FLOW CYTOMETRIC ANALYSIS OF EFFECTS OF 1,3–DINITROBENZENE ON RAT SPERMATOGENESIS

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Exposure of 100-d old rats to 1,3-dinitrobenzene (m-DNB) at dosages up to 48 mg/kg resulted in disruption of spermatogenesis as measured by flow cytometry (FCM) of acridine orange-stained sperm and testis cells. One day (d 1) after a single exposure to 48 mg/kg m-DNB, FCM measurements of caput epididymal fluid cells demonstrated the presence of testicular germinal epithelial cells apparently sloughed off into the epididymis. Also, at d 1 after the same exposure, a decrease in pachytene spermatocytes was observed. By d 16 after exposure to 32 or 48 mg/kg, testicular damage was evidenced by an alteration of cell type ratios in FCM-analyzed populations of testicular cells. Extensive recovery of cell type ratios occurred by d 32. At d 16, dosages of 32 and 48 mg/kg caused alterations of sperm chromatin structure as determined by the flow cytometric sperm chromatin structure assay (SCSA); 48 mg/kg caused alterations at both d 16 and d 32.

Exposure to m-DNB caused a dose response increase in percent sperm head morphology abnormalities (%ABN) assessed in cauda epididymal and vas sperm. A slightly higher correlation existed between dose and SCSA α_t values (d 16, .78; $p < .01$) than between dose and %ABN (d 16, .70; $p < .01$). Also, a higher correlation existed between standard deviation of α_t ($SD\alpha_t$) values and %ABN (.97; $p < .01$) than between dose and %ABN (.70; $p < .01$).

This study demonstrated rapid and unique FCM procedures originally derived for reproductive toxicology studies in mice to be equally useful for studies in rats.

INTRODUCTION

1,3-Dinitrobenzene (m-DNB) is a chemical intermediate used in the synthesis of various dyes and manufacture of plastics and explosives. The chemical is readily absorbed through the skin (Ishihara et al., 1976) and its vapors are very toxic (Hamblin, 1963). The LD50 of m-DNB for rats is 83 mg/kg (Cody et al., 1981); for humans the probable LD50 is 5–50 mg/

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kg (Gosselin et al., 1984). Exposure to m-DNB causes formation of methemoglobin leading to cyanosis and hypoxia (Beard and Noe, 1981). Other symptoms in humans are reduced vision, hearing loss, and ringing ears (NIOSH, 1977).

m-DNB is a potent testicular poison in laboratory animals. Subchronic exposure to m-DNB by oral gavage caused decreased sperm production at a dosage of 1.5 mg/kg·d and produced infertility at 3 mg/kg·d (Linder et al., 1986). Testicular atrophy was noted in rats after subchronic exposure to m-DNB in drinking water providing about 2.6–13 mg/kg·d (Cody et al., 1981). A single oral dose has been shown to cause marked rat testicular changes (Blackburn et al., 1988; Hess et al., 1988), and reduced sperm quantity and quality as well as fertilizing ability (Linder et al., 1988). Histological studies of acutely exposed rats (Hess et al., 1988) as well as in vitro studies (Foster et al., 1987; Blackburn et al., 1988) suggest that the chemical's primary mode of action is likely on Sertoli cells, which lose their ability to protect maturing sperm. Degenerative changes in pachytene spermatocytes and Sertoli cells have been reported within only 24 h of exposure (Blackburn et al., 1988; Hess et al., 1988).

Using flow cytometric measurements, recent studies (Evenson et al, 1985, 1986, 1987) have characterized alterations of mouse testicular cells and sperm cells induced by toxic chemicals. The purpose of the present work was to determine whether FCM procedures previously used to characterize mouse germ cells, particularly those having sperm chromatin structure altered by toxic chemicals, could be used to determine toxic effects of m-DNB in rats.

MATERIALS AND METHODS

Experimental Animals

Sixty-day-old male Sprague-Dawley rats (Sasco Inc., Omaha, Neb.) were acclimatized to our facilities for 40 d prior to treatment. Animals were fed an ad libitum diet of Wayne Rodent Blox (Continental Grain Co., Chicago, Ill.) and deionized water. Rats were housed individually in 7 × 9 1/2 × 7 in. stainless steel wire mesh cages suspended on mobile racks (Hazelton Systems, Inc., Aberdeen, Md.), maintained at constant room temperature (21 ± 2°C), and kept on a 0700–1900-h lighting schedule.

Chemical Treatment

At 100 d of age, rats were weighed and randomly assigned to 5 treatment groups (16 animals per group). A stock solution of m-dinitrobenzene (m-DNB: Sigma Chemical Co., St. Louis, Mo.) was prepared in certified 99.5 mol % pure acetone (Fisher Scientific, Fair Lawn, N.J.) at a final concentration of 600 mg/ml. Serial dilutions of stock m-

DNB solution were made in corn oil (Sigma Chemical Co., St. Louis, Mo.). Each animal received a single oral dose (2.5 ml) of m-DNB-corn oil solution delivering either 0, 8, 16, 32, or 48 mg m-DNB/kg body weight. Doses were administered by oral intubation using an 18-gauge 1 1/2-in. curved feeding needle with 2 1/4-mm ball tip (Popper and Sons, New Hyde Park, N.Y.). Dosages were based on mean population weight (301 ± 18 g).

Tissue Sampling

Four animals from each dosage group were killed on d 1, 4, 16, and 32 posttreatment by cervical dislocation. Times were selected to (a) detect early (d 1) testicular changes and follow these through the epididymis, (b) determine incidence of abnormal cell types, and (c) look for evidence of recovery. Germinal cells from testes, caput epididymides (caput), and cauda epididymides (cauda) tissues were sampled from each animal. Body weights were recorded on d 1, 4, 16, and 32 after treatment, while on d 16 and 32 testis weights were recorded and vas deferens (vas) sperm were sampled.

Cell Preparation

Excised testes were stripped of the tunica albuginea and the approximate middle third of one testis from each rat was minced in 2 ml Hanks balanced salt solution (HBSS, Gibco Laboratories, Grand Island, N.Y.). Minced testes tissues were pipetted into 12 × 75-mm conical-bottom polystyrene tubes (Fisher Scientific, Pittsburgh, Pa.), tissue clumps were allowed to settle, and cell suspensions were filtered through 53-μm Swiss nylon monofilament filters (Tetko Inc., Elmsford, N.Y.). All procedures were performed at 4°C.

Caput epididymides were separated from testes, blotted dry, and slit lengthwise with single-edged razor blades. Ductus epididymis contents were gently washed into suspension with HBSS (4°C) and a sample was retained for analysis by flow cytometry (FCM) and by light microscopy. The remaining cell suspension was admixed with an equal volume of ultrapure glycerol (Bethesda Research Laboratories, Gaithersburg, Md.), cooled to −20°C for 2 h, and stored at −100°C.

Vas deferens (vas) from animals killed at d 16 and 32 were placed into 2.0 ml of HBSS upon excission, and sperm were extruded from them by pressing a blunt probe along the length of the ductus. "Cords" of sperm were transferred to 12 × 75 mm plastic tubes, and samples of sperm suspensions were retained for microscopic examination and FCM measurements. Vas samples for FCM were sonicated but not treated with RNAse prior to measurement.

Microscopy

Unstained samples of cells recovered from caput, cauda, and vas (suspended in buffer) were placed on glass slides, coverslipped, and immediately examined by phase-contrast microscopy to determine the presence of immature and/or unusual cell types. Two aliquots of whole caput and vas sperm suspensions from each rat were stained with an equal volume of 1% (w/v) aqueous eosin-Y, smeared on glass slides, and air dried. Slides were dipped into methanol to remove excess stain and mounted with coverslips using Permount (Fisher Scientific, Fair Lawn, N.J.). Phase-contrast optics were used with a ×100 oil-immersion objective lens to score 1000 sperm heads for morphological abnormalities. The heads were scored as normal or abnormal without subclassification of shape.

Sonication and RNAse Digestion

A portion (1.5 ml) of the frozen caput sperm suspended in HBSS and glycerol was thawed, transferred to a 16 × 125 mm Falcon tube (Dickinson and Co., Oxnard, Calif.), and immersed in an ice-water slurry (4°C). The sperm suspension was then sonicated to separate sperm heads from tails using a 3/8-in. diameter sonic probe attached to a Biosonik IV ultrasonic homogenizer (VWR Scientific, San Francisco, Calif.). Cells were sonicated (30 s power, 30 s cooling, 30 s power) at a power setting of 50-low, which disrupts all somatic cells, separates sperm heads from tails and breaks most tails. Two-hundred-microliter aliquots of sonicated sperm suspension were incubated 30 min (22°C) with 3000 units RNase A/ml (Cooper Biomedical, Malvern, Pa.) as previously described (Darzynkiewicz et al., 1979) and kept at 4°C until FCM measurements.

Cell Staining

Testicular Cells Two hundred microliters of the testicular cell suspensions, containing approximately 2×10^5 cells, was combined with 400 μl solution of 0.08 *N* HCl, 0.15 *M* NaCl, and 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.), pH 1.2. After 30 s, 1.20 ml of acridine orange (AO, chromatographically purified; Polysciences, Warrington, Pa.) staining solution [6 μg AO/ml of buffer containing 0.2 *M* Na_2HPO_4, 0.1 *M* citric acid buffer (pH 6.0), 1m*M* EDTA, 0.15 *M* NaCl] was admixed with the samples according to the procedure of Darzynkiewicz et al. (1976). Triton X compromises cell membrane integrity, which facilitates entry of dye molecules, while low pH dissociates histones from DNA in histone-containing testicular cells (Evenson et al., 1980b). Excitation by blue laser light (488 nm) causes AO molecules intercalated into double-stranded (ds) nucleic acid to fluoresce green (F_{530}) (Lerman, 1963) and those bound to single-stranded (ss) DNA or RNA to fluoresce red ($F_{>630}$) (Bradley and Wolf, 1959).

Epididymal Sperm Nuclei The same AO staining procedure as

above, termed the sperm chromatin structure assay (SCSA; Evenson, 1986), was used to stain epididymal sperm nuclei. In contrast to somatic cells, the low pH apparently causes partial denaturation of DNA in abnormal sperm chromatin to the same extent observed previously with thermal denaturation (Evenson et al., 1980a, 1985, 1986). Two-hundred-microliter aliquots of buffer containing about 2×10^5 whole unsonicated, sonicated, or sonicated plus RNAse-digested epididymal sperm were stained as above with AO.

Flow Cytometry

Immediately after AO staining, flow cytometric analysis of testicular cells and epididymal sperm samples was performed. At a flow rate of about 200 cells/s, samples were passed through the quartz flow channel of a Cytofluorograf II interfaced to a 2150 Data Handler (Becton Dickinson, Westwood, Mass.) and a Tektronics 4612 hard copier (Tektronics, Inc., Beaverton, Ore.) The Lexel 100-mW laser delivered 35 mW at 488 nm. Recorded measurements of 5×10^3 cells/sample began 3 min after staining. Red ($F_{>630}$) fluorescence and green (F_{530}) fluorescence emitted from each cell were separated optically, and the digitized signals were recorded in list mode on the computer disk.

The extent of DNA denaturation in sperm nuclei was quantitated for each cell and expressed as α_t [= red/(red + green) fluorescence] (Darzynkiewicz et al., 1975). The variation of α_t, expressed as standard deviation of α_t ($SD\alpha_t$), has previously shown an excellent correlation with extent of toxic insult to germ cells (Evenson, 1989; Evenson et al., 1985, 1986, 1987, 1989).

Data Analysis

Data were analyzed using the general linear models procedure (GLM) contained in the Statistical Analysis System (SAS; Goodnight et al., 1988). Independent variables were dose m-DNB, rat nested within dose, day posttreatment, and the interaction of dose with day. Rat nested within dose was used as the error term for testing the effect of dose. The residual was used to test the effect of rat within dose, day, and the interaction of dose with day. Data for some dependent variables were transformed, and the method of Kirk (1982) was used for selection of the appropriate transformation. Round spermatid, early pachytene spermatocyte, and abnormal caput epididymis sperm data were transformed using $\log_{10}$ (observation), diploid testicular cell data were transformed using 1/observation, elongating spermatid data were transformed using $\sqrt{\text{observation}}$, and abnormal vas deferens sperm data were transformed using 1/(observation + 1). Results are reported as least-square means ± standard errors; transformed means are reported as such unless otherwise indicated. Preplanned comparisons of treatment to control values for all variables were performed using orthogonal contrasts.

RESULTS

Body and Testis Weights

Rat body weights were not significantly affected by dose, rat nested within dose, or the interaction of dose with day posttreatment. Body weight progressively increased with time, being significantly greater on d 16 and 32 than on d 1 and 4 (364.5 ± 5.3 g and 409.8 ± 5.9 g vs. 299.7 ± 5.3 g and 301.3 ± 5.5 g, respectively; $p < .01$).

Rat testis weight did not differ significantly from control at 16 or 32 d posttreatment with 8, 16, or 32 mg m-DNB/kg. Significantly lower than control testis weights resulted on d 16 and 32 with 48 mg m-DNB/kg (Table 1).

Testicular Cells

The ratios of cell types present in minced testicular samples were determined using the seven distinct clusters of AO-stained cells, shown for example in Fig. 1.

Tetraploid Cells The proportion of total tetraploid testicular cells relative to other testicular cells in any dose-day subclass did not significantly differ from their respective control values. Of the variations that did occur within the population of tetraploid cells (Fig. 1, boxes 1 and 2), the percentage in box 2 was significantly reduced compared to control at d 1 and 16 following exposure to 32 or 48 mg m-DNB/kg (Fig. 2). The population of cells occupying box 2 has been previously identified as pachytene spermatocytes (Evenson et al., 1986).

Diploid Cells Relative percentages of diploid testicular cells varied significantly with dose-day subclass. At d 1 posttreatment, no dosage of m-DNB resulted in diploid cell levels that differed significantly from control (Fig. 3a). Levels of diploid cells significantly greater than control were evident at d 4 and 16 with 32 and 48 mg m-DNB/kg. At d 32, only the 48 mg/kg dose resulted in greater than control value for diploid cells.

Haploid Cells The percentage of total haploid testicular cells ap-

TABLE 1. Testicular Weights of Rats at 16 and 32 Days after Exposure to 0, 8, 16, 32, and 48 mg m-DNB/kg Body Weight[a]

	Day	
Dose (mg/kg)	16	32
0	3.21 ± 0.17	3.92 ± 0.17
8	3.62 ± 0.17	3.46 ± 0.17
16	3.64 ± 0.17	3.71 ± 0.17
32	2.81 ± 0.17	3.75 ± 0.17
48	1.89 ± 0.17[a]	3.16 ± 0.17

[a]Significance: $^{**}p < .01$.

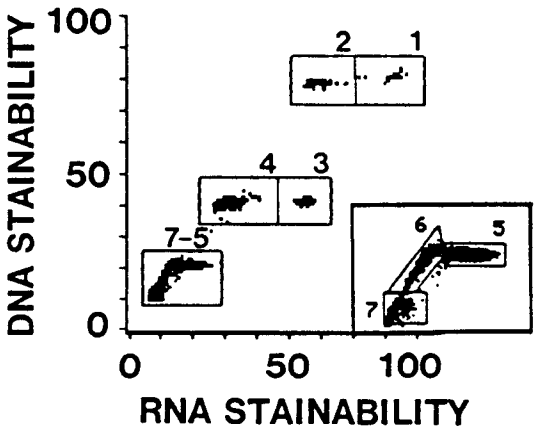


FIGURE 1. Dual-parameter (DNA vs. RNA) FCM distribution of AO-stained control rat testicular cells. Boxes 1,2 and 3,4 correspond to tetraploid and diploid cells with differing RNA contents, respectively. Diploid levels were determined by measuring AO-stained blood lymphocytes. Boxes 5, 6, and 7 correspond to round, elongating, and elongated spermatids, respectively; the inset is a computer amplification of the signals to enhance resolution between the subsets of haploid spermatids.

peared to respond inversely to m-DNB treatment relative to diploid cells. On d 16 after treatment with 32 and 48 mg/kg m-DNB, levels of haploid testicular cells significantly lower than control were observed (Fig. 3*b*).

Round Spermatids No significant differences existed between treatment groups and their respective controls for levels of round spermatids on d 1 or 4 posttreatment for any dosage of m-DNB administered (Fig.

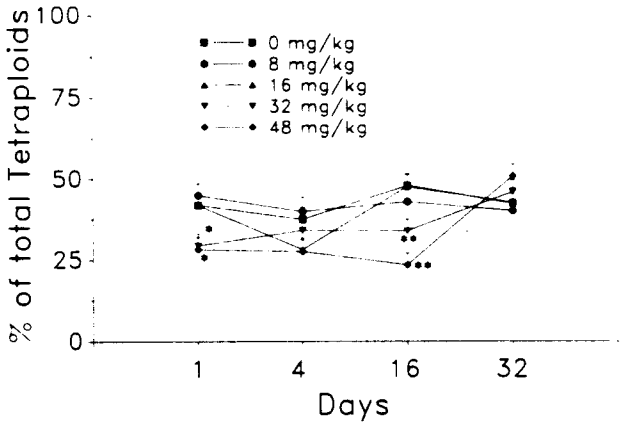


FIGURE 2. Low-RNA-containing tetraploid cells (pachytene spermatocytes) as a percent of total tetraploid cells, as a function of dose and time after exposure to m-DNB. Significance: $*p < .05$, $**p < .01$. These cells are represented in box 2 of Fig 1.

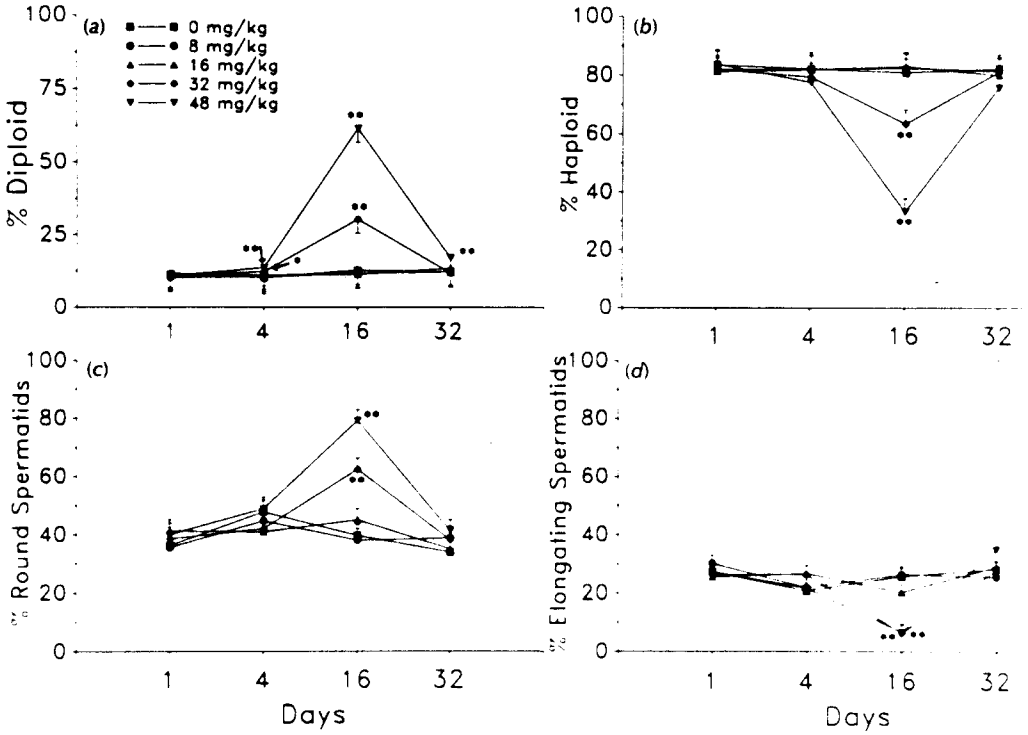


FIGURE 3. *Relative* percentage of testicular cell types determined by FCM at d 1, 4, 16, and 32 posttreatment with a single oral dose of m-DNB-corn oil solution ($n = 4$). (*a*) Diploid. (*b*) Total haploid. (*c*) Round spermatids. (*d*) Elongating spermatids. Significance: $^{*}p < .05$, $^{**}p < .01$.

3*c*). On d 16, percentages of round spermatids were significantly greater than control values after administration of 32 and 48 mg m-DNB/kg. By d 32, proportions of round spermatids did not differ significantly from control values for all doses.

Elongating Spermatids The percentage of elongating spermatids did not differ between treatment and control groups on d 1 and 4 (Fig. 3*d*). Levels of these cell types significantly lower than control resulted on d 16 in both the 32- and 48-mg m-DNB/kg treatment groups.

Elongated Spermatids Day posttreatment and the interaction of dose with day posttreatment were nonsignificant sources of variation for proportions of elongated spermatids. When data were pooled across day posttreatment, significantly lower proportions of elongated spermatids existed in the 48-mg m-DNB treatment group than in the control (24.9 ± 1.9% vs. 35.0 ± 1.9%; $p < .01$).

Presence of Unusual Cell Types in the Caput Epididymis Following m-DNB Treatment

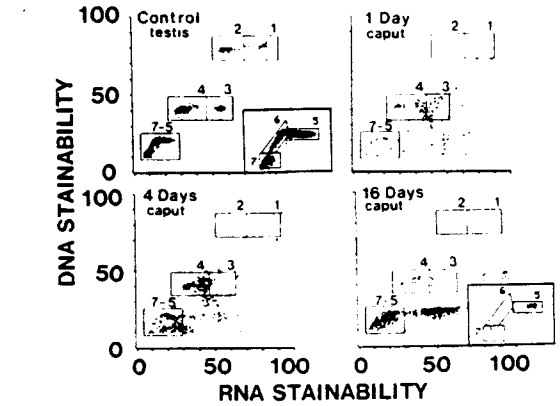
To determine whether m-DNB resulted in release of immature testicular cells into the epididymis, the PMT gains of the flow cytometer were set at a level used to measure AO-stained testicular cells similar to the method previously described (Evenson and Melamed, 1983). It was generally observed that m-DNB treatment resulted in a day- and dose-dependent increase of germinal cell types (haploid, diploid, and tetraploid) or unusual cell types (indicated by signals exhibiting higher red and/or lower green fluorescence than the three normal testicular populations) associated with caput epididymal contents (Fig. 3*a* and *b*). An increase in immature germ cell types and/or unusual cell types was first observed on d 1 after 48 mg/kg m-DNB (Fig. 4*a*; indicated by points outside boxes 1-7) and observed at d 4 at dosages $\geq$16 mg/kg (Fig. 4*b*). Elevated numbers of these cell types persisted through d 32 (32 and 48 mg/kg), although the greatest numbers were observed at d 16 (Fig. 4*a*). Due to debris present in the samples, it was not possible to determine the percent of each cell type present. Estimates of mean percentage diploid cells present are 9 ± 2, 8 ± 2, 14 ± 7, 12 ± 4, and 19 ± 2% at 0, 8, 16, 32, and 48 mg/kg, respectively. The presence of these cell types was confirmed by phase microscopic examination of the unstained samples; however, they were not specifically identified or quantitated.

Sperm Chromatin Structure

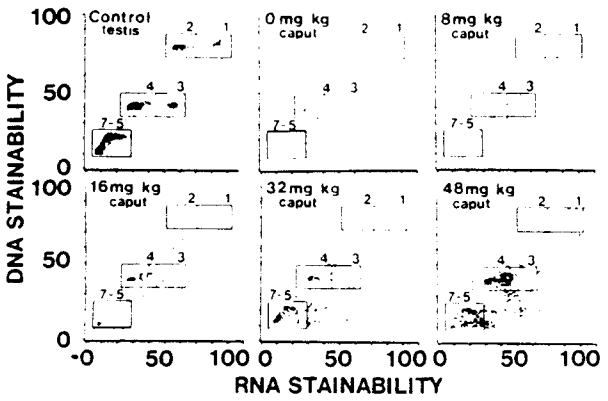
Caput Sperm The standard deviation of α_t ($SD\alpha_t$) for caput sperm did not vary significantly between control and treatment groups on d 1 (Fig. 5*a*). However, values significantly greater than control for $SD\alpha_t$ resulted on d 16 from 32 mg m-DNB and on d 4, 16, and 32 from 48 mg m-DNB. There was notable uniformity in $SD\alpha_t$ values among the lower dosages (0, 8, 16 mg/kg m-DNB) across the time span of the experiment.

Cauda Sperm No dose of m-DNB elicited a response in $SD\alpha_t$ that varied significantly from control on d 1 and 4 (Fig. 5*b*). Significantly greater than control values for $SD\alpha_t$ resulted on d 16 with 32 and 48 mg m-DNB/kg. On d 32, only 48 mg m-DNB/kg yielded a value significantly larger than control for $SD\alpha_t$.

Vas Sperm The $SD\alpha_t$ for vas sperm on d 16 did not differ from control at 8, 16, 32, or 48 mg m-DNB/kg (22.9 ± 9.2, 22.4 ± 7.1, 22.3 ± 7.1, 42.7 ± 7.1, and 31.0 ± 7.1, respectively; $p > .05$). Only the 48 mg/kg dosage resulted in an $SD\alpha_t$ greater than control (65.5 ± 7.1 vs. 23.2 ± 7.1; $p < .01$) at d 32; the $SD\alpha_t$ values resulting from the 8, 16, and 32 mg/kg dosages did not differ from control (20.8 ± 7.1, 22.3 ± 9.2, and 24.8 ± 7.1, respectively).



(a)



(b)

FIGURE 4. Comparison between FCM distribution of AO-stained rat testicular cells (control) and AO-stained cells isolated from the lumen of caput epididymi removed from rats (*a*) at d 1, 4, and 16 following exposure to 48 mg/kg m-DNB, and (*b*) at d 4 following exposure to 0, 8, 16, 32, and 48 mg/kg m-DNB. All samples were measured at the photomultiplier tube settings used for measuring testicular cells.

Effects of Sonication and RNAse Digestion on Caput Sperm

Sonication of sperm with or without RNAse treatment had no significant effect on the $SD\alpha_t$ of samples recovered from DNB-treated animals. Figure 6 provides an example from rats sampled on d 16 after treatment with 0, 8, 16, 32, and 48 mg/kg. The data indicate that the increased $SD\alpha_t$ values were not due to chemical-induced changes in cellular RNA content.

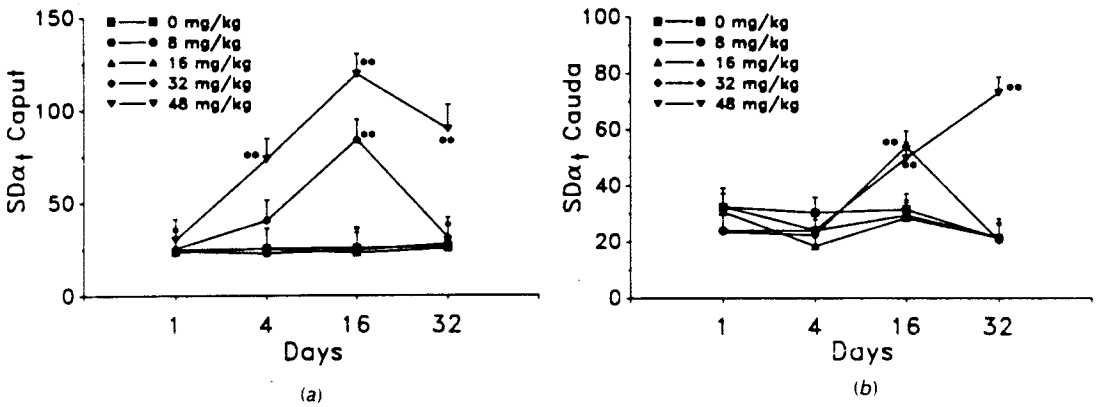


FIGURE 5. Relationship between $SD\alpha_t$, dose of m-DNB, and time of recovery for (a) caput and (*b*) cauda sperm samples; $n = 4$; Significance: $^{**}p < .01$.

Sperm Head Morphology

Caput Compared to control, a significantly greater proportion of sperm had abnormal heads at (% ABN) d 16 after treatment with 32 mg m-DNB/kg (Fig. 7*a*). Treatment with 48 mg/kg rendered the caput oligospermic at d 16; although flow cytometry measure 5×10^3 sperm, the concentration of caput sperm was too low at this day to obtain meaningful %ABN data using the microscope. The 48-mg m-DNB/kg dosage resulted in significantly greater %ANB values 32 d posttreatment (Fig. 7*b*).

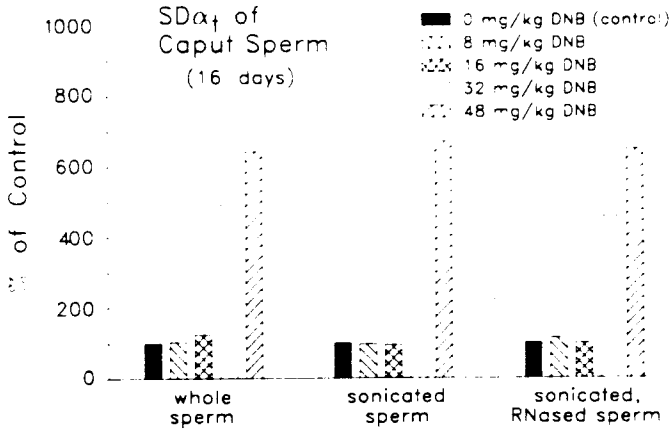


FIGURE 6. Bar graph of $SD\alpha_t$ values derived from the SCSA on d 16 caput sperm samples from rats exposed to 0, 8, 16, 32, or 48 mg m-DNB/kg body weight and subjected to (1) no treatment, (2) sonication, or (3) sonication + RNAse digestion prior to the SCSA protocol. Measurements were made from a single animal sample, and raw data values were normalized to those of corresponding controls.

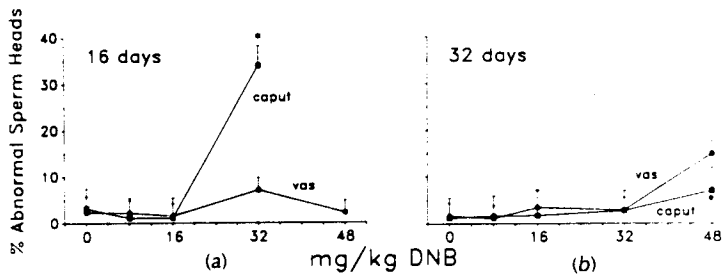


FIGURE 7. Mean percent (±SD) abnormal sperm head morphology in caput epididymal (■) and vas deferens (●) samples at (a) d 16 and (b) d 32 after exposure to 0, 8, 16, 32, and 48 mg m-DNB/kg body weight; Significance: $^*p < .05$.

Vas Analysis of variance showed that the proportion of vas sperm having abnormal heads was not significantly affected by m-DNB dose, rat nested within dose, day posttreatment, or the interaction of dose with day posttreatment. Nonetheless, it is interesting to note that mean percentage abnormal vas sperm was 9 times that of control values at d 32 after treatment with 48 mg/kg (Fig. 7b).

Detachment of Sperm Heads and Tails

Light microscopic examination of caput and vas sperm recovered from rats on d 16 and 32 showed that caput sperm remained intact (Fig. 8). Dose, rat nested within dose, day, and the dose by day interaction were nonsignificant sources ($p \geq .30$) of variation for decapitated caput sperm. Decapitated vas sperm, on the other hand, exhibited greater levels on d 32 than 16 (33.8 ± 6.9 vs. 5.9 ± 6.5, $p < .05$) when data were pooled across doses. When data were pooled across days, only the 48 mg/kg dosage elicited a greater than control value for percentage of detached sperm heads (54.9 ± 9.1 vs. 10.7 ± 9.6, $p < .01$); percentages

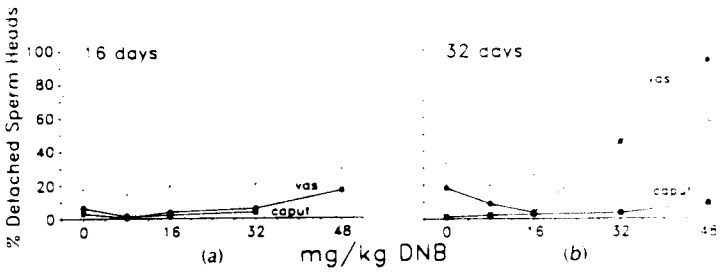


FIGURE 8. Percent detached caput (■) and vas (●) sperm heads in samples from rats at d 16 and 32 after exposure to 0–48 mg m-DNB/kg body weight; 10^3 sperm were evaluated for all points plotted. Caput samples (d 16) following 48 mg/kg treatment contained too few cells for accurate microscopic examination.

resulting from 8, 16, and 32 mg/kg (4.5 ± 9.1, 3.8 ± 9.1, and 25.5 ± 9.6, respectively) did not differ significantly from control.

Relation between Sperm Head Morphology and SDα_t Compared on a Single-Animal Basis

The relation between sperm head morphology and SDα_t was determined for all samples of caput and vas sperm collected at d 16 and 32. Table 2 and corresponding Fig. 9 show the relationship between sperm head morphology and the SDα_t for the same population of sperm. Note in Table 2 that the correlation between dose and SDα_t was always higher than between dose and %ABN. Also, the correlation between SDα_t and %ABN was always higher than dose and %ABN, suggesting, in both cases, that SDα_t was a more sensitive indicator of toxin-induced damage than deterioration of sperm head morphology.

Figure 9 plots the relationship between %ABN and SDα_t for each animal killed at d 16 or 32. The 3 highest value points for caput samples at d 16 and 32 are numbered. The numbers for the vas values correspond to the same rat in the caput value. Note the general correspondence between high levels of abnormalities observed in the caput and the vas samples from the same rat. Differences in levels of abnormality between caput and vas can be ascribed to the time lag between m-DNB exposure and arrival of sperm into the caput versus the vas.

DISCUSSION

This study confirms previous (Cody et al., 1981; Hess et al., 1985, 1988; Linder et al., 1986, 1988; Strader et al., 1987) studies indicating that m-DNB exerts a very strong toxic effect on male rat reproductive function. In addition, the present study shows effects on m-DNB on loss and recovery of seven testicular cell types and effects of sperm chromatin structure and altered sperm head morphology. Although the effects of

TABLE 2. Correlations of SDα_t of Caput and Vas Sperm with Percentage of Abnormal Sperm Head Morphology (%ABN) and m-DNB Dosage at 16 and 32 days Posttreatment[a]

	16 d			32 d		
	Dose	SDα_t	%ABN	Dose	SDα_t	%ABN
			Caput (n = 16)			
Dose	1.0	0.78**	0.70**	1.0	0.62**	0.59**
SDα_t		1.0	0.97**		1.0	0.90**
			Vas (n = 19)			
Dose	1.0	0.40	0.21	1.0	0.67**	0.59**
SDα_t		1.0	0.88**		1.0	0.96**

[a]Significance: $^{**}p < .01$.

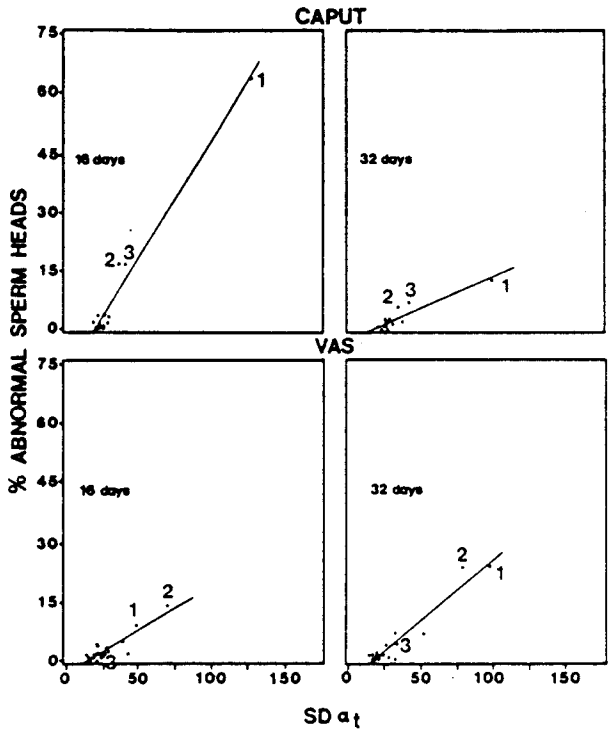


FIGURE 9. Comparison between percent sperm head morphology abnormalities and SDα, in caput and vas samples obtained at d 16 or 32 after treatment with 48 mg/kg m-DNB and the SDα_t of the same samples. The numbers 1, 2, 3 in the upper figures (caput) correspond to samples from the same rat in the lower (vas) figures.

chemical exposure were both time and dose responsive, some variability between response of individual rats was observed. These differences are not explained simply by slight variations in initial weights of rats, but may be related to pharmokinetic and pharmodynamic factors.

Primary m-DNB-induced reproductive damage appeared to occur in the testis, resulting in abnormal sloughing of seminiferous epithelium which moved into the caput epididymis by d 1 (Fig. 4*a*) following exposure to 48 mg/kg with resultant loss of other cells and testicular weight by d 16. Work by Hess et al. (1988) and Foster et al. (1987) suggests that primary targets of m-DNB are the Sertoli cells, which then lose their protective role for the developing testicular germ cells. Pachytene spermatocytes are particularly susceptible to many forms of damage (Setchell, 1978).

Flow cytometric measurement of cells obtained from parenchymal samples showed that m-DNB caused altered ratios of normal testicular cell types (Fig. 3*a–d*). Similar to the effect seen after exposure of mice to

thiotepa (Evenson et al., 1986), the first noticeable loss was the percent of pachytene spermatocytes (Fig. 2), which is consistent with the histological observations of Blackburn et al. (1988) and Hess et al. (1988). In the present study, the reduction in the proportion of elongating spermatids (Fig. 3*d*) on d 16 was the first indication of loss of testicular haploid cells, an effect also reported in mice following exposure to ethylnitrosourea (Evenson et al., 1985). Thus, the kinetics of loss of testicular cell types in rats resulting from m-DNB exposure was similar to our observations of the effects of two potent alkylating agents in mice. By d 16 the dramatic reduction in haploid cell types (Fig. 3*b*) corresponds to a sharp reduction in testicular weight (Table 1). Significant recovery of cell type ratios had occurred by d 32 for all doses, although the testicular weights had not recovered in rats exposed to 48 mg/kg m-DNB (Table 1). This corresponds with the time when testicular damage was also apparent histologically (Hess et al., 1988). The present study demonstrates the advantage of flow cytometry in quickly and easily measuring the loss of particular testicular cell types or the abnormal passage of testicular cells into epididymal ducts following trauma to the testis.

m-DNB induced alterations of sperm chromatin structure, defined as an increased sensitivity to acid-induced DNA denaturation in situ and expressed as increased α_t values derived by the sperm chromatin structure assay. Since these effects were not seen in caput epididymal sperm at d 1 and 4, rat chromatin structure is likely resistant to m-DNB damage after passage to the epididymis. Significant effects were seen in caput sperm at d 16 (Fig. 5*a*), suggesting either an action directly with sperm or more likely an interaction with germ cells and Sertoli cells (Hess et al., 1988; Foster et al., 1987) that prevents normal sperm chromatin packaging. Hess et al. (1985) observed on d 1 after m-DNB exposure, that the nuclei of round spermatids had a "halo" appearance in some tubules due to the movement of chromatin to the margin of the nucleus. This manifestation could possibly lead to the altered chromatin structure detected as increased α_t values for sperm in the present study.

Whether the increase of α_t values of sperm is related to loss of cell viability is a question under further study in our laboratory. However, it should be noted that semen samples of excellent quality for sperm numbers, motility, and morphology have also had high α_t values (Evenson, 1986). Other studies (Evenson et al., 1989) have shown the high α_t values resulting from mutagenic chemical exposure in mouse sperm may persist for months after exposure and long after induced cell death. Thus, the weight of evidence suggests that increased α_t values for surviving sperm are due to genetically or, more likely here, developmentally induced abnormal chromatin structure.

m-DNB induced an increased percentage of abnormal shaped sperm heads (Fig. 7*a,b*). Although the response was variable, at d 16 following a 32 mg/kg exposure 34% of the epididymal sperm heads were morpholog-

ically abnormal. This increase was generally in parallel to the increase in the α_t values for altered chromatin structure similar to that observed in previous mouse studies (Evenson, 1986; Evenson et al., 1985, 1986). Table 2 (also Fig. 5*a,b*) provides evidence, though not extensive, that SDα_t is a more sensitive indicator for detecting response to toxic chemical exposure than sperm head morphology.

Of interest is the observation that at d 32 following exposure, the vas sperm had up to 90% detached sperm heads from tails (Fig. 8). In contrast, caput samples at that time were not different from controls in the percentage of detached heads, apparently due to the testis recovering within that time frame. Previous studies (Evenson et al., 1986) have shown a relationship between α_t values for sperm chromatin structure and the percentage of detached heads, suggesting that one defect in sperm structure is often reflected by another abnormality. Note in Fig. 9, however, that at d 32 the caput sperm had a high SDα_t value at 48 mg/kg whereas the percentage of detached sperm heads (Fig. 8) was normal. At the same time point and exposure level the vas sperm had a very high percentage of detached sperm heads but moderately increased α_t values. Thus, the relationship between α_t values and detachment of sperm heads and tails is not consistent.

At d 32, 48 mg/kg produced a significant ($p < .01$) elevation in SDα_t of caput sperm (Fig. 5a). This effect is likely not due to a direct effect of m-DNB but may be characteristic of recovery similar to the abnormal chromatin structure found in the first wave of spermatogenesis during puberty (Janca et al., 1986, and companion paper).

This study demonstrated that flow cytometry protocols developed for toxicological studies with mice are readily useful for studies on rats. No differences were encountered except that whole rat sperm tended to clog the flow cytometer sample tubing and filter to a greater extent than whole mouse sperm, probably due to larger tail structure of rat sperm. Particularly interesting was the similarity in dose response, of rats in this study and mice in previous studies, as measured by flow cytometry and compared to sperm head morphology abnormalities. Thus, the sperm chromatin structure assay appears equally useful for reproductive toxicology studies on rats.

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