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Six month oral toxicity study of trinitrotoluene in beagle dogs*

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Summary

This study was conducted to evaluate the toxicity of the munitions compound 2,4,6-trinitrotoluene (TNT; CAS Reg. No. 118-96-7) in beagle dogs when administered daily for 26 weeks by capsule. Groups of six dogs per sex received TNT at doses of 0 (vehicle controls), 0.5, 2, 8, or 32 mg/kg/day. Toxicologic endpoints included clinical signs, body weights, food consumption, clinical biochemistry, hematology, urinalyses, organ weights, and gross and tissue morphology. The major toxic effects following the oral administration of TNT to dogs included hemolytic anemia, methemoglobinemia, liver injury, splenomegaly with accompanying histologic lesions, and death. Only the highest dose given proved to be lethal. Hepatocytic cloudy swelling and hepatocytomegaly were apparent at all doses tested. Thus, a no observable effect level was not established in this investigation.

Key words: 2,4,6-Trinitrotoluene; Beagle dogs; Hemolytic anemia; Hepatotoxicity

Introduction

Trinitrotoluene (TNT) is a commonly used explosive which poses toxic hazards to various segments of the environment. This includes factory workers who are engaged in its manufacture, filling of munitions and washing out of expired or rejected end items [1]. In addition, wastewaters which are discharged from these plants pose hazards to the general environment.

Studies in our laboratory have examined the subchronic toxicity of TNT in F344 rats and B6C3F1 mice [2,3]. The primary toxic effects of TNT in these animals included hemolytic anemia, methemoglobinemia, liver injury, and testicu-

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lar atrophy. Similar toxicologic responses to TNT intoxication have been observed in other rodent strains [4]. In addition, fatal and non-fatal human poisonings have generally demonstrated similar effects to those identified in rodents, however additional target organs including the CNS and urogenital system were suggested [1,5,6].

The short-term toxicity of TNT in beagle dogs has been previously examined [4]. The results of that experiment were generally consistent with subchronic rodent toxicity studies of TNT in our laboratory. The present study was initiated to gain insight into the chronic toxicity of TNT in a non-rodent, and to further define dose-response relationships.

Materials and methods

Chemicals

Trinitrotoluene (2,4,6-trinitrotoluene; TNT), 99.1 ± 0.4% purity as determined by high performance liquid chromatography, was obtained from stocks at the IIT Research Institute, Kingsbury Ordnance Plant Explosive Facility, LaPorte, IN.

Animals

Beagle dogs, obtained from Marshall Research Animals, Inc., North Rose, NY, were used in this study. They were 4 months old upon arrival and were held in quarantine for approximately 2 months. The animals were individually housed in stainless steel cages in an air-conditioned room (21—23 °C) at ambient relative humidity (approx. 30—50%), and on a 12 h light/12 h dark cycle. The cage size conformed to the upper weight range recommended in the Guide for the Care and Use of Laboratory Animals, DHEW, NIH No. 78.23.

All animals received daily rations of 400 g of Purina Dog Chow, Ralston Purina Co., St. Louis, MO from arrival until termination, except during a 16—18 h fast prior to either blood collection or routine kill. Tap water was available ad libitum.

Experimental design

Following the quarantine period, test-eligible animals were randomly assigned, within sex, into five treatment groups by a restricted randomization procedure (stratified by weight; blocked design). Animals were considered test-eligible on the basis of clinical examinations, endoparasite tests, and clinical biochemistry, hematology and urinalyses data collected in Week −3. Six animals per sex received either 0.0 (empty gelatin capsules), 0.5, 2.0, 8.0 or 32.0 mg/kg/day for 6 months. Test capsules were prepared on the basis of the most recent body weight for each dog.

Animals were observed daily for pharmacologic and/or toxicologic signs. Physical examinations, which included body weights and palpations for masses, were conducted weekly. Twenty-four hour food consumption measurements were also performed once weekly.

Clinical biochemistry, hematology, and urinalyses tests were performed during

Weeks −3, −1, 3, 8, 12, 17, 22 and 26. Blood samples were collected from the femoral vein after a 16—18 h fast. Clinical biochemistry parameters were measured on a centrifugal analyzer (Centrifichem 300, Union Carbide, Pleasantville, NY) and included glucose, urea nitrogen (BUN), SGOT, SGPT, alkaline phosphatase, triglycerides, cholesterol, LDH, CPK, calcium, sodium, potassium, chloride, bilirubin, total protein, and albumin. Globulin was calculated as total protein less albumin. Hematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), erythrocyte count, total and differential leukocyte counts, reticulocyte counts, platelet counts, prothrombin time, clotting time, and methemoglobin were measured by standard clinical laboratory procedures which included an electronic particle size counter (Model S Coulter Counter, Coulter Electronics, Hialeah, FL). Urinalyses measurements included specific gravity, estimates of pH, protein, ketones, bilirubin, blood and glucose (Multistix, Miles Laboratories, Elkhart, IN), and microscopic examination of spun sediment.

All animals received ophthalmic and electrocardiogram examinations during Weeks −1, 13 and 25. Heart rates and PQ and QRST intervals were measured from lead AVF.

Necropsies were conducted for all animals which either survived the 26 week treatment period or died during the study. Major organs of all killed animals were weighed. Approximately 50 tissues for all animals were collected and fixed in 10% neutral buffered formalin. Eyes and testes were fixed in 3% glutaraldehyde and Bouins solution, respectively, prior to being stored in ethanol. All tissues collected were examined microscopically.

Statistical analysis of quantitative data was accomplished by two-way (sex × dose) 'fixed effects' analysis of variance tests. The analyses were performed on change scores, i.e. test week minus baseline, for body weight, food consumption, clinical biochemistry, hematology and electrocardiography data, and on M:E ratios and absolute and relative organ weight data. A P value of $\leqslant 0.05$ was considered statistically significant and Dunnett's t-test was used for pair-wise comparisons when appropriate [7].

Results

Clinical observations and mortality

Ataxia was observed for some of the males and females receiving 32 mg/kg/day. This was primarly seen from the onset of dosing for approximately 6 weeks, and only infrequently thereafter. Orange/brown urine and orange/red feces were present throughout the dosing period for several dogs administered 32 and to a much lesser extent 8 mg/kg/day. This was apparently a consequence of highly colored metabolic and/or photolytic decomposition products although urinalyses demonstrated elevated bilirubin levels at doses of 2 mg/kg/day or greater. Other signs of toxicity, which were confined to the high dose animals from approximately Week 10 and occasionally thereafter, included darkening of the tongue and/or gums, and evidence of jaundice.

One high dose female was considered to be moribund during Week 14 and was

TABLE I

EFFECTS OF ORAL ADMINISTRATION OF TNT TO BEAGLE DOGS ON BODY WEIGHT (kg)[a]

		Dose (mg/kg/day)				
		0	0.5	2	8	32
Week 26	Males	11.0 ± 0.5	10.4 ± 1.2	10.3 ± 0.9	9.2[b] ± 1.0	9.3[b] ± 0.5
	Females	11.0 ± 0.5	10.4 ± 1.2	10.3 ± 0.9	9.2[b] ± 1.0	9.3[b] ± 0.5
Weight Δ[c]	Males	0.9 ± 0.7	0.2 ± 0.7	0.4 ± 0.3	−0.9[b] ± 1.5	−0.5[b] ± 0.7
	Females	1.1 ± 1.1	0.6 ± 1.3	0.4 ± 1.6	0.6 ± 0.5	−0.1[b] ± 0.4

[a]Mean ± S.D.; $N = 6$.

[b]Mean significantly different from appropriate control group mean, $P < 0.5$.

[c]Week 26 minus Week −1.

immediately sacrificed. The animal showed signs of dehydration and emaciation, had a depressed body temperature, and was in an advanced icteric state. A second high dose female was found dead during Week 16. Prior to death, it showed considerable weight loss, diarrhea and ataxia. No other deaths occurred.

Body weight/food consumption

Treatment-related reductions in body weight gains and/or body weight losses were apparent for TNT-treated animals of both sexes throughout the 26-week treatment period (Table I). Statistically significant results (body weight losses) occurred at the 8 (males only) and 32 mg/kg/day dose levels, however body weights were, in general, lower for all TNT treatment groups compared to respective controls. The observed body weight losses for both sexes were generally progressive throughout the treatment period. Reductions in food consumption for animals receiving 32 mg/kg/day were slight but were seen during most of the 26-week treatment period.

Clinical pathology

Dose-dependent anemia (decreased hematocrit, hemoglobin, and erythrocyte counts) was observed for TNT-treated dogs. These parameters were affected to approximately the same extent, and the results for hematocrit and hemoglobin are shown in Table II. The severity of the anemic state was similar at all observation periods with animals at the high dose demonstrating approximate 20—30% reductions in the hematocrit. Methemoglobinemia was also apparent at the highest dose (Table II).

Compensatory responses which occurred as a result of the anemic state included reticulocytosis, macrocytosis, and elevated levels of nucleated erythrocytes. Macrocytic erythrocytes were marginally hypochromic (Table II), although absolute mean corpuscular hemoglobin levels were unaffected. In addition, bone

TABLE II

EFFECTS OF ORAL ADMINISTRATION OF TNT TO BEAGLE DOGS FOR 26 WEEKS ON HEMATOLOGY PARAMETERS[a]

		Dose (mg/kg/day)				
		0	0.5	2	8	32
Hematocrit (%)	Males	44.3 ± 1.9	44.0 ± 1.3	42.8 ± 2.3	37.5[b] ± 3.5	38.2[b] ± 4.2
	Females	46.5 ± 3.0	47.6 ± 2.6	43.9 ± 2.8	42.8 ± 1.9	38.8[b] ± 2.9(4)
Hemoglobin (G%)	Males	15.6 ± 0.8	15.4 ± 0.7	14.8 ± 0.8	12.5[b] ± 1.4	11.3[b] ± 1.3
	Females	16.6 ± 1.3	16.8 ± 0.9	15.1 ± 1.0	14.2[b] ± 0.7	11.8[b] ± 1.5(4)
MCV (μ^3)	Males	67 ± 2	66 ± 1	67 ± 2	70[b] ± 2	76[b] ± 1
	Females	68 ± 1	68 ± 2	68 ± 1	71[b] ± 1	74[b] ± 3(4)
MCHC (G%)	Males	36.4 ± 0.3	35.9 ± 0.7	35.8 ± 0.5	34.5[b] ± 1.0	32.7[b] ± 0.7
	Females	36.5 ± 0.6	36.2 ± 0.2	35.5 ± 0.4	34.7[b] ± 0.3	32.9[b] ± 1.2(4)
Methemoglobin (G%)	Males	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	1.0[b] ± 0.4
	Females	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.8[b] ± 0.2(4)
Platelets ($10^3/mm^3$)	Males	332 ± 67	316 ± 103	458 ± 94	557[b] ± 126	585[b] ± 254
	Females	430 ± 106	397 ± 126	407 ± 145	522[b] ± 117	569[b] ± 105

[a]Week 26 data (Mean ± S.D.; $N = 6$ unless otherwise noted in parentheses).
[b]Mean significantly different from appropriate control group mean, $P \leq 0.05$.

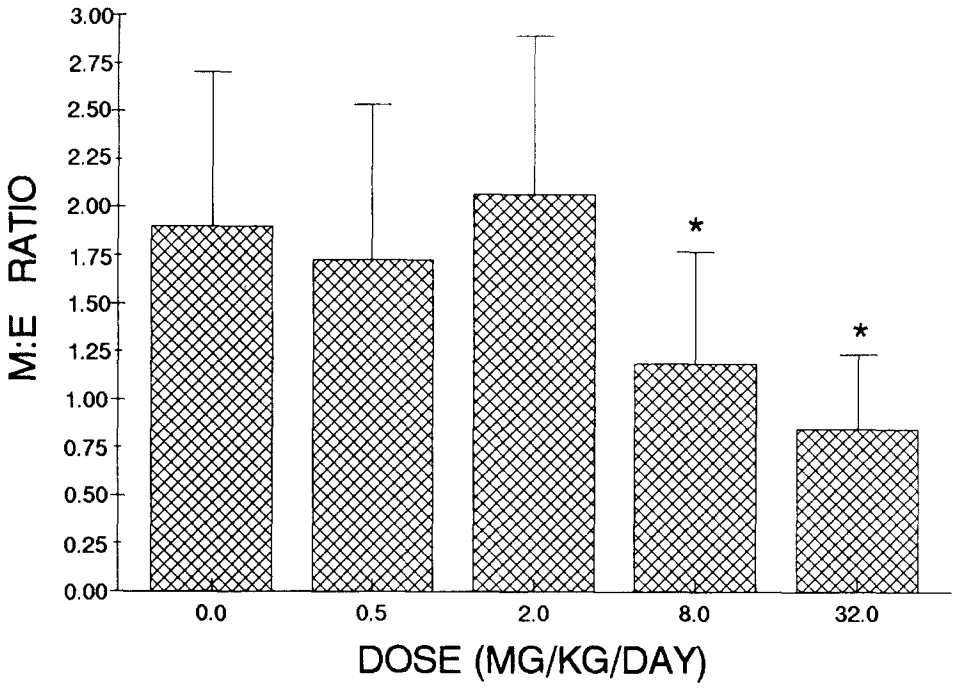

Fig. 1. Effect of oral administration of TNT to beagle dogs on myeloid:erythroid ratios. Samples were collected in Week 27 at scheduled kill. The results for males and females are combined as a sex effect was not apparent. Each bar represents the mean and S.D. for 12 animals. *Mean significantly different from control group mean, $P < 0.05$.

marrow M:E ratios were significantly reduced for dogs receiving 8 or 32 mg/kg/day (Fig. 1). Thrombocytosis was also observed for animals receiving 32 or 8 mg/kg/day (Table II).

Dose-dependent decreases in SGPT were seen for both sexes throughout the 26-week treatment period and the results for week 26 are shown in Table III. Following an initial decrease, SGPT levels were generally stable, suggesting a possible effect on synthesis of the enzyme. Although serum protein levels were essentially unaffected in TNT-treated dogs, albumin levels were decreased and globulin levels were increased in high dose male but not female dogs resulting in decreased albumin/globulin ratios (Table III). As anticipated with hemolytic anemia, serum total bilirubin levels were routinely elevated at the high dose, and typical results are shown in Table III. TNT treatment also resulted in a lowering of serum glucose levels. This was observed at the high dose at all of the sampling points, and the data from Week 26 are depicted in Table III.

As previously mentioned, the urine of dogs receiving 32 and to a significantly lesser extent 8 mg/kg/day was light to dark brown in appearance throughout the 26-week treatment period. Urinary bilirubin levels were significantly elevated at 32, 8 and possibly 2 mg/kg/day, commencing at Week 17 and thereafter. In

TABLE III

EFFECTS OF ORAL ADMINISTRATION OF TNT TO BEAGLE DOGS FOR 26 WEEKS ON SELECTED CLINICAL CHEMISTRY PARAMETERS

		Dose (mg/kg/day)				
		0	0.5	2	8	32
SGPT (IU/l)	Males	19 ± 6	20 ± 4	16 ± 7	7[b] ± 2	3[b] ± 2
	Females	22 ± 7	16 ± 5	12[b] ± 4	10[b] ± 3	5[b] ± 3
Alb./Glob.	Males	1.3 ± 0.2	1.2 ± 0.2	1.1[b] ± 0.1	1.1[b] ± 0.0	0.9[b] ± 0.3
	Females	1.3 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.4 ± 0.3	1.1[b] ± 0.1
Total Bilirubin (mg/dl)	Males	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.5[b] ± 0.2
	Females	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5[b] ± 0.2
Glucose (mg/dl)	Males	94 ± 8	87 ± 9	83 ± 9	78 ± 16	73[b] ± 10
	Females	88	89	81	84	67[b]

[a]Week 26 data (Mean ± S.D.; $N = 6$ unless otherwise noted in parentheses).
[b]Mean significantly different from appropriate control group mean, $P \leq 0.05$.

TABLE IV

EFFECTS OF ORAL ADMINISTRATION OF TNT TO BEAGLE DOGS ON ORGAN WEIGHTS (% BODY WEIGHT)[a]

		Dose (mg/kg/day)				
		0	0.5	2	8	32
Liver	Males	2.79 ± 0.10	3.28 ± 0.36	2.99 ± 0.29	3.67[b] ± 0.34	4.42[b] ± 0.71
	Females	2.92 ± 0.47	2.92 ± 0.50	3.06 ± 0.48	3.33 ± 0.11	4.37[b] ± 0.32
Kidneys	Males	0.59 ± 0.09	0.62 ± 0.09	0.52 ± 0.03	0.61 ± 0.14	0.61 ± 0.15
	Females	0.47 ± 0.09	0.51 ± 0.07	0.45 ± 0.03	0.50 ± 0.04	0.59[b] ± 0.08

[a]Mean ± S.D.; $N = 6$.
[b]Mean significantly different from appropriate control group mean, $P < 0.5$.

addition, trace levels of urobilinogen were observed during this time for 32 mg/kg/day treated dogs. Urinary protein levels appeared to be increased at Test Week 26 for dogs receiving 8 or 32 mg/kg/day. No other clinical pathology parameters appeared to be affected by TNT administration.

Ophthalmology/electrocardiography

Ophthalmology examinations and analysis of ECG tracings failed to detect changes related to TNT treatment. Decreases in heart rate and increases in PQ and QT intervals with time were observed for all groups, but were not dose-related.

Pathology/organ weights

At scheduled necropsy, four of six male and three of four female dogs receiving 32 mg/kg/day demonstrated enlarged livers. In addition, two of the livers for each sex appeared friable. Statistical analyses of liver weights confirmed hepatomegaly for dogs receiving 8 or 32 mg/kg/day (Table IV).

Hepatocytic cloudy swelling, which generally increased in severity as a function of dose, was present in all TNT treatment groups, but was not seen for control animals (Table V). At 0.5 mg/kg/day, the animals demonstrated trace to mild lesions. By contrast, 32 mg/kg/day resulted in moderate to marked severity for this morphologic alteration. The female which spontaneously died and the moribund female which was killed did not show evidence of this lesion, suggesting a minimum exposure period for expression of this microscopic change.

Hepatocytomegaly of trace to mild severity was observed in males and females receiving 0.5 mg/kg/day. None of the control animals demonstrated this lesion. As in the case of hepatocytic cloudy swelling, hepatocytomegly increased in severity as a function of dose. For dogs administered 32 mg/kg/day, moderate to marked severity was noted.

Hemosiderosis in Kupffer's cells was seen for one female at 2 mg/kg/day (trace) and all animals at 8 or 32 mg/kg/day, except for the two females necropsied prior to schedule. The severity of this lesion increased as a function of dose. Microscopic evidence for hepatic cirrhosis was observed for animals which received 8 or 32 mg/kg/day.

Enlargement of the spleen appeared to be related to TNT administration. This was subsequently confirmed from statistical analyses of organ weight data. Histologically, marked to severe generalized congestion of the spleen was observed primarily for males and females receiving either 8 or 32 mg/kg/day. On the basis of incidence and/or severity, splenic hemosiderosis appeared to be related to the administration of 2 mg/kg/day or greater; the results at 0.5 mg/kg/day were equivocal. High dose treatment group dogs also demonstrated splenic extramedullary erythropoiesis.

Enteritis involving at least one level of small intestine appeared to be related to TNT treatment. Although not apparent at necropsy, this lesion was characterized microscopically by retention of villous tips and the presence of an inflammatory serous and cellular exudate within the body of villi. The incidence and severity of this morphologic alteration were similar at all dose levels with the possible excep-

TABLE V

HISTOPATHOLOGIC CHANGES FOLLOWING ORAL ADMINISTRATION OF TNT TO BEAGLE DOGS

Organ/Lesion	Dose (mg/kg/day)				
	0	0.5	2	8	32
LIVER					
Hepatocytomegaly					
Males	0	5	3	6	5
Females	0	2	5	6	3
Hemosiderosis					
Males	0	0	0	6	5
Females	0	0	1	6	4
Cirrhosis					
Males	0	0	0	1	6
Females	0	0	0	0	1
Cloudy swelling					
Males	0	5	5	6	5
Females	0	2	6	6	4
SMALL INTESTINE					
Membranous enteritis					
Males	0	2	3	2	5
Females	0	3	3	3	2
SPLEEN					
Sinusoidal congestion					
Males	2	0	1	6	6
Females	0	0	2	5	2
Hemosiderosis					
Males	1	2	2	6	5
Females	0	3	3	4	5
Erythropoiesis					
Males	0	0	0	0	4
Females	0	0	1	1	1
BONE MARROW					
Erythrocytic hypoplasia					
Males	0	1	2	5	2
Females	0	1	3	4	4

[a]Number of animals demonstrating lesions; $N = 6$.

tion of a higher incidence at 32 mg/kg/day. Enteritis was not seen for any of the control animals.

Erythroid hypoplasia was observed for some dogs of all treatment groups receiving TNT. It was not seen for any of the control animals. The incidence and/or severity of this change generally increased as a function of dose. Myeloid:Erythroid ratios were subsequently determined from bone marrow smears,

and suggested that 32 and possibly 8 mg/kg/day resulted in a reduction of this parameter for male but not female dogs.

Gross pathologic changes of the thyroids were not apparent although bilateral C-cell hyperplasia was seen for animals in all groups including controls. All of the females (6/6 in each group) demonstrate this morphologic change, with the frequency for males ranging from 4/6 to 6/6. The severity of this microscopic change appeared to be greater for animals receiving 32 mg/kg/day than for control animals, and males may have been more affected than females.

Enlarged pigmented lymph nodes were observed at necropsy for one 8 mg/kg/day and three 32 mg/kg/day females. In addition, a slight but statistically significant increase in renal weights was seen for high dose females but not males. No microscopic changes corresponded to these observations.

Discussion

This study examined the oral toxicity of TNT in beagle dogs following daily administration for 6 months. TNT was found to be lethal at the highest dose tested (32 mg/kg/day), as one female was sacrificed in a moribund state during Test Week 14 while another female died in Test Week 16. Prior to these conditions, these animals became dehydrated, emaciated, ataxic, icteric and hypothermic. Additional clinical signs of toxicity observed at this lethal dose level included orange-brown urine and feces, and darkening of the tongue and/or gums. In addition, high dose dogs showed reduced food intake with associated loss of body weight. Slight reductions in body weight gains were apparent at lower dose levels.

The presence of icterus at the 32 mg/kg/day dose level was supported by elevated bilirubin levels in serum and urine, and increased urobilinogen values. This was consistent with the observed anemic state for animals receiving either 8 or 32 mg/kg/day. Physiologic compensatory responses to anemia at these doses included reticulocytosis, macrocytosis, and elevated numbers of nucleated RBCs. Methemoglobinemia was also seen at the 8 and 32 mg/kg/day dose levels. A hemosiderin-like pigment in macrophages of the spleen and liver, and sinusoidal congestion of the splenic red pulp with accompanying splenomegaly were seen primarily at the higher doses. These observations suggested that TNT-induced anemia was hemolytic in origin. Methemoglobin production indicative of the oxidizing nature of TNT and/or its metabolites supports this concept. Erythrocytic hypoplasia of bone marrow was seen which may have been due to a direct hemolytic effect on RBC precursors. Hemolytic anemia with its subsequent splenic 'lesions' and methemoglobinemia were generally consistent with observations in rodent toxicity studies of TNT in our laboratories [2,3]. The beagle dog, however, appears to be somewhat more sensitive than rodents to TNT intoxication. This was also suggested from a recent short-term toxicity test in beagles by other investigators [4].

Liver injury following the administration of TNT was primarily observed from histologic examination. Hepatocytomegaly and hepatocytic cloudy swelling were seen at all dose levels tested with the incidence and/or severity of these lesions

generally increasing as a function of dose. Hepatomegaly and microscopic evidence of cirrhosis were also seen, but were restricted to the 32 and to a lesser extent 8 mg/kg/day dose levels. Histologic evidence of liver injury has not been seen in prior studies in rats, mice or beagles [2—4]. Additional observations in the study which may have been suggestive of hepatotoxicity included slight increases in LDH at 32 mg/kg/day and dose-dependent reductions of SGPT. This latter effect, seen at all doses except 0.5 mg/kg/day, the lowest dose level tested, may have suggested interference with SGPT synthesis by hepatocytes. As serum alkaline phosphatase levels were uneffected by TNT, it appears that the elevated serum bilirubin levels previously discussed were apparently due to hemolytic changes and not to cholestasis.

Additional toxic effects were seen primarily at 32 and to a lesser extent 8 mg/kg/day. These included thrombocytosis and bone marrow erythrocytic hypoplasia. Renal weights were increased for females but not males receiving 32 mg/kg/day. Urinary protein levels were elevated at 8 and 32 mg/kg/day, however, neither histologic changes in the kidneys nor glycosuria (in the presence of hypoglycemia) were in evidence. Morphologic changes in this organ have not been apparent in other short-term toxicity tests [2—4]. Although serum glucose values were significantly reduced for TNT-treated dogs, no other indication of altered carbohydrate metabolism was seen and microscopic examination of pancreas was non-remarkable. The utilization of glucose by RBCs in the production of NADH, a cofactor for methemoglobin reductase, may have accounted for a mild hypoglycemic response.

In summary, the major toxic effects following the oral administration of TNT to dogs included hemolytic anemia, methemoglobinemia, liver injury, splenomegaly with accompanying histologic lesions, and death. Only the highest dose given (32 mg/kg/day) proved to be lethal. Although the results of a previously reported short-term toxicity study in beagles suggested that 2 mg/kg/day was essentially a no effect level, hepatocytic cloudy swelling and hepatocytomegaly in the present study were apparent at all doses tested. Thus, a no observable effect level was not established in this investigation.

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