TOXICOLOGY AND APPLIED PHARMACOLOGY 94, 466–472 (1988)

Reproductive Toxicity of 2,4-Dinitrotoluene in the Rat[1]

ERIC BLOCH,*,† BERNARD GONDOS,‡ MICHAEL GATZ,§
SANTOSH K. VARMA,* AND BENJAMIN THYSEN*,§

Department of Obstetrics and Gynecology, †Department of Biochemistry, and §Department of Laboratory Medicine, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461, and ‡Sansum Medical Research Foundation, Santa Barbara, California 93105

Received January 15, 1988; accepted March 18, 1988

Reproductive Toxicity of 2,4-Dinitrotoluene in the Rat. BLOCH, E. GONDOS, B., GATZ, M., VARMA, S. K., AND THYSEN, B. (1988). *Toxicol. Appl. Pharmacol.* **94**, 466–472. The present study was undertaken to evaluate the effects of the chemosterilant 2,4-dinitrotoluene (DNT) on the rat testis. Adult male rats were fed control, or 0.1%, or 0.2% DNT for 3 weeks. An ultrastructural study of the testes was performed, serum was assayed for testosterone and gonadotropins, and sperm reserve count was determined. A marked change in Sertoli cell morphology was found after 3 weeks of 0.2% DNT exposure. Varying sized vesicles associated with swollen mitochondria and distended endoplasmic reticulum were visible in cells from DNT-treated animals. Circulating levels of follicle stimulating hormone and luteinizing hormone were increased in 0.2% DNT-treated animals. Reduced weights of the epididymides and decreased epididymal sperm reserves were observed in DNT-treated animals. These results indicate that DNT is capable of inducing testicular injury, of directly or indirectly disturbing pituitary function, and of exerting a toxic effect at the late stages of spermatogenesis. These findings suggest that a locus of DNT action is the Sertoli cell, resulting in both inhibition of spermatogenesis and changes in testicular–pituitary endocrine activity.

Dinitrotoluene (DNT) is an important intermediate widely used in the production of dyes, munitions, and explosives. The principal use of DNT, however, is in the synthesis of toluene diisocyanate, the building block of polyurethane foams, coatings, and elastomers.

In animals, the reproductive toxic effects of exposure to 2,4-DNT and related isomers on reproduction have been well established (Lee *et al.*, 1976a,b; Ellis *et al.*, 1979; Soares and Lock, 1980; Thysen *et al.*, 1985a,b). Such compounds are of special interest because of their potential hazard to humans.

The site(s) of action at which these agents disrupt spermatogenesis is unknown. Therefore, the present investigation was undertaken in an attempt to clarify the mechanism by which DNT induces infertility in experimental rats. To evaluate spermatogenic effects, cauda epididymal sperm numbers were quantitated. To determine whether the agent affects the reproductive endocrine system, blood levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone were measured. Testis morphology was studied using electron microscopy (EM).

[1] Supported by Grant OH01520 from the National Institute for Occupational Safety and Health. The authors express their appreciation to the National Pituitary Agency, NIADDK, NIH, for making available RIA kits for measuring rLH and rFSH. Requests for reprints should be addressed to Dr. Eric Bloch, Department of Obstetrics and Gynecology, 1300 Morris Park Avenue, Bronx, NY 10461.

0041-008X/88 $3.00

TABLE 1

BODY AND ORGAN WEIGHTS[a] OF RATS FED 2,4-DINITROTOLUENE FOR 3 WEEKS

Treatment	N^b	Weights		Body weight (g)	
		Testis (mg)	Epididymis (mg)	Initial	Final
Control	10	1561 ± 147	561 ± 48	390 ± 6	465 ± 12
0.1% DNT	10	1555 ± 171	520 ± 64	389 ± 12	437 ± 26[c]
0.2% DNT	9[e]	1346 ± 651	474 ± 83[d]	391 ± 17	417 ± 18[c]

[a] Mean ± SD. Left member of each pair of organs was weighed.
[b] Number of animals.
[c] Comparison with control, $p < 0.01$, as determined by ANOVA and Fisher's protected t test.
[d] Comparison with control, $p < 0.02$, as determined by ANOVA and Fisher's protected t test.
[e] One rat died of unknown causes during treatment.

METHODS

Male Sprague–Dawley rats (Charles River Breeding Laboratories) weighing 300–325 g were randomly distributed into groups of 9–10 rats each, and kept at 24°C on a light–dark cycle of 12 hr, with lights on from 0600 to 1800 hr. All animals were conditioned in our animal quarters for 2 weeks prior to use. Rats were fed Purina Law Chow admixed with 2,4-dinitrotoluene (97% pure: Aldrich Chemical Co.) in concentrations of 0, 0.1, or 0.2% for 3 weeks. All animals were housed 1 per cage and given their respective diets and tap water *ad libitum*. Just prior to euthanasia with CO_2 gas, cardiac blood was collected. Samples were collected between 0900 and 1100 hr to avoid the complicating effects of circadian variation. Serum was prepared and stored frozen at −20°C until assayed for LH, FSH, and testosterone.

LH and FSH concentrations were measured with the double antibody radioimmunoassay (RIA) kits kindly provided by the National Pituitary Agency, National Institutes of Health. The standard reference preparations were rat LH-RP-2 and rat FSH-RP-2. Testosterone was determined by RIA using reagents purchased from Diagnostic Products, Los Angeles, California. The intraassay coefficients of variation for LH at 0.8 ng/ml, FSH at 13 ng/ml, and testosterone at 5.7 ng/ml were 5.3, 5.9, and 4.2%, respectively. Each specimen was analyzed in duplicate.

After death, the testes and epididymides were removed and the left member of each pair was weighed. Portions of testicular tissue from each of five rats per treatment group were fixed in 2.5% glutaraldehyde buffered with Na cacodylate and washed in 0.2 M phosphate buffer. Samples were postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon. Sections cut at 0.5-μm thickness with a Porter-Blum MT2-B ultramicrotome were stained with toluidine blue and examined by light microscopy. Thin sections for ultrastructural study were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

Cauda epididymides were minced and homogenized for 2 min in 200 ml saline-Triton-Merthiolate solution (Robb *et al.*, 1978). Sperm reserves were assessed by hemocytometric counting of spermatozoal heads from quadruplicate determinations.

The data were analyzed by one-way analysis of variance. When significant differences between groups were found, means were compared with Fisher's protected t test. Significant levels were set at $p < 0.05$ for all comparisons.

RESULTS

Body and Organ Weights

No clinical signs of toxicity were observed in rats treated with DNT at the administered dosages. Body weight in all animals increased during the course of the study (Table 1). However, the final body weights were reduced in animals treated with 0.1% ($p < 0.01$) or 0.2% 2,4-DNT ($p < 0.01$) compared to controls.

While testis weights were not significantly affected after 3 weeks treatment, a significant decrease in epididymal weight was seen at the 0.2% DNT dose level (Table 1).

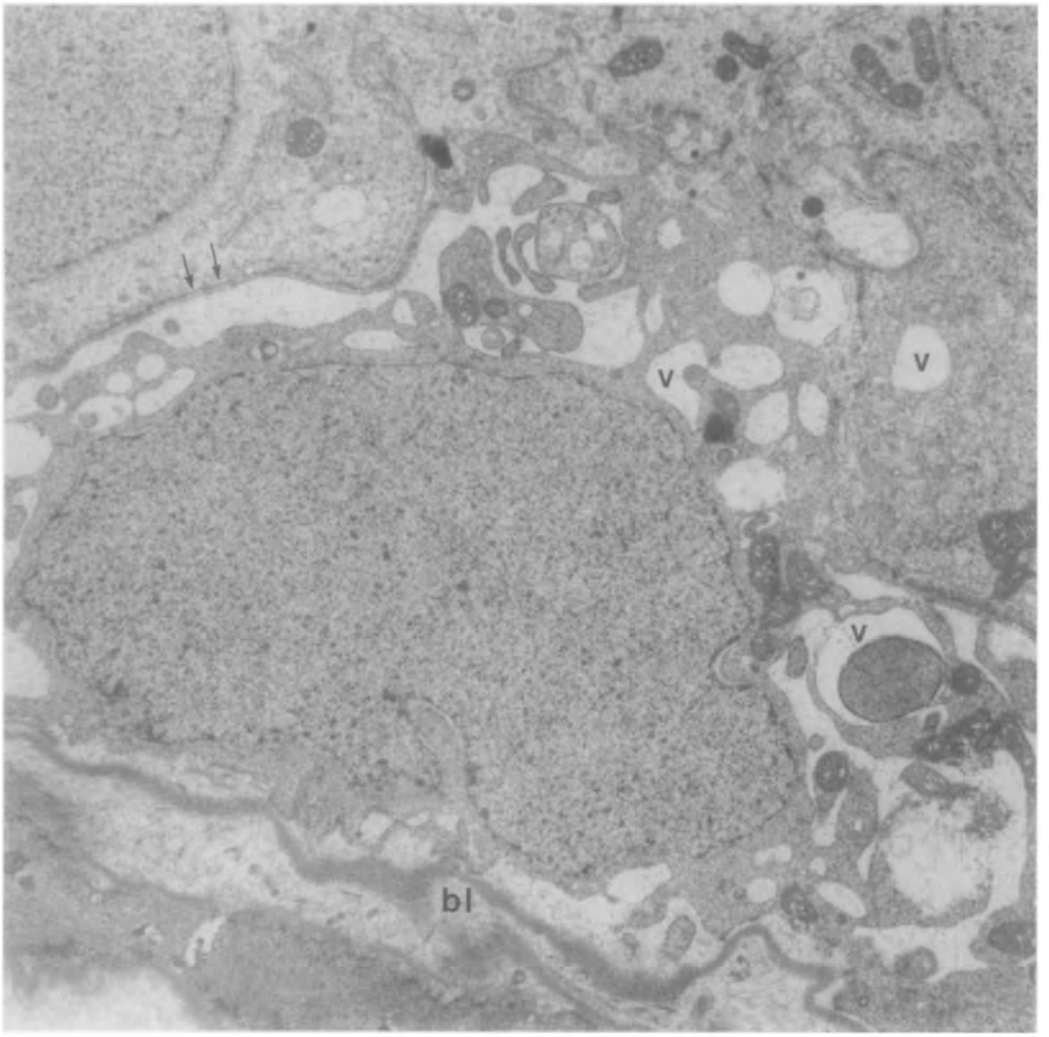

FIG. 1. Electron micrograph of affected testis showing extensive Sertoli cell damage following dinitrotoluene treatment. Much of the cytoplasm is occupied by varying sized vesicles (v) associated with swollen mitochondria and distended endoplasmic reticulum. Portions of Sertoli cell junctions (arrows) can be seen. The basal lamina (bl) has become frayed and thickened and there is general distortion of the peritubular tissue. ×8,800.

Ultrastructural Observations

Electron microscopy was preceded by examination of 0.5-μm-thick sections stained with toluidine blue in the light microscope. Testes from animals treated with 0.1% DNT did not differ from those of the controls. Animals in the 0.2% treatment group demonstrated extensive disruption of spermatogenesis, irregularity of the peritubular tissue, and widespread vacuolization of Sertoli cells. No Leydig cell abnormalities were evident in either of the treatment groups.

Electron microscopic examination showed focal alterations at the 0.1% dose level, including fine vacuolization and lipid accumu-

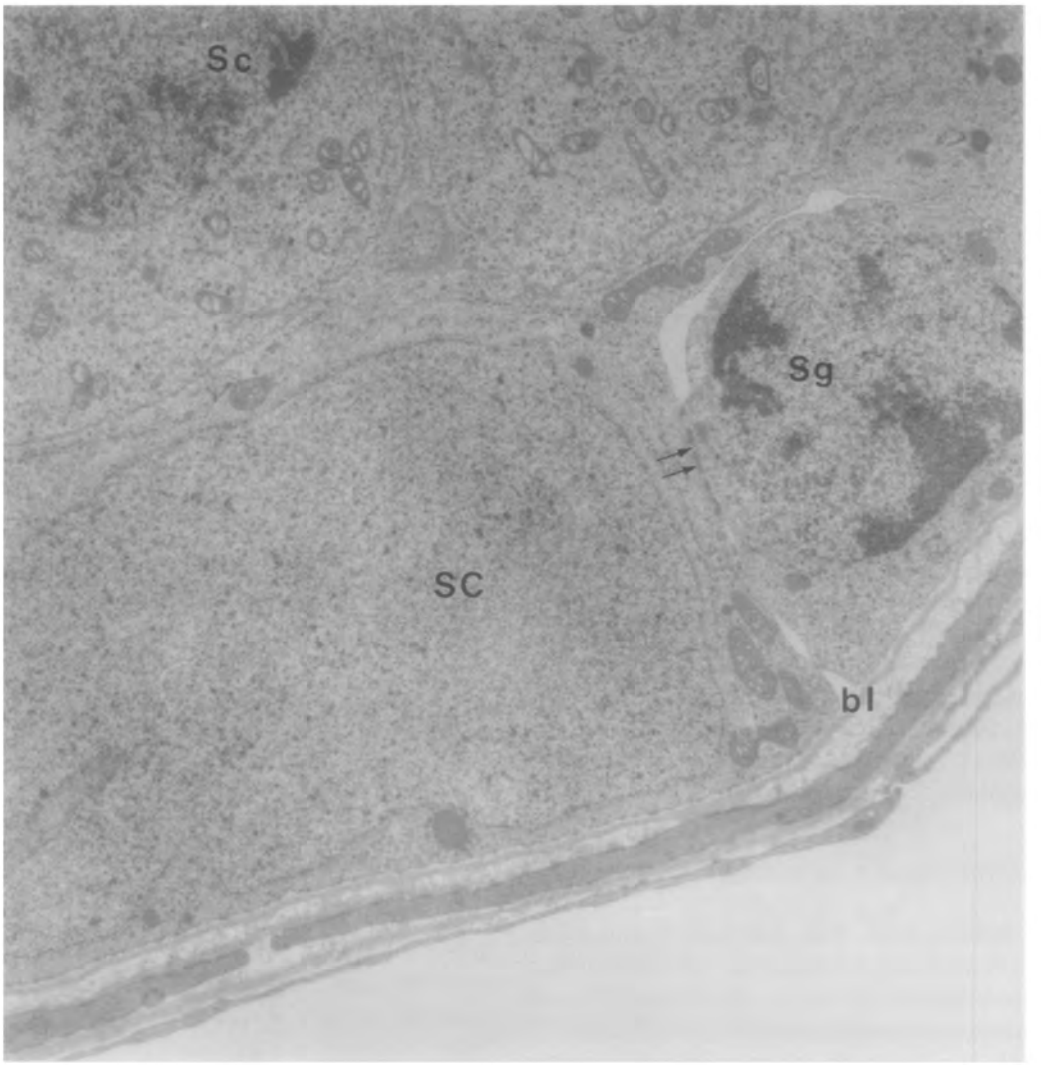

FIG. 2. Electron micrograph of control testis showing normal Sertoli cell (SC), adjacent type B spermatogonium (Sg) and portions of spermatocytes (Sc). Sertoli cell cytoplasm includes elongated mitochondria, strands of rough endoplasmic reticulum, and small droplets. Junctional attachment plaques (arrows) are seen between Sertoli cell and spermatogonium. Note the delicate peritubular tissue with smooth basal lamina (bl). ×8,800.

lation in Sertoli cells, prominence of multinucleated spermatids in some tubules, and mild irregularity of the basal lamina. These changes were limited and variable with most samples demonstrating patchy damage.

At the 0.2% dose level, Sertoli cells throughout showed distinct cytoplasmic alterations. These consisted of varying sized vesicles, swollen mitochondria, and distended endoplasmic reticulum (Fig. 1). Extensive degenerative changes were present in spermatocytes and spermatids, and more mature forms were generally lacking. The basal lamina was thickened and frayed with irregu-

TABLE 2

CAUDA EPIDIDYMAL SPERM RESERVES AND SERUM HORMONE LEVELS[a] IN RATS FED 2,4-DINITROTOLUENE FOR 3 WEEKS

Treatment	N[b]	Total sperm count ($\times 10^6$/100 mg cauda)	Serum hormones (ng/ml)		
			LH	FSH	Testosterone
Control	10	119 ± 24	0.66 ± 0.25	12.9 ± 3.0	2.82 ± 1.50
0.1% DNT	10	99 ± 23	0.97 ± 0.44	13.5 ± 2.6	3.38 ± 0.96
0.2% DNT	9[e]	46 ± 18[c]	1.30 ± 0.82[d]	16.2 ± 3.9[d]	2.76 ± 1.68

[a] Mean ± SD.
[b] Number of animals.
[c] $p < 0.01$, by ANOVA and Fisher's protected t test, comparison with control.
[d] $p < 0.05$, by ANOVA and Fisher's protected t test, comparison with control.
[e] One rat died of unknown causes during treatment.

lar infolding and distortion of the peritubular tissue. Leydig cell ultrastructure was similar to that of controls.

In control animals, Sertoli cells had a normal appearance with elongated mitochondria, delicate strands of rough endoplasmic reticulum, and scattered small lipid droplets (Fig. 2). Basal lamina was smooth and regular.

Sperm Concentration and Hormone Analysis

Sperm, FSH, LH, and testosterone measurements are presented in Table 2. Reduced cauda epididymal sperm counts by 63% were found in animals treated with 0.2% DNT ($p < 0.01$). These effects on epididymal sperm stores were accompanied by a doubling of serum LH ($p < 0.05$) and a 25% increase in FSH levels ($p < 0.05$). Animals receiving DNT for 3 weeks showed no treatment-related effects in the levels of testosterone. Rats fed 0.1% DNT showed no significant effects on sperm concentrations or serum hormone levels.

DISCUSSION

Lee *et al.* (1976a) found that a dose of 0.2% DNT produced a retardation in weight gain in male rats as a result of reduced food consumption. In the present study, rats tolerated exposure to this concentration well for the 3-week treatment period. The only indication of systemic toxicity was a modest reduction in weight gain. Animals appeared to be in good health. Their response to handling was normal, and there were no signs of lethargy or disorientation.

Feeding of 0.2% DNT for 13 weeks has been shown to decrease spermatogenesis in rats (Lee *et al.*, 1976a). We have demonstrated that antispermatogenic activity can be induced at this concentration following a 3-week exposure, suggesting that the site of action is at the late stages of spermatogenesis. These findings indicate that the more mature testicular elements, i.e., spermatids and possibly spermatocytes may be particularly sensitive to this compound.

Fine structural alterations of Sertoli cells accompanied the diminished cauda epididymal sperm count in the animals acutely exposed to 0.2% DNT. Other deleterious effects caused by the agent included frayed basal lamina and distortion of peritubular tissue. It seems likely that the structural changes in the Sertoli cells may be precipitating events responsible for the spermatogenic disruption noted in the 0.2% DNT-treated rat. It has been suggested that germ cell depletion may

occur as a direct consequence of functional impairment of Sertoli cells (Creasy *et al.*, 1983; DeMartino *et al.*, 1975; Fang and Anderson, 1976; Flores and Fawcett, 1972; Hausler and Hodel, 1979; Lamb and Chapin, 1985). Since the Sertoli cells play a major role in maintaining spermatogenesis in the testis (Setchell and Waites, 1975; Wright *et al.*, 1981), it is possible that the reduced sperm reserves seen in the DNT-treated animals reflect a change in Sertoli cell function.

Several studies have linked FSH levels to Sertoli cell function (Krueger *et al.*, 1974; Means *et al.*, 1976; Steinberger and Steinberger, 1966). Raised serum levels of FSH have been reported to be frequently, if not always, associated with Sertoli cell malfunction (de Kretser and Kerr, 1983). The increased FSH concentrations seen in sera of 0.2% DNT-exposed rats also were associated with marked changes in Sertoli cell ultrastructure. The Sertoli cells have been suggested as the source of inhibin (Steinberger, 1981), a nonsteroidal hormone believed to modulate (inhibit) FSH secretion. The severe damage to these cells as described in our study may have disrupted inhibin secretion, resulting in the observed elevation of FSH concentration. It should be noted, however, that a direct effect on the hypothalamic–hypophyseal axis is not precluded, since testosterone concentrations remained within the normal range, whereas LH levels were elevated in our DNT-treated animals.

In summary, our data provide evidence that DNT affects pituitary function, alters Sertoli cell structure, and appears to affect maturation of spermatozoa. The results suggest that the inhibition of spermatogenesis may be mediated through Sertoli cell injury.

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