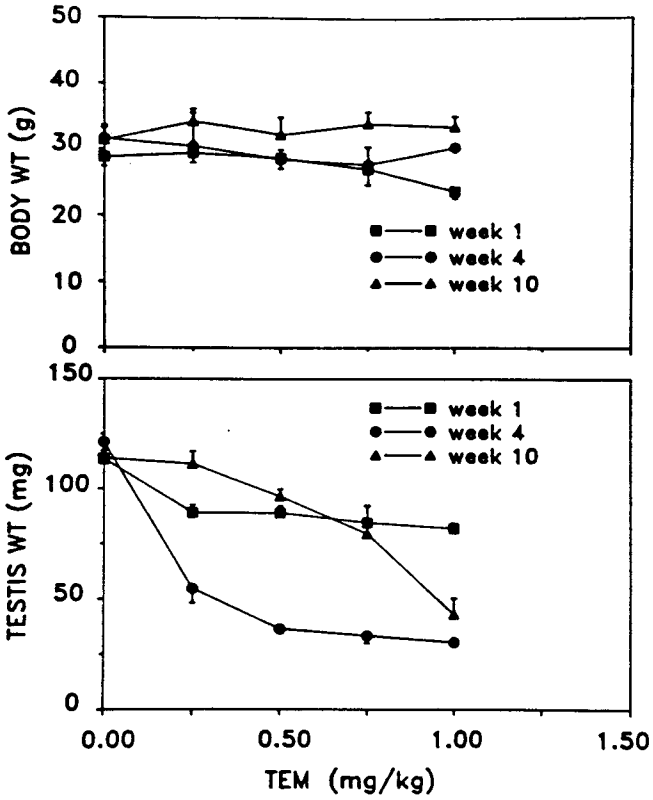

Fig. 1. Mean body and testis weights (± SD) of mice 1, 4, and 10 wk after exposure to 0, 0.25, 0.50, 0.75, and 1.0 mg/kg (daily × 5) TEM.

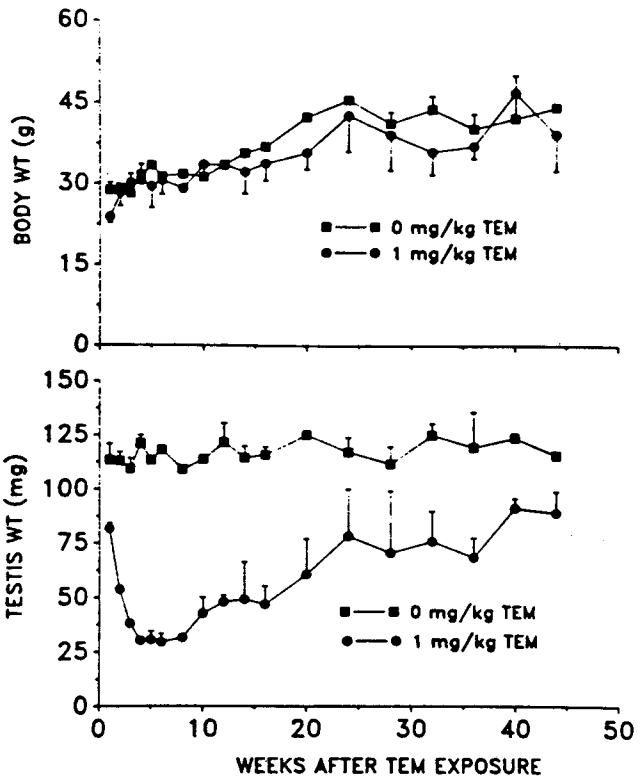

Fig. 2. Mean body and testis weights (± SD) of control mice and mice 1–44 wk after exposure to 1.0 mg/kg (daily × 5) TEM.

44 wk

As shown in Figure 2, body weights of mice exposed to 1.0 mg/kg TEM remained generally below corresponding control values over the 44 wk period. Testis weights dropped to 28% ($P<.01$) below control on wk 1 to 75% below from wk 4 to 6. Testis weights were still below, but nonsignificantly, control values on wk 44. Due to nonlinearity between dose and testis weight, the overall correlation of the two variables ($n=58$) is only .68 ($P<.001$). Correlation coefficients for wk 1–4 and 5–44 are −.96 and .81 (both $P<.001$), respectively.

Testicular Kinetics

Controls

As shown in Figure 3, dual parameter green (DNA) vs. red (RNA) fluorescence flow cytometry measurements of AO-stained mouse testicular cells easily resolved cell populations with tetraploid, diploid, and haploid DNA content; mouse diploid lymphocytes were used as a ploidy reference marker. 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 *stainability*) fluorescence, were discernible. Due to a loss of resolution between the video display and the hard copier, the clusters differing in red fluorescence are less obvious on the printout (Fig. 3) than on the display screen. Acridine orange stainability of tetraploid cell, diploid cell, and round spermatid DNA is very close to being proportional to the ploidy level [Evenson et al., 1986b]. However, spermatid stages occurring after the round spermatid stage have reduced DNA *stainability* resulting from chromatin condensation making DNA less accessible to the dye [Gledhill et al., 1966; Evenson et al., 1986b]. Elongating and elongated spermatids have reduced levels of red fluorescence due to loss of RNA during cell differentiation.

Photomultiplier tube gains set to display all 1n to 4n cells do not easily allow discrimination between round, elongating, and elongated spermatids. A software program produces an enhanced signal of the haploid cells permitting easy computer quantitation [Evenson et al., 1986a]; these cell types have been flow sorted and verified by light microscopy. The percentage of each different cell type identified by discrete clusters was determined by boxing each population on the computer screen and then using software that calculates the percentage of each cell type.

1, 4, and 10 wk

Flow cytometry measurements of testicular cells at 1, 4, and 10 wk after TEM exposure demonstrated that TEM

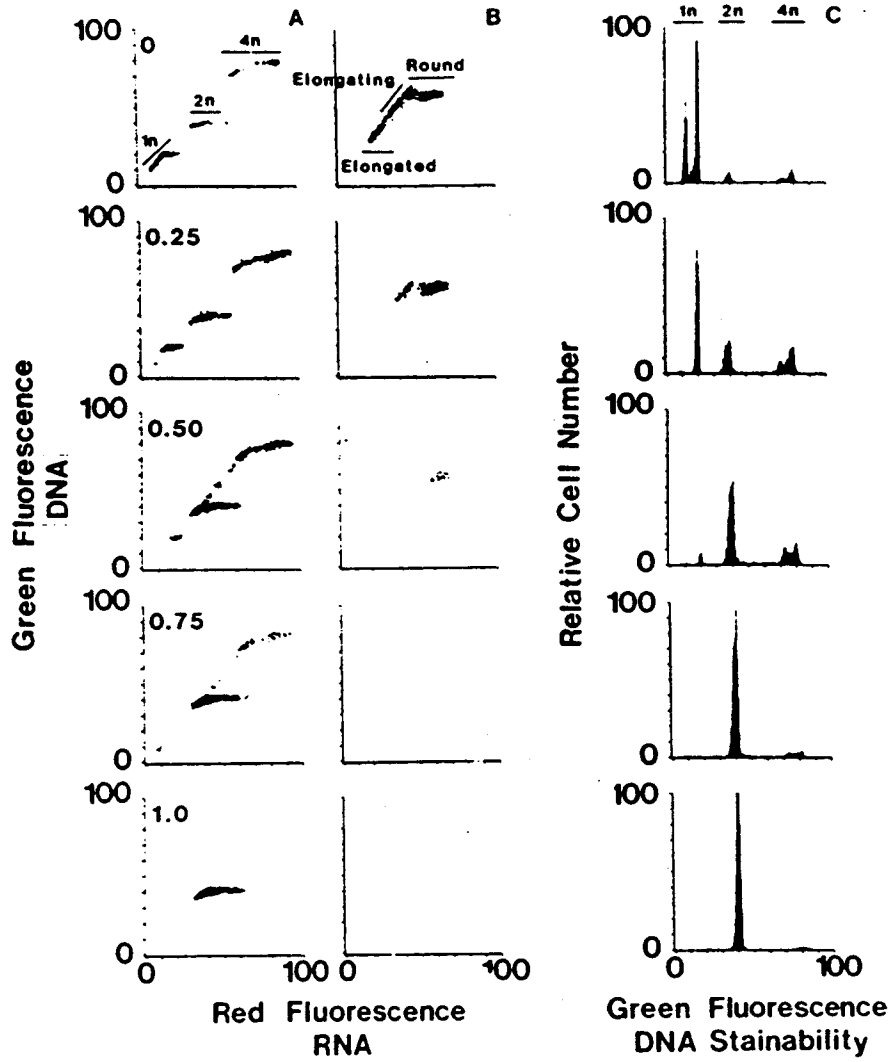

Fig. 3. Flow cytometry cytograms showing effect of TEM (0–1.0 mg/kg daily × 5) on DNA and RNA distributions of acridine orange stained (A) total testicular cells and (B) haploid cells from mice 4 wk after exposure. C: The corresponding frequency histograms of testicular cell DNA stainability.

severely affected the ratios of testicular cell types present (Fig. 3). A partial to almost complete loss of haploid and tetraploid cell types occurred with increasing dosage. Figure 4 is a plot of relative percent cell types present 1, 4, and 10 wk after exposure to 0–1.0 mg/kg. At 1 wk, the tetraploid population, normally about 5% of the total testicular germ cells, was eliminated (<1%, $P<.01$) by all TEM dosage levels, resulting in a relative increase in haploid cells while the diploid population remained fairly constant. Within the haploid population itself, round spermatids decreased ($P<.05$) with 0.25 mg/kg TEM.

At 4 wk after TEM exposure the ratios of testicular populations present were altered. All dosages significantly decreased ($P<.01$) the haploid population. The relative increase in percent diploid and tetraploid cells was likely due to the real decrease in percent haploid cells. With 0.25 mg/kg TEM, elongated spermatids ($P<.01$) and elongating spermatids ($P<.05$) were significantly decreased.

Ten weeks following exposure to 1.0 mg/kg TEM the percent haploid cells was reduced ($P<.01$) from control values. The percent elongated spermatids decreased significantly ($P<.01$) with exposure to 0.5 and 0.75 mg/kg TEM, but not 1.0 mg/kg TEM.

44 wk

1.0 mg/kg TEM eliminated the tetraploid population 1–3 wk following exposure (Fig. 5). This population was present again at 4 wk and stabilized by 5 wk. Haploid cells disappeared by wk 4 and returned to normal levels by wk 32. Within the haploid populations, round spermatid numbers were decreased by 1 wk and were 0% for wk 3 and 4.

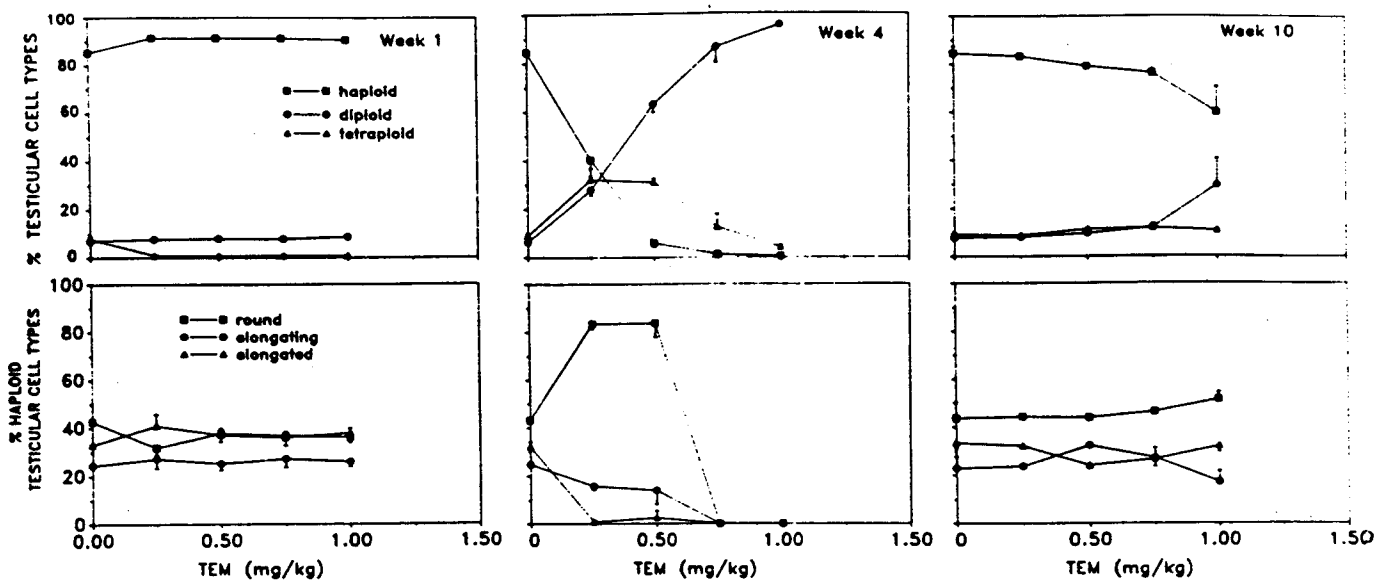

Fig. 4. Relative percentage testicular cell types present 1, 4, and 10 wk following exposure to 0–1.0 mg/kg TEM. **Top:** The percentage of haploid, diploid and tetraploid cell types. **Bottom:** The percentage of round, elongating, and elongated spermatids which compose the haploid population.

At wk 5 the haploid population, most of which were round spermatids, only composed 4% of the total testis population. Percent elongated spermatids remained low until wk 8. Routine histology of hematoxylin and eosin-stained sections of paraffin embedded testes showed that TEM affected all stages of spermatogenesis. By wk 36, the testes appeared to have returned to near normal with <5% tubules devoid of germ cell proliferation.

Effect of TEM on Sperm Chromatin Structure and Sperm Head Morphology

Figure 6 provides an example of raw SCSA data and computer generated α_t frequency histograms of sperm samples obtained from treated and untreated mice. The elongated green fluorescent (DNA) stainability signal is due to an artifact described by Gledhill et al. [1979] and has no effect on α_t values. Sample data from treated mice show some cells outside the main population of cells resistant to DNA denaturation (COMPα_t) with an increased red and concomitant decreased green fluorescence indicative of an increased sensitivity to DNA denaturation. Note in the frequency histograms that the difference between control and exposed sample is primarily a shift to increased α_t values with increased SDα_t.

Fresh Sperm at 1, 4, and 10 wk

At 1 wk following TEM exposure, no significant differences were detectable between controls and treated animals in SDα_t or percentage abnormal sperm head morphology

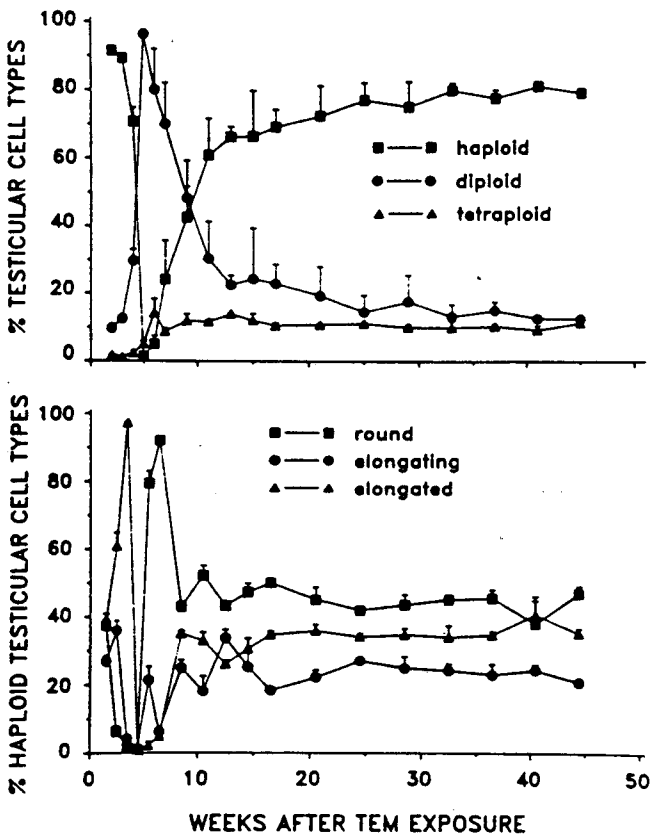

Fig. 5. Relative percentage testicular cell types present from 1 to 44 wk after exposure to 1.0 mg/kg TEM (daily × 5). **Top:** The percentage of haploid, diploid and tetraploid cell types. **Bottom:** The percentage of round, elongating, and elongated spermatids which compose the haploid population.

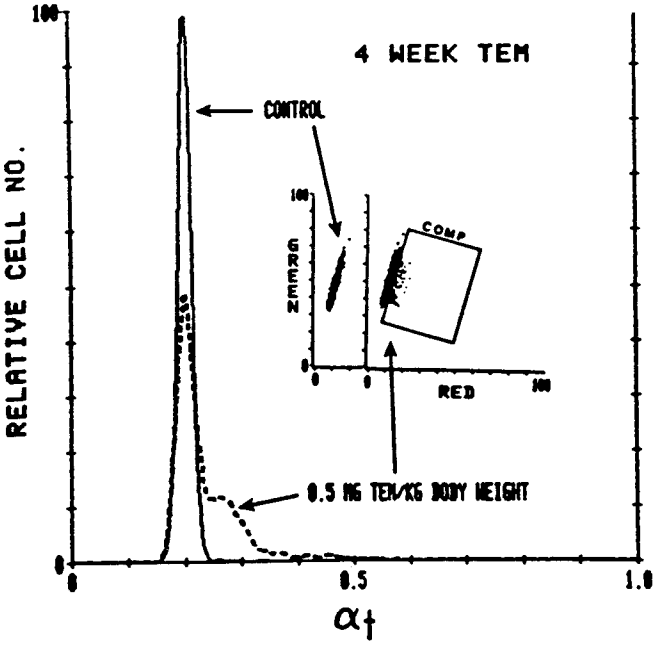

Fig. 6. Flow cytometric cytograms (inset) of green and red fluorescence distributions and corresponding α_t histograms of caudal sperm from a control mouse and a mouse exposed 4 wk earlier to 0.5 mg TEM/kg (daily × 5) measured by the SCSA. Sperm with an increased sensitivity to DNA denaturation correspond to the events in the COMP box and the skewed distribution of the α_t frequency histogram.

(ABN) (Fig. 7). By 4 wk all dosages caused significant ($P<.01$) elevations in both parameters. The $SD\alpha_t$ for all treatment levels was at least 2× greater ($P<.01$) than the control values. Note that the effect of 0.25 mg/kg TEM exposure was as great or greater than the higher dosages. At 10 wk, both curves rise in a more dose-response fashion. $SD\alpha_t$ for 0.75 and 1.0 mg/kg TEM dosages were significantly ($P<.01$) higher than control, and %ABN was 7 and 9× greater than the control for the same treatment levels. Previous studies [Evenson et al., 1985, 1986a,b] have shown SCSA measurements on isolated nuclei, with or without incubation with RNAse, do not significantly alter the results indicating that increased α_t values are not due to residual RNA. On a within-dose, across-week basis, correlations of %ABN with $SD\alpha_t$ were .00, .94, .91, .99, and .95 for 0, 0.25, 0.50, 0.75, and 1.0 mg/kg TEM, respectively.

Fresh Sperm Over 44 wk (1.0 mg/kg)

Figure 8A,B, and D shows the relationship between ABN, $SD\alpha_t$, and $COMP\alpha_t$ for fresh sperm. In contrast to controls, a sharp rise in all these curves occurred during the first 6–12 wk. The maximum $COMP\alpha_t$ occurred by 6 wk, whereas the maximum $SD\alpha_t$ occurred at 12 wk and the maximum %ABN at 8–12 wk.

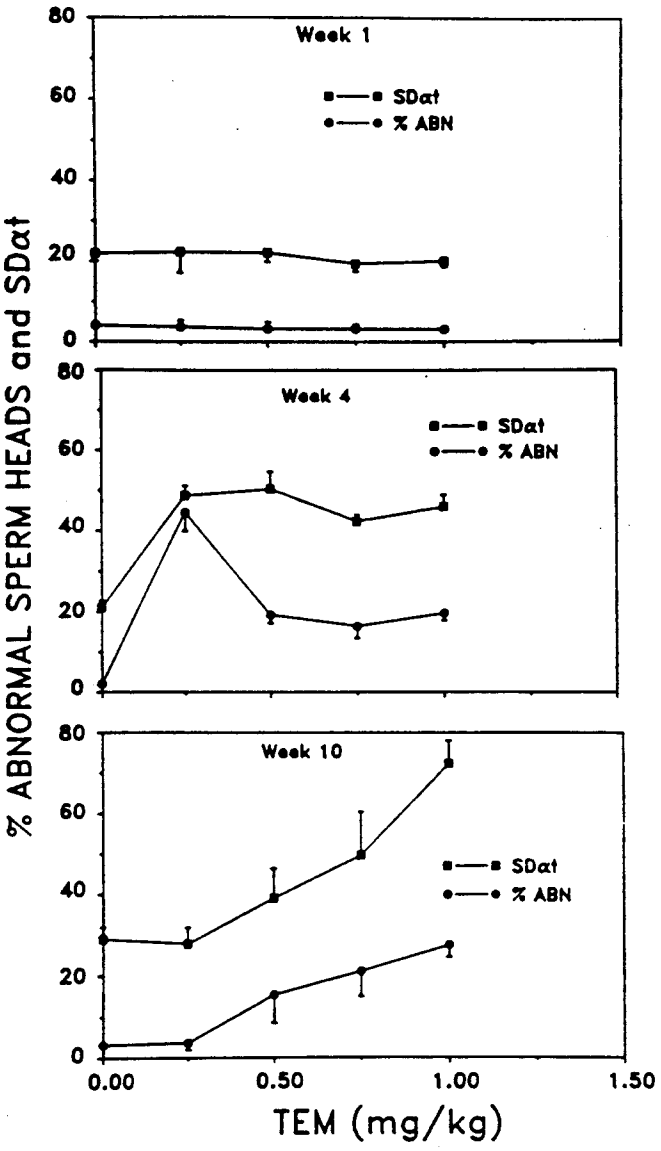

Fig. 7. Effect of 0, .25, .50, .75, and 1.0 mg TEM/kg body weight on percentage ABN and $SD\alpha_t$ at 1, 4, and 10 wk following exposure.

Frozen/Thawed Sperm

In order to assess the reliability of flow cytometric measurements of fresh samples collected on different days, an aliquot of each sperm sample, collected and measured as a fresh sample, was frozen and measured later at a single time period. Data shown in Figure 8C and E are very similar to data from weekly measurements (Fig. 8B,D, respectively). Note the very similar curve patterns with few points having any major differences. These data suggest that freezing samples had little to no effect on susceptibility of DNA to denature in situ. The data also show that the flow cytometer can be accurately calibrated many times over 44 wk. Correlations of all dose data between fresh and frozen sperm

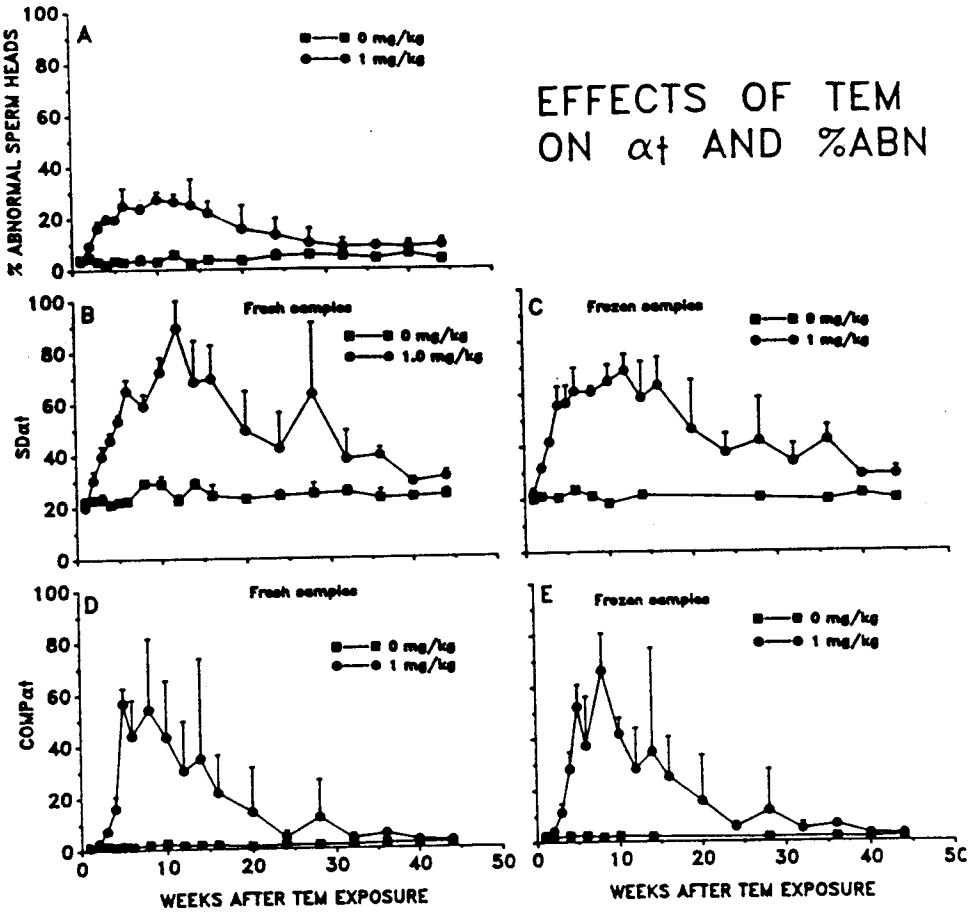

Fig. 8. Effects of 1.0 mg/kg (daily × 5) TEM on percentage ABN, $SD\alpha_t$ and $COMP\alpha_t$ over a 44 wk period. A shows %ABN values. B and D show SCSA values derived from fresh caudal sperm compared with C and E showing SCSA values from the same samples frozen, stored, thawed, and measured at one time period.

collected at 1, 4, and 10 wk for $SD\alpha_t$ and $COMP\alpha_t$ were .92 and .87, respectively ($P<.001$). Correlations of $SD\alpha_t$ and $COMP\alpha_t$ between fresh and frozen sperm for 1.0 mg/kg treated mice (n = 55) collected over 44 wk (no controls included) were .88 and .93, respectively (all $P<.001$).

Effect of TEM on Caudal Sperm Reserves

10 wk

Cauda sperm reserves in mice 10 wk after exposure were determined both by hemacytometer and flow cytometry; the correlation coefficient between the counts for the two methods was .97 ($P<.001$). Relative mean caudal reserves were 85, 87, 49, and 10% of controls for 0.25, 0.50, 0.75, and 1.0 mg/kg, respectively by FCM measurements. Table I shows high correlations between dose, ABN, $SD\alpha_t$ and cell counts by hemacytometer and flow cytometry.

TABLE I. Correlations Among TEM Dose, Abnormal Sperm Head Morphology (ABN), Standard Deviation of Alpha t ($SD\alpha_t$), and Caudal Sperm Reserves Counted by Flow Cytometry (FCM) and Hemacytometer (H)*

	DOSE	ABN	$SD\alpha_t$	FCM	H
DOSE		.91	.89	−.89	−.91
ABN			.92	−.87	−.85
$SD\alpha_t$				−.93	−.91
FCM					.97

*All $P<.001$.

44 wk (1.0 mg/kg TEM)

Caudal reserves were determined by flow cytometry and hemacytometer beginning at 5 wk recovery. The correlation of hemacytometer to flow cytometer values was 0.94. Figure 9 shows the relative caudal reserve in control and treated mice over this time period. The caudal reserve of mice treated with 1.0 mg/kg dosage level is decreased by 5 wk and begins to recover by wk 12.

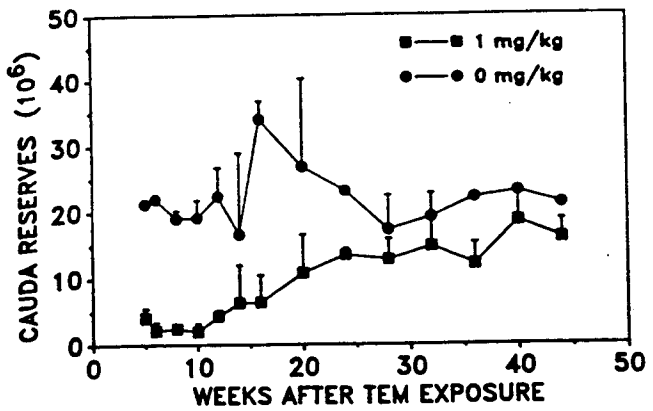

Fig. 9. Number of sperm from pairs of cauda epididymi of control mice and mice treated at the 1.0 mg/kg dosage level and diluted into 2.0 ml buffer. Each point represents the flow cytometric measured mean number of sperm (± SD) of >3 mice per time point over 44 wk.

DISCUSSION

TEM severely impaired mouse spermatogenesis with acute effects seen during the first and/or subsequent spermatogenic cycles and recovery proceeding thereafter. Exposure to 1.0 mg/kg daily × 5 days caused a 75% decrease in testis weight, which was reflected histologically by depletion of maturation stages of spermatogonia in the seminiferous tubules. Despite slight reductions in testes weights and cauda sperm reserves, testicular cell type ratios and histological patterns returned to an apparent normal pattern by 34–44 wk. However, both sperm head morphology and chromatin structure abnormalities remained elevated above controls. Since TEM has been shown to be mutagenic far below its toxic level [Matter and Generoso, 1974] these residual abnormalities may be a result of mutations.

This study demonstrated that the sperm chromatin structure assay can detect dose responses and provides data that are highly correlated with sperm head morphology abnormalities. This correlation has been observed in other studies [Evenson et al., 1985, 1986a, 1989]; and the data suggest measurements by FCM, which are more easily obtainable on large numbers of sperm, may be an alternative to sperm head morphology measurements, especially since the samples can be frozen or fixed [Evenson et al., 1985] and measured at a convenient time. Of significance is the high correlation between sperm samples measured weekly and those accumulated each week and then measured at one time period. This evidence shows that instrument settings over the 11 mo study period can be readjusted to provide repeatable measurements. COMPα_t values are relatively stable through minor instrument alterations, however, SDα_t values are very sensitive to outlying cells which strongly influence this parameter. Minor changes in photomultiplier tube (PMT) settings of both green and red fluorescence signals can cause significant shifts in SDα_t. We have observed in this study that setting the flow cytometer to the same SDα_t values for control sperm samples provides the necessary precision for high correlation between day-to-day measurements. Previously "quarter-bright" and half-bright" fluorescent beads (Coulter Electronics, Hialeah, FL) had been used to set the PMT gains to the same red and green fluorescent channel numbers [Ballachey et al., 1987]. Due to sensitivity of SDα_t measurements to slight changes in photomultiplier settings, frozen samples measured at a single time period likely provide more accurate information than fresh samples measured at a number of time periods. However, given the similarity of the two data sets, either method is valid. Frozen samples not only allow more flexibility in scheduling for FCM measurements but are more convenient to handle than fixed samples [Evenson et al., 1985].

The relationship among sperm head morphology abnormalities, chromatin structure abnormalities, and potential mutations remains unclear. The proportion of morphologically abnormal sperm (ABN) is known to vary dramatically among inbred lines of mice [Beatty and Sharma, 1960;Beatty 1972; Wyrobek, 1979; Krzanowska, 1981]. A lower incidence of ABN is observed in F_1 crosses relative to inbred parental lines [Krzanowska, 1976; Wyrobek, 1979], indicating the existence of heterosis for the trait. Sperm head morphology and nuclear chromatin structure assayed by the SCSA were compared on sperm obtained from a diallel cross of mice, in which some parental lines had naturally occurring high ABN [Ballachey et al., 1986]. Some sperm samples with high ABN (68%) had low COMPα_t (5%). Nevertheless, the positive correlation (.75, $P<.01$) obtained between the two measures indicates disruptions of spermatogenesis leading to abnormal sperm are associated with increased chromatin heterogeneity in a small proportion of sperm cells. As noted below, the main population could also contain a defect in chromatin structure that was not detectable by the level of physical stress used to potentially denature the DNA. Wyrobek et al. [1983] evaluated the relationship between changes in sperm morphology and germ cell mutagenicity by comparing the effects of 41 agents on mouse sperm shape to available data from three different mammalian germ-cell mutational tests (specific locus, heritable translocation, and dominant lethal). The mouse sperm morphology test was found to be highly sensitive to germ-cell mutagens with 100% of the known mutagens correctly identified as positives in the sperm morphology test. In this EPA Gen-Tox Report, Wyrobek et al. [1983] concluded that the mouse sperm morphology test has potential use for identifying chemicals that induce spermatogenic dysfunction and perhaps heritable mutations. Staub and Matter [1976, 1977] showed that all TEM-induced sterile translocation carriers had relatively high incidences of sperm abnormalities. On the other hand, sterile

non-translocation carriers had sperm abnormalities within the normal range. These authors concluded that there seemed to be no definite correlations between sperm abnormalities and presence of chromosome aberrations, ability to produce normal litters or incidence of dead implantations. Our studies [Evenson et al., 1985, 1986; Ballachey et al., 1987, 1988] on the relationship between sperm head abnormalities, sperm chromatin structure and fertility suggest the following relationships. First, a high correlation has been found between chemical-induced sperm head abnormalities and $SD\alpha_t$. Likewise in two studies [Ballachey et al., 1987, 1988] on bulls correlating these same parameters, a correlation (.58 and .94, $P<.01$) exists between fertility level and $SD\alpha_t$, but not with morphology. In these studies, $SD\alpha_t$ correlated closer to fertility than did $X\alpha_t$ and $COMP\alpha_t$. This observation suggests that the variation (SD) may reflect other unmeasured alterations of chromatin structure of cells in the main population. Increased DNA denaturing conditions—i.e., lower pH, increased heating, lower ionic strength buffers, etc.—would likely increase the α_t values, but whether these values would have a greater level of correlation with chemical dose or fertility is unknown. In other words, evidence from reproductive toxin and bull fertility studies suggest that sperm with abnormal chromatin structure and subfertility potential [Ballachey et al., 1987, 1988; Evenson et al., 1986a] likely exist in the "main" population to the extent that the abnormality is reflected in the variation of the α_t distribution.

This study demonstrates the usefulness of flow cytometry to assess genotoxin action on spermatogenesis. Dual-parameter (DNA,RNA) measurements of testicular cell suspensions can be accomplished within a few minutes and provide information on reduction or elimination of various cell types. This study as well as another more extensive study (manuscript in preparation) has shown that flow cytometry provides a very rapid, accurate, and efficient assessment of sperm concentration that is much less time consuming than use of a hemacytometer and also discriminates between sperm cells and debris in the sample. Finally, flow cytometry is the only practical means to measure increased susceptibility to DNA denaturation in situ, which is a measure of toxin interference with normal spermatogenesis.

REFERENCES

Balhorn R (1982): A model for the structure of chromatin in mammalian sperm. J Cell Biol 93:2998–2305.

Ballachey BE, Evenson DP, Saacke R (1988): The sperm chromatin structure assay relationship with alternate tests of sperm quality and heterospermic performance of bulls. J Androl 9:109–115.

Ballachey BE, Hohenboken WD, Evenson DP (1986): Sperm head morphology and nuclear chromatin structure evaluated by flow cytometry in a diallel cross in mice. Can J Genet Cytol 28:954–966.

Ballachey BE, Hohenboken WD, Evenson DP (1987): Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol Reprod 36:915–925.

Beatty RA (1972): The genetics of size and shape of spermatozoan organelles. In Beatty RA, Gluecksohn-Waelsh (eds). "Proceedings of the International Symposium: The Genetics of the Spermatozoan." Edinburgh: University of Edinburgh, pp 97–115.

Beatty RA, Sharma KN (1960): Genetics of gametes. III. Strain differences in spermatozoa from eight inbred strains of mice. Proc R Soc Edinburgh 68:25–53.

Cattanach BM (1957): Induction of translocations in mice by triethylenemelamine. Nature 180:1364, 1365.

Cattanach BM, Edwards RG (1958): The effects of triethylenemelamine on the fertility of male mice. Proc R Soc Edinburgh B 67:54–64.

Cox BD, Lyon MF (1975): The mutagenic effect of triethylenemelamine (TEM) on germ cells of male golden hamsters and guinea pigs. Mutat Res 30:293–298.

Darzynkiewicz Z (1979): Acridine orange as a molecular probe in studies of nucleic acids *in situ*. In Melamed M, Mullaney P, Mendelsohn M (eds): "Flow Cytometry and Sorting." New York: John Wiley & Sons, pp 285.

Darzynkiewicz Z, Traganos F, Sharpless T, Melamed M (1975): Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res 90:411–428.

Darzynkiewicz Z, Traganos F, Sharpless R, Melamed MR (1976): Lymphocyte stimulation: A rapid multiparameter analysis. Proc Natl Acad Sci USA 73:2881–2884.

Evenson DP (1986): Flow cytometry of acridine orange stained sperm is a rapid and practical method for monitoring occupational exposure to genotoxicants. In Sorsa M, Norppa H (eds): "Monitoring of Occupational Genotoxicants." New York: Alan R. Liss, Inc., pp 121–132.

Evenson DP (1989): Flow cytometry evaluation of male germ cells. In Yen A (ed): "Flow Cytometry: Advanced Research and Clinical Applications," Volume I. CRC Press (in press).

Evenson DP, Baer RK, Jost LK, Gesch RW (1986a): Toxicity of thiotepa on mouse spermatogenesis as determined by dual-parameter flow cytometry. Toxicol Appl Pharmacol 82:151–163.

Evenson DP, Darzynkiewicz Z, Jost L, Janca F, Ballachey B (1986b): Changes in accessibility of DNA to various fluorochromes during spermatogenesis. Cytometry 7:45–53.

Evenson DP, Higgins PH, Grueneberg D, Ballachey BE (1985): Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 6:238–253.

Evenson DP, Janca FC, Jost LK, Baer RK (1989): Flow cytometric analysis of effects of 1,3-Dinitrobenzene on rat spermatogenesis. J Toxicol Envir Health (in press).

Evenson DP, Melamed MR (1983): Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J Histochem Cytochem 31:248–253.

Gledhill BL, Gledhill MP, Rigler R, Jr, Ringertz NR (1966): Changes in deoxyribonucleoprotein during spermiogenesis in the bull. Exp Cell Res 47:652–665.

Gledhill BL, Lake S, Dean PN (1979): Flow cytometry and sorting of sperm and other male germ cells. In Melamed MR, Mullaney PF, Mendelsohn ML (eds): "Flow Cytometry and Sorting." New York: John Wiley and Sons, pp 471–484.

Grimes SR, Meistrich ML, Platz RD, Hnilica LS (1977): Nuclear protein transitions in rat testis spermatids. Exp Cell Res 110:31–39.

Hitotsumachi S, Kikuchi (1977): Chromosome aberrations and dominant lethality of mouse embryos after paternal treatment with triethylenemelamine. Mutat Res 42:117–124.

Krzanowska H (1976): Inheritance of sperm head abnormality types in mice—the role of the Y chromosome. Genet Res 28:189–198.

Krzanowska H (1981): Sperm head abnormalities in relation to the age and strain of mice. J Reprod Fertil 62:385–392.

Matter BE, Generoso WM (1974): Effects of dose on the induction of

dominant-lethal mutations with triethylenemelamine in male mice. Genetics 77:753–763.

Monesi V (1965): Synthetic activities during spermatogenesis in the mouse: RNA and protein. Exp Cell Res 39:197–224.

SAS (1988 Version 5.18): "SAS User's Guide." Cary, NC: SAS Institute Inc.

Schmid W (1976): The micronucleus test for cytogenetic analysis. In Hollaender A (ed): "Chemical Mutagens, Principles and Methods for Their Detection," Vol 4. New York: Plenum Press, pp 31–53.

Staub JE, Matter BE (1976, 1977): Heritable reciprocal translocations and sperm abnormalities in the F_1-offspring of male mice treated with triethylenemelamine (TEM). Arch Genet 49/50:29–41.

Stewart CC, Steinkamp JA (1982): Quantitation of cell concentration using the flow cytometer. Cytometry 2:238–243.

Vogel E, Sobels FH (1976): The function of *Drosophila* in genetic toxicology testing. In Hollaender A (ed): "Chemical Mutagens, Principles and Methods for Their Detection," Vol 4. New York: Plenum Press, pp 93–142.

Wyrobek AJ (1979): Changes in mammalian sperm morphology after X-ray and chemical exposure. Genetics 92:s105–s119.

Wyrobek AJ, Bruce WR (1975): Chemical induction of sperm abnormalities in mice. Proc Natl Acad Sci USA 72:4425–4429.

Wyrobek AJ, Gordon LA, Burkart JG, Francis MW, Kapp RW, Jr, Letz G, Malling HV, Topham JC, Whorton MD (1983): An evaluation of the mouse sperm morphology test and other sperm tests in nonhuman mammals: A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat Res 115:1–72.