

Spermatotoxicity Associated with Acute and Subchronic Ethoxyethanol Treatment

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Investigations of the male reproductive toxicity of ethoxyethanol (ethylene glycol monoethyl ether) have been restricted exclusively to histopathological assessments of the testes. The present study consisted of two experiments designed to document the spermatotoxicity of ethoxyethanol (EE) as reflected in evaluations of ejaculated rat semen. The basic strategy involved the evaluation of individual ejaculates recovered from the genital tract of a female rat prior to exposure of the males to EE. Repeated assessments of the ejaculate were made during the experimental phase according to specified protocols.

Adult Long-Evans hooded male rats received 0, 936, 1872 or 2808 mg/kg (PO) of EE for five consecutive days. Semen evaluations were then conducted at weeks 0, 1, 4, 7, 10 and 14 after exposure. Males in the highest two dose groups showed declines in sperm counts by week 4 and were essentially azoospermic by week 7. At this time, males receiving the lowest dose of EE also exhibited decreases in ejaculated sperm counts. An increase in abnormal sperm shapes was also observed. Over the ensuing weeks all males exhibited varying degrees of recovery as reflected by increasing ejaculated sperm counts. In a second experiment, males were treated with 0 or 936 mg/kg of EE daily (5 days/week) for 6 weeks with semen evaluations conducted weekly. By week 5, sperm counts were significantly depressed and there was an increase in the number of abnormal shapes. Sperm motility was depressed by week 6. The temporal trends seen in these experiments suggested a differential sensitivity of the spermatocyte stage to EE toxicity. Histopathological assessments confirmed this observation while distinguishing the pachytene spermatocytes as the most sensitive cells. Subchronic treatment did not appear to alter the spermatogonial population, suggesting the potential for recovery as witnessed following acute EE administration.

The majority of work evaluating the male reproductive toxicity of glycol ethers has been with methoxyethanol (ethylene glycol monomethyl ether). Studies on ethoxyethanol (ethylene glycol monoethyl ether, EE) have been limited and are summarized in Table 1. These studies have been restricted essentially to histopathological evaluations of the testes. No published reports are available on other spermatogenic endpoints or fertility aside from the report by Lamb in these proceedings (1). Ethoxyethanol appears to produce the same pattern of testicular toxicity as that seen with the methyl derivative, although higher doses of EE are required (2). The present study consisted of two experiments. The first experiment employed acute EE treatment and was designed to determine the sensitivity of the various stages of spermatogenesis to EE insult as well as to estimate recovery from such toxicity. The second investigation examined the spermatotoxicity and associated testicular histopathology accompanying subchronic EE treatment using the lowest dose which produced effects in Experiment I. Both studies utilized

evaluations of ejaculated rodent semen to assess spermatotoxicity.

Materials and Methods.

Ethoxyethanol was obtained from Aldrich Chemical (99.9% pure) and its purity confirmed by gas chromatography. EE was diluted with distilled water such that all animals received equivalent volumes of fluid.

Treatment Protocol

Male and female Long-Evans hooded rats (Charles River, Wilmington, MA) were obtained at approximately 70 to 80 days of age. All animals were kept on a reverse 10:14 light-dark cycle with the lights turned off at 0900 hours. Purina Lab Chow #5001 and tap water were available *ad libitum*. Males were single-caged while females were housed three per cage.

The same basic protocol was employed in Experiments I and II. The experimental strategy involved semen evaluations on ejaculates recovered from the genital tract of a female rat, 15 min after copulation. This approach allowed for the semen evaluations to be performed on each male prior to exposure (baseline) and then repeatedly throughout the experimental phase.

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Table 1. Summary of effects of ethoxyethanol on the male reproductive system.

Species	Dose	Route	Duration	Effects	Reference
Mice	0.5, 1.0, 2.0%	Drinking water	ca. 100 days	Total infertility at 2.0% ↓ live pups/litter at 1.0% ↓ epididymal sperm motility ↓ abnormal sperm ↑ testes weight at 2.0% ↓ abnormal forms at 1.0% ↑	(1)
Rat	250–1000 mg/kg	PO	11 days	Testes weight and testicular atrophy ↓ Pachytene spermatocytes most sensitive	(2,4)
Mice	500–4000 mg/kg	PO	5 weeks	Marked degeneration of germinal epithelium	(3)
Rat	1.45%	Diet	2 years	Testicular edema and atrophy of germinal epithelium	(5)
Rat	94–750 µg/kg	SC	4 weeks	Testicular edema, absence of more mature sperm cell stages	(6)
Rat	94–750 µg/kg	PO	13 weeks	Similar pattern to that seen with SC injection	(6)
Dog	50–20 µL/kg	PO	13 weeks	Similar pattern to that seen in rats	(6)

Prior to the treatment phase, the males were allowed to mate with ovariectomized, hormonally primed females on several occasions over a 2-week period. Females received 0.1 mg/0.1 mL of estradiol benzoate (SC) (Sigma) 48 hr prior to mating followed by 0.1 mg/0.1 mL (SC) of progesterone (Lilly, Indianapolis, IN) 4 hr before mating. These initial mating trials were necessary in order to insure a high level of mating proficiency by the time the experiment was initiated. All the males were mated on a weekly basis for the duration of the experiment in order to maintain a constant interval between ejaculations. At the beginning of the experimental period, the males were intubated with distilled water for 5 consecutive days. This procedure was employed in order to acclimate the animals to the procedure of intubation. On the fifth day of the water intubation, the males were mated with ovariectomized hormonally primed females in order to obtain pre-exposure baseline measures on each animal. Mating behaviors were observed and at 15 min post-ejaculation, the females were sacrificed. The semen was recovered from the uterine horns and processed for determinations of sperm count, percent motile sperm and sperm morphology (see below). The sperm count data were also used to match males for assignment to treatment groups. The males were rank-ordered by sperm counts and assigned to treatment groups such that the mean sperm count of each group was essentially equivalent.

Copulatory Behaviors

Evaluations of copulatory behaviors were conducted between 1300 and 1500 hr during the dark cycle. The male was placed in a Plexiglas observation cage (33 × 28 × 23 cm) and allowed to habituate for approximately 15 min. An ovariectomized, hormonally primed female was then introduced into the cage and copulatory behaviors monitored. Four behaviors were recorded: (1) mount latency, defined as the elapsed time between introduction of the female and the first mount; (2) number of mounts; (3) number of intromissions, distinguished from mounts by increased vigorous thrusting, backpedaling following desengagement, and immediate geni-

tal grooming; and (4) ejaculation latency, defined as the elapsed time between the initial mount and ejaculation. The presence of a copulatory plug upon sacrifice in 100% of the females confirmed our accuracy in determining time of ejaculation.

Sperm Evaluation

Approximately 15 min after copulation, the female was sacrificed by CO₂ asphyxiation. The uterine horns were exposed, and the contents of the uteri (semen and uterine fluid) aspirated into a syringe. Subsequently, the uteri and vagina were excised, cut open and flushed to obtain residual sperm, and the copulatory plug was removed, washed and weighed.

A drop of the sample was placed on a slide and a subjective estimate was made of the degree of sperm concentration (low, moderate, high) in order to dilute the sample for motility evaluation. An aliquot of the sample was diluted with phosphate-buffered saline. The buffer and slides were maintained at 37°C. A 10 µL portion of this diluent was pipetted onto a slide, cover-slipped and placed on the microscope stage maintained at 37°C by an air curtain incubator. In Experiment I, percent motility was then determined for 100 sperm viewed in three to four areas of the slide. In Experiment II, three fields on the slide were videotaped and this tape subsequently scored for percent motile.

In Experiment I, approximately 50 µL (one drop) of the diluted sample was stained with Eosin Y and subsequently scored for sperm morphology (i.e., percent normal shapes seen in 200 sperm). In Experiment II, a stain was used that was developed by Bryan (?). This consisted of air drying an unstained sperm smear on a slide and staining for 40 min in an acidic Fast Green, Eosin Y and Naphthol Yellow solution. The slides were then rinsed in 1% acetic acid, cleared in xylene and mounted. This stain allowed for a more detailed inspection of the head and tail structures.

The remaining undiluted sample was transferred to a graduated cylinder. The residual sperm from the uteri, vagina and copulatory plug were added to the gradua-

ted cylinder along with washings of the syringes, pipette tips, and slides. The sample was then diluted to a final volume of 50 mL. Both chambers of a hemocytometer were charged with 10 μ L of sample and sperm counts were determined. If counts differed by more than 10% the chamber was recharged and sperm count was redetermined.

Data Analyses

For both experiments, a two-way repeated measures analysis of variance (ANOVA) with one between factor (treatment) and one within factor (weeks) was used for analysis of body weights, sperm counts, sperm motility, plug weights, mount latency, ejaculation latency, number of mounts and intromissions. The latency measures were subjected to a logarithmic transformation prior to analysis to reduce heterogeneity of variance.

Percent of baseline was calculated for each animal by dividing values obtained during the experimental phases by his pre-exposure baseline values. These data were also analyzed employing a two-way repeated measures ANOVA. The morphology data were transformed using an arc sine transformation to normalize distributions and analyzed by a one-way ANOVA. Data collected at sacrifice (see below) were analyzed employing one-way ANOVA's to test for the presence of treatment-related effects. All significant trends were further tested with univariate ANOVA's to contrast the various treatment groups.

Experiment I

Method

Two days following baseline semen evaluations, males were intubated with either 0, 936, 1972, or 2808 mg/kg of EE (four males/group) for five consecutive days. At the cessation of EE exposure (week 1, post-baseline), the animals were again mated and copulatory behaviors and semen samples evaluated. The males were subsequently sampled at weeks 4, 7, 10 and 14. Sample weeks 1, 4, 7 and 10 corresponded to the transit time required in the rat for germ cells in the various spermatogenic stages (i.e., spermatozoa, spermatids, spermatocytes, and spermatogonia) to appear in the ejaculate subsequent to exposure. A sample was also obtained one month later (week 14), since week 10 samples had indicated that some of the animals exposed to EE were demonstrating recovery (i.e., elevated sperm counts). Animals were mated in the interim weeks, but samples were not recovered. At week 16, all animals were sacrificed and weights of the liver, kidney, testis, epididymis (total), cauda epididymis, vas deferens, ventral prostate, and seminal vesicles were obtained. A cauda epididymis was processed to obtain a sperm count. The other cauda epididymis and one testis were fixed in Bouin's solution, dehydrated, embedded in

paraffin, sectioned, stained with hematoxylin and eosin by routine procedures and examined by light microscopy.

Results

All groups showed significant increases in body weights during the course of the experiment ($p \leq 0.01$). However, animals in the high dose group exhibited less weight gain relative to the other groups. This trend was only significant at week 1 ($p \leq 0.05$), reflecting concurrent EE treatment.

EE produced no effects on copulatory behaviors or seminal plug weights. However, EE treatment did result in a significant decline in ejaculated sperm counts in all groups (Fig. 1). At the fourth week post exposure, males in the two highest dose groups had significantly decreased sperm counts and by the seventh week the males were essentially azoospermic. At this time males in the lowest dose group also exhibited significant decreases in ejaculated sperm relative to the controls.

Sperm counts were so depressed in the middle and high dose groups that assessments of sperm motility

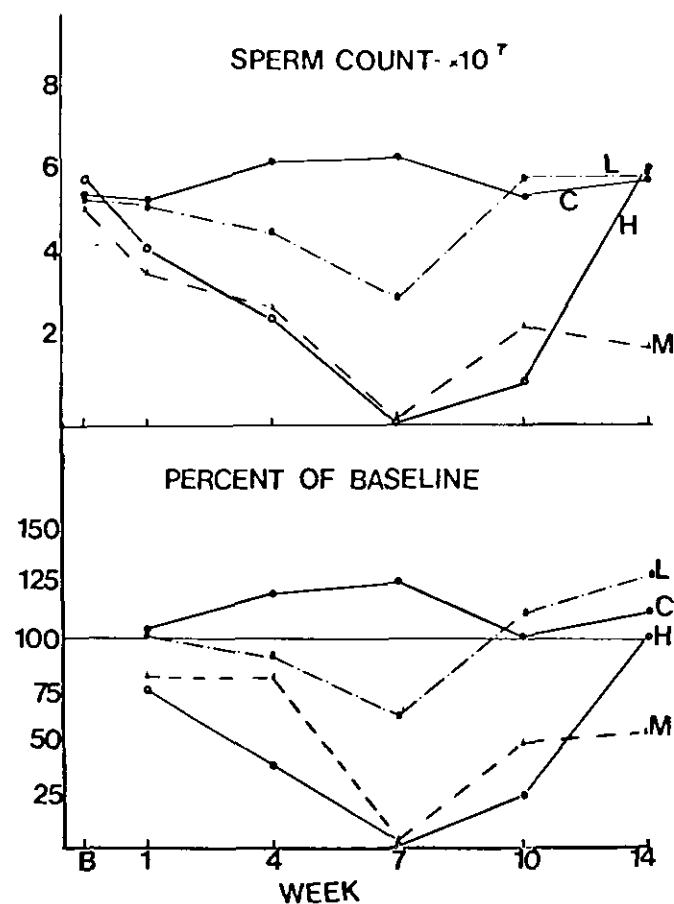


FIGURE 1. Group mean sperm counts expressed both as (A) raw values and (B) as percent of each animal's baseline. Letters L, M and H represent low (936 mg/kg), medium (1872 mg/kg) and high (2808 mg/kg) doses of ethoxyethanol administered (PO) for 5 consecutive days.

and morphology were negated. The low dose group showed a significant decrease in normal forms at week 7 compared to controls (mean \pm SD = 95.8 ± 2.9 vs. 99.4 ± 0.7 , respectively). These abnormal forms were unlike any seen previously in the laboratory and reflected marked deformation of the sperm head (Fig. 2). Motility, although lower in this group, was not significantly different from controls.

All treated males evidenced some degree of recovery by week 10 post-exposure. By week 16, only males in the middle dose group lagged behind in recovery as reflected in ejaculated sperm count. Histological evaluations of the testes of rats in this group suggested the presence of some residual lesions. These lesions were characterized by depleted or disorganized seminiferous tubules interspersed among normal tubules.

Data collected at sacrifice are presented in Table 2.

Analyses of organ weights revealed no differences between the groups except in the case of the epididymis ($p \leq 0.01$). In this instance, epididymal weights in the middle dose group were significantly lower than controls ($p \leq 0.05$). Differences between controls and high dose animals approached significance ($p \leq 0.10$). Although not significant, similar trends were observed in the cauda epididymal weights and caudal sperm counts for these two groups again indicating that recovery had not been totally achieved by this time.

The data from Experiment I suggested that EE exerted its most dramatic effects at week 7 corresponding to a heightened sensitivity of the spermatocyte stage to this agent. Moreover, the spermatogonia appear to remain intact as witnessed by the high levels of recovery even in animals rendered azoospermic by EE treatment.

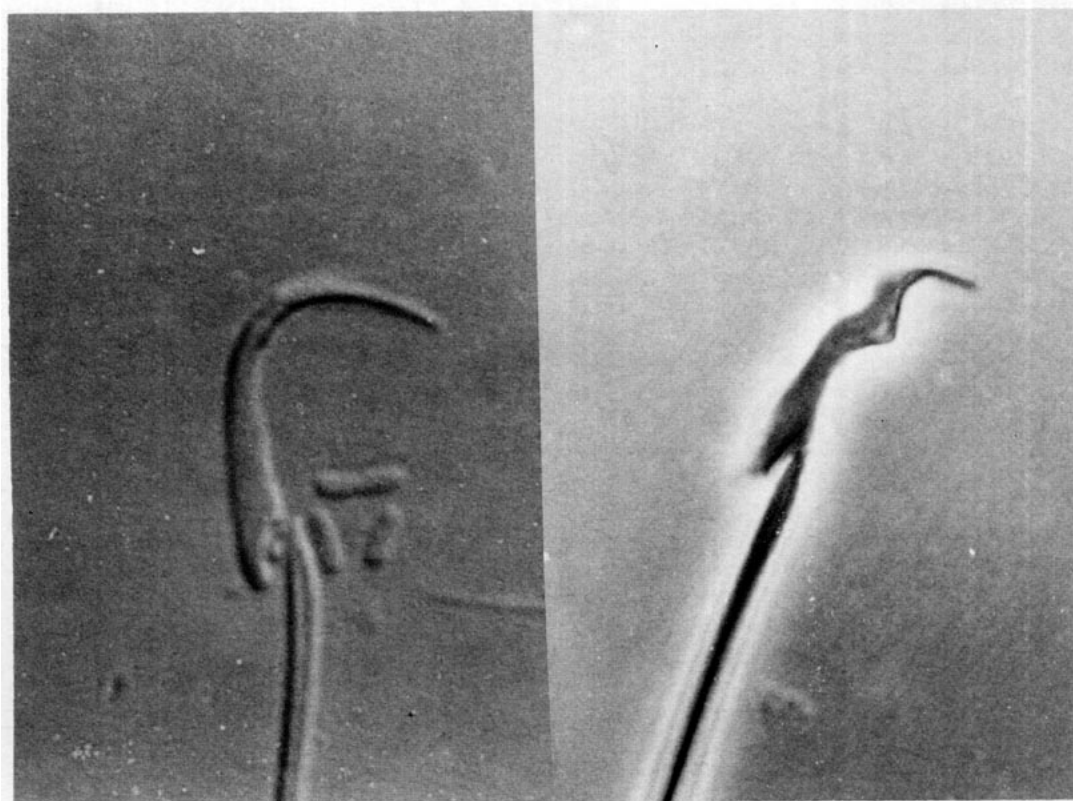


FIGURE 2. Photomicrograph of sperm heads: (right) high dose animal (2808 mg/kg) at week 7; (left) normal shape seen in a non-exposed animal. $\times 1000$ Nemarski.

Table 2. Body weight, organ weight and cauda epididymal sperm counts obtained at sacrifice following acute ethoxyethanol exposure.

Treatment (PO), mg/kg	Body and organ weight, g (mean \pm SD)				Cauda sperm count $\times 10^6$
	Body	Testis	Epididymis (total)	Cauda epididymis	
0 (control)	555 ± 37.4	1.65 ± 0.10	0.633 ± 0.07	0.263 ± 0.09	161 ± 30
936	540 ± 59.1	1.85 ± 0.18	0.650 ± 0.04	0.238 ± 0.03	187 ± 25
1872	594 ± 40.6	1.57 ± 0.09	$0.542 \pm 0.03^*$	0.213 ± 0.01	129 ± 27
2808	517 ± 16.0	1.64 ± 0.15	0.543 ± 0.06	0.190 ± 0.04	134 ± 36

* $p < 0.05$ significantly different from controls.

In light of these data, Experiment II was designed to utilize subchronic exposure and weekly semen evaluations so as to better document the development of the spermatotoxicity. Moreover, histological evaluations of the testes were done concurrently in a subpopulation of males.

Experiment II

Method

The experimental strategy was identical to that employed in Experiment I with the one exception: that ejaculated semen samples were evaluated weekly. After obtaining a baseline semen evaluation on each male, treatment was initiated. Males were intubated with either 0 or 936 mg/kg EE daily (5 days/week), for 6 weeks (nine males/group). At the end of the sixth week, all animals were sacrificed and weights were obtained on all major organs. One of the cauda epididymides was processed for determination of sperm count and sperm morphology. The hematocrit and hemoglobin levels were also determined at this time.

Fourteen additional males were used for histological evaluations. Twelve males received 936 mg/kg EE (PO) 5 days/week for up to 6 weeks. Two control males were intubated with equivalent volumes of water. Two treated males were sacrificed at weekly intervals through week 6. At this time, the control males were sacrificed. The males were sacrificed by pentobarbital overdose, perfused with a 1% procaine saline solution, followed by a 5% glutaldehyde fixative. For light microscopy, testicular tissue was embedded in paraffin, sliced in 5 μ m sections and stained with hematoxylin and eosin.

Results

Subchronic EE treatment produced a significantly slower rate of weight gain relative to the controls (Fig. 3). This difference increased over weeks, suggesting the gradual appearance of a more pronounced systemic toxicity. Evidence of a generalized toxicity was also seen in a number of other endpoints. Data obtained at sacrifice (Table 3) revealed that EE animals had elevated brain and spleen weights either expressed as absolute values or as organ/body weight ratios ($p \leq 0.05$). Hemoglobins and hematocrits were significantly

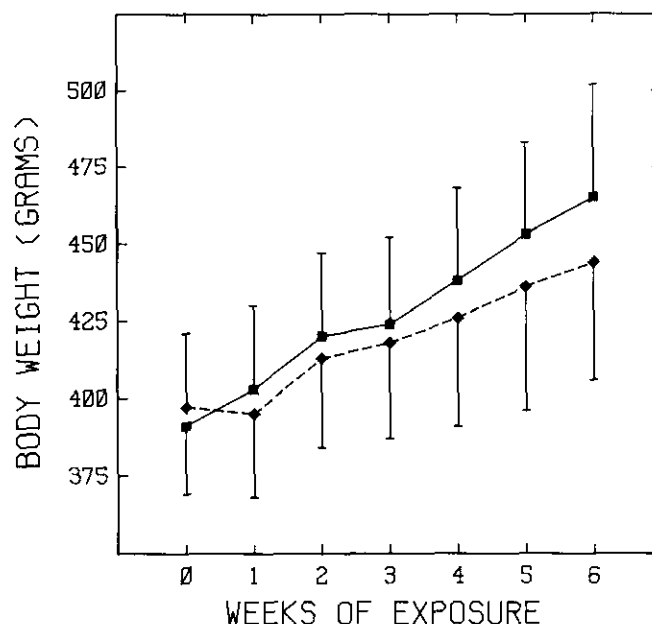


FIGURE 3. Plots of body weight gain (■) in controls and (◆) in animals treated with 936 mg/kg ethoxyethanol (PO) for 6 weeks.

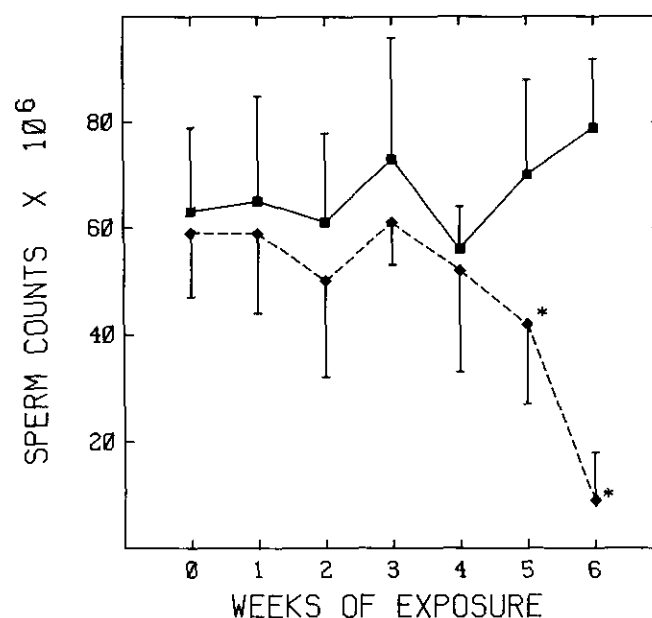


FIGURE 4. Alterations in ejaculated sperm counts associated with subchronic ethoxyethanol treatment (936 mg/kg, PO): (■) controls; (◆) treated with EE; * $p \leq 0.01$.

Table 3. Body weight, organ weights and hematologic measures obtained at sacrifice following subchronic ethoxyethanol exposure.

Treatment, mg/kg	Body and organ weight, g (mean \pm SD)					Hematocrit, %	Hemoglobin, mg/dL
	Body	Brain	Spleen	Liver	Kidneys		
0	487 \pm 39	1.78 \pm 0.17 (0.365) ^a	0.79 \pm 0.21 (0.160)	17.0 \pm 2.28 (3.49)	3.65 \pm 0.55 (.746)	46.1 \pm 1.62	14.2 \pm 0.70
936	470 \pm 39	2.05 \pm .17* (0.438)*	1.12 \pm 0.17* (0.239)*	16.94 \pm 2.06 (3.61)	3.62 \pm 0.43 (0.771)	44.3 \pm 1.80*	13.5 \pm 0.70*

^aOrgan weight/100 g body weight.

* $p < 0.05$.

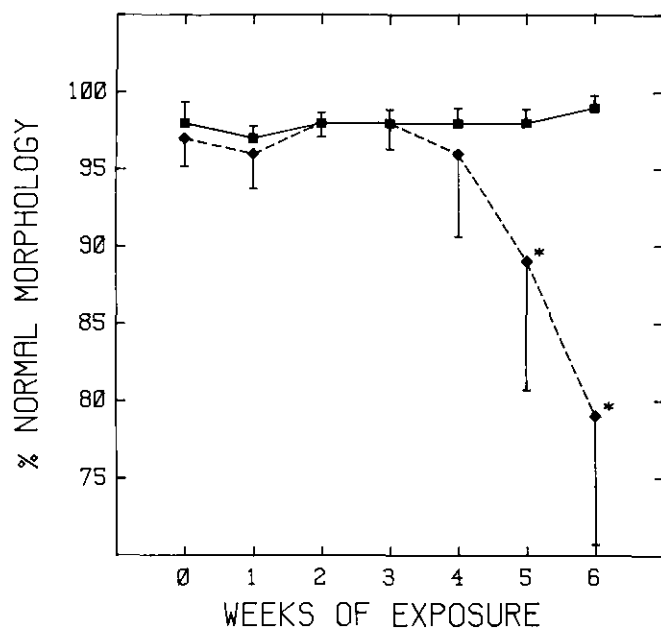


FIGURE 5. Alterations in sperm morphology associated with subchronic ethoxyethanol treatment (936 mg/kg, PO): (■) controls; (◆) treated with EE; * $p \leq 0.01$.

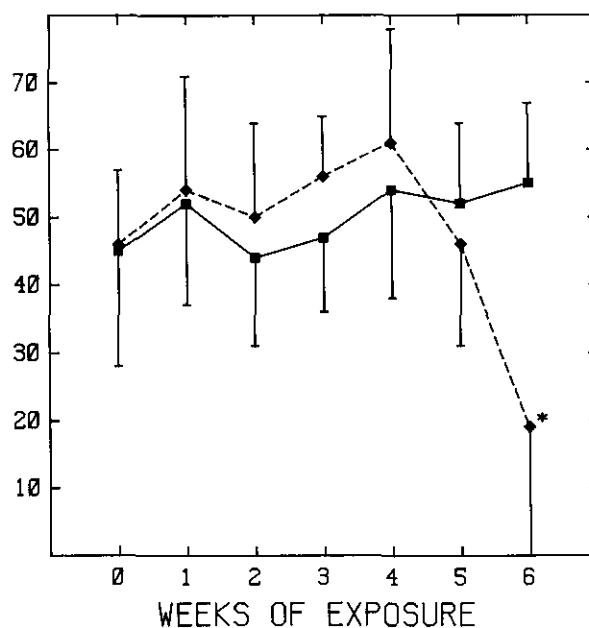


FIGURE 6. Alterations in sperm motility associated with subchronic ethoxyethanol treatment (936 mg/kg, PO): (■) controls; (◆) treated with EE; * $p \leq 0.01$.

Table 4. Reproductive organ weights and sperm parameters obtained at sacrifice following subchronic ethoxyethanol exposure.

Treatment, mg/kg	Reproductive organ weight, g (mean \pm SD)						Caudal sperm count $\times 10^6$	Normal morphology, %
	Prostate	Seminal vesicle	Vas deferens	Testes	Epididymides	Cauda epididymis		
0	0.84 \pm 0.15 (0.174) ^a	2.21 \pm 0.24 (0.455)	0.26 \pm 0.03 (0.054)	3.33 \pm 0.36 (0.690)	1.22 \pm 0.11 (0.251)	0.15 \pm 0.03 (0.031)	114 \pm 26	95 \pm 2.5
936	0.70 \pm 0.14 (0.150)	2.41 \pm 0.31 (0.516)	0.24 \pm 0.03 (0.051)	1.37 \pm 0.03* (0.292)*	0.75 \pm 0.08* (0.159)*	0.09 \pm 0.01* (0.018)*	11 \pm 7*	61 \pm 14*

^aOrgan weight/100 g body weight.

* $p < 0.01$.

depressed ($p \leq 0.05$). Other investigators have reported similar hematopoietic effects (3).

All sperm parameters were adversely affected by EE treatment. Significant declines in sperm count (Fig. 4) and percent normal forms (Fig. 5) were seen at weeks 5 and 6, and a significant decline in motility was seen at week 6 (Fig. 6). The abnormal shapes were similar to those observed in Experiment I.

Data on the reproductive organs obtained at sacrifice (Table 4) further supported these observations. While accessory organ weights were unaffected, testicular and epididymal weights were severely depressed. Cauda epididymal sperm counts were only 10% of that seen in controls and the percent of abnormal forms was markedly increased.

The time course for appearance of spermatotoxicity (as assessed in ejaculates) corresponded closely to that seen with acute exposure in Experiment I. This supported the previous observation of the heightened sensitivity of the spermatocytes to EE insult. The more profound depression in sperm counts reflected the extended dosing period allowing for a more complete encompassing of cells passing through this stage.

Histological evaluations as early as week 1 confirmed the sensitivity of the spermatocyte stage. At this time the testes showed scattered lesions primarily seen as vacuolization of the tubules directly above the Sertoli cell nuclei and spermatocytes with pyknotic nuclei. By the second and third weeks, the damage to the seminiferous tubules was widespread, and a number of tubules showed exfoliation of developing germ cells. The testes showed little, if any progressive damage from week 4 to week 6. There was no apparent damage to the Leydig cells, Sertoli cells, spermatogonia, or early spermatocytes (i.e., leptotene stage). However, there was a total absence of maturing germ cells beyond the leptotene-zygotene spermatocyte stages. These observations are essentially identical to those reported by Foster et al. (2,4).

Discussion

The data from these studies emphasize the remarkable sensitivity of the testes to EE toxicity. Few clinical signs were observed at the end of the 6 weeks of dosing

in spite of marked testicular insult. The similar patterns of insult observed across Experiments I and II would suggest the potential for recovery of testicular function within the dose and time period examined in these studies. Histological data would support this view since the spermatogonial population remained intact in the males exposed to EE for 6 weeks. Germ cell repopulation has also been noted by Foster et al. (2). Such recovery would argue against the hypothesis that EE produces its toxicity by interfering with mechanisms regulating cell division (3).

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