Table 3
Developmental toxicity of dinoseb in rabbits

Strain	Dose (mg/kg)	Exposure time	Developmental effect observed	Reference
Gavage				
Chinchilla rabbit	10	GDs 6–18	External, internal and skeletal defects	[47]
Dermal				
NZ white rabbit	3	GDs 7–19, 6 h/day	Hydrocephaly, anophthalmia	[45]
	9		†Dead and resorbed fetuses, cleft palate, microcephaly, microphthalmia	

18.8 mg/kg bw/day. At these doses, maternal death was not observed. Pretreatment with SKF-525A in combination with dinoseb at 15.8 mg/kg bw/day caused fetal anomalies, potentiated dinoseb-induced resorptions, and produced maternal mortality. SKF-525A in combination with dinoseb at 17.7 mg/kg bw/day was markedly lethal maternally, however developmental parameters could not be analyzed because of the small number of litters surviving. Therefore, it is likely that a proximate toxicant for maternal toxicity is dinoseb itself.

Swiss–Webster mice were treated with dinoseb on GDs 9–11 and maintained in an increased environmental temperature (32 °C) for 24 h or a decreased temperature (0–6 °C) for 1.5–4 h [44]. Exposure to 32 °C enhanced adverse effects of dinoseb; it increased maternal mortality, decreased fetal body weight, and increased the incidence of fetal anomalies at 7.5 mg/kg bw/day. Fetal body weight and the frequency of malformations were generally the same in groups exposed to low temperature and maintained at room temperature at 15.8–17.7 mg/kg bw/day. Maternal mortality was observed at doses which caused fetal toxicity. Based on these results, higher temperature enhanced the maternal and developmental toxicity of dinoseb.

2.2.3. Subcutaneous studies in mice

Dinoseb was subcutaneously administered to pregnant Swiss–Webster mice during GDs 8–16, 10–12 or 14–16 at 0, 10 or 17.7 mg/kg bw/day [15]. Adverse effects were observed only at 17.7 mg/kg bw/day. Dinoseb on GDs 14–16 induced increases in resorption rate and the incidence of cleft palate and decreases in the number of live fetuses, fetal CRL and fetal body weight. At this dose, one out of eight dams died. Dinoseb on GDs 10–12 induced an increase in the incidence of fused ribs/vertebrae and absent or not ossified sternebrae, and on GDs 8–16 induced supernumerary ribs/vertebrae, absent or not ossified sternebrae, decreased fetal body weight and decreased fetal CRL without maternal toxicity. The authors concluded that an s.c. dose of dinoseb was not teratogenic and cleft palate induced by treatment of dinoseb was not considered as a toxicological response because this anomaly was not found in any i.p.-treated groups, described in Section 2.2.2, or in other s.c. treatment groups given 17.7 mg/kg bw/day. However, this anomaly can be considered as a toxic effect because the incidence of cleft plate was statistically significant, and other studies showed that i.p. dose of dinoseb induced cleft plate in mice [44] and in rabbits [45]. These later reports were not referred to in Gibson's report (1973) [15]. Moreover, a recent survey by international experts in the field of reproductive/developmental toxicology resulted in high agreement that fused ribs and vertebrae are considered as a malformation [46]. Therefore, it can be concluded that an s.c. dosing of dinoseb in mice may have the potential to produce teratogenic effects in the same way as i.p. dosing of dinoseb.

2.3. Developmental toxicity in rabbits

Table 3 shows the results of developmental toxicity of dinoseb in rabbits. There are gavage and dermal dose studies. The data were reviewed by routes of administration, in order of the most likely route of human intake. Only statistically significant effects are summarized unless noted otherwise.

2.3.1. Gavage studies in rabbits

In a rabbit teratology study, 16 Chinchilla rabbits were exposed by gavage to dinoseb at 0 (vehicle corn oil), 1, 3 or 10 mg/kg bw/day on GDs 6–18 [47]. There were no differences in fetal body weight and sex ratio between the dinoseb-treated and control groups. In the highest dose group, there were neural tube defects including dyscrania associated with hydrocephaly, scoliosis, kyphosis, malformed or fused caudal or sacral vertebrae and/or encephalocele in a total of 40 fetuses in 11/16 litter. Eleven fetuses showed only hydrocephalus and/or micro- or anophthalmia, and four fetuses showed only skeletal abnormalities. No maternal death occurred. Body weight gain and food consumption in dams and number of implantations were not affected. Rabbits were more susceptible to teratogenic influence by dinoseb than most of rats and mice.

2.3.2. Dermal studies in rabbits

In a New Zealand white rabbit study, 16–17 pregnant rabbits were dermally given dinoseb at 0, 1, 3, 9 or 18 mg/kg bw/day on GDs 7–19 [45]. Six hours after the dinoseb (no vehicle was used) was dermally applied to rabbits wearing Elizabethan collars, the application site was wiped and then dried. Because overt maternal toxicity was observed at 18 mg/kg bw/day and animals were also dying in the 9 mg/kg bw/day group, animals treated with dinoseb at the high dose were reassigned to the 9 mg/kg bw/day dose group and did not contribute to the evaluation. The mean number of implantations was not affected by treatment. There were increased incidences of anophthalmia and hydrocephaly at 3 and 9 mg/kg bw/day. Dead and resorbed fetuses and fetuses with cleft palate, microphthalmia and microcephaly were increased at 9 mg/kg bw/day. At 3 mg/kg bw/day and higher, hyperthermia and reduced body weight were observed in maternal rabbits.

3. Discussion and conclusions

A difficulty lies in the risk assessment of chemical compounds for developmental toxicity because there are many variable factors in the manifestation of developmental toxicity of chemicals. Species difference is one of the definitive factors in the developmental toxicity of chemicals. In this review, we showed that developmental toxicity of dinoseb was also remarkably different between animal species used in experiments. Susceptibility to developmental toxicity caused by dinoseb was greater in rabbits than in rats and mice.

Doses of dinoseb in rats by gavage during organogenesis induced skeletal variations and growth retardation at maternally toxic levels (10–20 mg/kg bw/day) [16]. Malformations such as microphthalmia or hypoplastic tail were observed when dinoseb was given in diet (10.86–15 mg/kg bw/day) or by gavage (15 mg/kg bw/day) with a certain composition of diet (protein 21%, fat 4.8%, fiber 4.2%, ash 8.5% and N-free extractives 61.5%) in CD rats [16,20,33]. In Wistar/Han rats, absence of thoracic vertebrae was observed at 3 mg/kg bw/day and higher without maternal toxicity. Prena-

tal i.p. dose of dinoseb did not induce teratogenicity but induced growth retardation at or above the maternally toxic level in rats [35,36]. These findings in rats suggest that the composition of diet used in experiments, genetic difference in strains of rats, and route of administration may influence developmental toxicity of dinoseb.

In mice, gavage dosing of dinoseb during organogenesis induced skeletal variations and growth retardation at or above maternally toxic levels (26–50 mg/kg bw/day). Teratogenic effects were observed without maternal toxicity at 50 mg/kg bw/day by gavage in CD-1 mice. Prenatal i.p. and s.c. doses of dinoseb induced growth retardation, embryolethality and/or teratogenicity at or over the maternally toxic dose levels (10–20 mg/kg bw/day) in Swiss–Webster mice. The maternal toxicity of dinoseb seems greater in rats than in mice, because dinoseb treatment (i.p.) during GDs 10–12 at 9.0 mg/kg bw/day caused 3/16 maternal deaths in rats [35] while no maternal toxicity was observed at 15.8 mg/kg bw/day after i.p. dosing of dinoseb during GDs 10–12 in mice [15]. This may explain why teratogenicity was induced in mice, but not in rats after i.p. dosing of dinoseb. It can be considered that maternal mice were tolerant to dose levels that can produce fetal malformations.

Gavage dosing of dinoseb during organogenesis in rabbits produced external, internal and skeletal malformations in fetuses without maternal toxicity at 10 mg/kg bw/day [13,17]. A dermal teratology study in rabbits also showed a markedly increased incidence of dead and resorbed fetuses [45]. The survivors evidenced a high incidence of external and soft-tissue malformations at application levels of dinoseb that were also maternally toxic. It appears that administration of dinoseb is teratogenic in rabbits.

The developmental toxicity of dinoseb was also influenced by administration methods. These effects are considered to be related to differences in absorption owing to concentration of the chemical, duration of exposure, and rate of release or to differences in metabolic fate and the nature of the metabolites reaching the embryo [48]. In fact, food deprivation for 24 h that enhanced external, soft and skeletal malformations slowed disappearance of dinoseb from the plasma, but phenobarbital that reduced developmental toxicity hastened disappearance of dinoseb from the plasma. SKF-525A pretreatment which enhanced both maternal and developmental toxicity decreased the rate of disappearance from the liver [43]. When pregnant mice were dosed with dinoseb by either i.p. at 17.7 mg/kg bw or gavage at 32 mg/kg bw, the amount of dinoseb and its metabolites present in the embryo was greater after i.p. than oral administration, and peak levels were reached much earlier after i.p. administration (8 min vs. 12 h for oral) [6]. Developmental effects of i.p. dosing of dinoseb in mice can be related to rapid and relatively extensive uptake of the compound or its metabolites by the embryo.

There is a recent paper that evaluated the relationship of maternal and fetal toxicity in rats, mice and rabbits by analyzing 125 developmental toxicity bioassays [49]. In these bioassays, most LOAELs were determined by reduced maternal gestational weight gain or fetal weight at term, and lower fetal weights were associated with reduced maternal weight gains due to reduced food consumption. The authors of this paper noted that these findings may indicate that some of the developmental adverse effects observed in these bioassays were general toxicity due to agents that disrupt food consumption rather than developmental toxicity. General toxicity and real developmental toxicity should be distinguished for the regulatory process. In our previous study [19], dinoseb produced a reduction in maternal body weight gain, but food consumption was increased. Dinoseb possesses agent-specific adverse effects as a metabolic activator, and the possibility of the observed developmental adverse effects resulting from general toxicity can be ruled out.

There is no clear understanding of the fundamental mechanism of developmental toxicity of dinoseb, but energy-deficient intra-uterine environment due to uncoupling of cellular oxidative phosphorylation may explain dinoseb-induced developmental toxicity. Oxidative metabolism is essential for embryonic/fetal development, and oxidative phosphorylation increases as gestation progresses [50,51]. Alternations in energy sources can contribute to developmental toxicity. For example, riboflavin deficiency [52] and folic acid deficiency [53], which produced fetal malformations, were considered to be associated with deficient mitochondrial activity and uncoupling of oxidation from phosphorylation, respectively. These examples may also explain the quality of diet modulating the toxic effects of dinoseb. However, it is difficult to identify the definitive dietetic factor involved.

In this paper, developmental toxicity including structural malformations, growth retardation, embryolethality, and functional abnormalities were reviewed. Recently, Hass [54] noted that the central nervous system appears to be especially susceptible to toxic insults during its development, and several developmental neurotoxicants have been identified in humans during the last decade. Investigations indicate that neurobehavioral dysfunction in children can be detected at a lower dose than malformations or in the complete absence of malformations. However, we are unaware of any developmental neurotoxicity studies of dinoseb. Further studies are needed to clarify the developmental neurotoxicity of dinoseb.

Over the years, many investigations have been done using laboratory animals to assess the risk to humans. This review paper reiterates the importance of the administration method to extrapolate laboratory results to the human situation. It is essential that the relevant mode of administration to human intake be used in laboratory studies for human risk assessment. Human exposure to dinoseb is anticipated to occur by direct contact, ingestion and inhalation for users and producers. In this review, we show that fetal malformations are produced by gavage and dermal exposure in rabbits. Confirmation of the adverse effects on reproduction and development in other species of mammals exposed by anticipated route of human exposure would be important for risk assessment in humans.

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Combined Repeated Dose and Reproductive/ Developmental Toxicity Screening Test of the Nitrophenolic Herbicide Dinoseb, 2-*sec*-Butyl-4,6-Dinitrophenol, in Rats

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ABSTRACT: In a combined repeated dose toxicity study with reproduction/developmental toxicity screening test, Crj:CD(SD)IGS rats were dosed with dinoseb, 2-*sec*-butyl-4,6-dinitrophenol, by gavage at 0 (vehicle), 0.78, 2.33, or 7.0 mg/kg bw/day. Six males per group were dosed for a total of 42 days beginning 14 days before mating. Twelve females per group were dosed for a total of 44–48 days beginning 14 days before mating to day 6 of lactation throughout the mating and gestation period. Recovery groups of six males per group and nonpregnant six females per group were dosed for 42 days followed by a 14-day recovery period. No deaths were observed in males of any dose group or in females of the recovery groups. At 7.0 mg/kg bw/day, eight females died and two animals were moribund during late pregnancy, and a significant decrease in body weight gain was found in both sexes. Hematocrit was significantly higher at 0.78 mg/kg bw/day and above in the main group males at the end of administration period. Reduction in extramedullary hematopoiesis in the spleen was significant at 2.33 mg/kg bw/day in the main group females. Sperm analysis revealed a decrease in sperm motility and an increase in the rates of abnormal sperm, abnormal tail, and abnormal head at 7.0 mg/kg bw/day. A number of dams delivered their pups and of dams with live pups at delivery was significantly lowered in the 7.0 mg/kg bw/day group. Based on these findings, the LOAEL for males and NOAEL for females were 0.78 mg/kg bw/day, and the NOAEL for reproductive/developmental toxicity was considered to be 2.33 mg/kg bw/day. © 2008 Wiley Periodicals, Inc. Environ Toxicol 23: 169–183, 2008.

Keywords: dinoseb; nitrophenolic herbicide; 2-*sec*-butyl-4,6-dinitrophenol; repeated dose toxicity; reproductive and developmental toxicity; screening test; testis toxicity; rat

INTRODUCTION

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7), was approved for sale as a nitrophenolic herbicide in the United States in 1948, and it is used in soybeans, vegetables, fruits, nuts, citrus, and other field crops for the selective control of grass and broadleaf weeds. It is also used as an insecticide in grapes and as a seed crop drying agent (EXTOXNET, 1996). Although the use of dinoseb as a pesticide was banned in the United States in 1986 and in Europe in 1991, based on the potential risk of birth defects and other adverse health effects in humans (EXTOXNET,

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1996; Rotterdam Convention, 2006), it is reported that dinoseb is a high volume chemical with production or importation exceeding 1000 ton/year in Organisation for Economic Cooperation and Development (OECD) member countries and still widely used (OECD, 2004; PAN, 2006). Dinoseb and dinoseb salts are banned in Japan but consented to import (PAN, 2006). It is estimated that the volume of dinoseb imported to Japan is 110 ton from April 2005 to March 2006 (NITE, 2007).

Dinoseb is well absorbed by the oral route in mice (Gibson and Rao, 1973) and the dermal route in rats (Shah et al., 1987; Hall et al., 1992) and can pass through the placenta into the fetus of mice (Gibson and Rao, 1973). In a dermal toxicity study, dinoseb was more absorbed in adult female rats than in young rats (Shah et al., 1987). Dinoseb shows strong acute toxicity with the dermal LD_{50} of 40 mg/kg in rabbits (US EPA, 2003). The inhalation LC_{50} is 33–290 mg/m^3 for 4-h exposure in rats (US EPA, 2003). The basic mechanism of toxicity is thought to be stimulation of oxidative metabolism in cell mitochondria by the uncoupling of oxidative phosphorylation (Leftwich et al., 1982). Toxicity of dinoseb is enhanced by physical activity and high ambient temperature such as in an outdoor agricultural environment (Leftwich et al., 1982; US EPA, 2007). Early symptoms of dinoseb exposure include hyperthermia, sweating, headache, and confusion. Other signs and symptoms include dyspnea, pulmonary edema, nausea, vomiting, abdominal pain, malaise, dehydration, and tachycardia. Severe exposure may result in restlessness, seizures, coma, and death (Leftwich et al., 1982; US EPA, 2006, 2007).

As for developmental toxicity, many studies were conducted in experimental animals in the 1970s and the 1980s. Dinoseb was reported to be teratogenic in mice when administrated intraperitoneally or subcutaneously (Gibson, 1973; Preache and Gibson, 1975a,b), but not by gavage administration (Gibson, 1973). Dietary administered dinoseb was also reported to induce several adverse effects on reproduction and development including teratogenic effects in rats (McCormack et al., 1980; Spencer and Sing, 1982; Giavini et al., 1986; Daston et al., 1988). In a rabbit teratology study, Chinchilla rabbits were exposed by oral gavage to dinoseb at levels of 0, 1, 3, or 10 mg/kg bw/day on days 6–18 of gestation. Teratogenic effects were observed at 10 mg/kg bw/day without maternal toxicity (Research and Consulting Company, 1986). This study, conducted by a laboratory in Switzerland, became a main trigger for the cancellation of dinoseb. A male reproductive toxicity study in rats showed decreased sperm counts and increased atypical spermatozoa when receiving 9.1 mg/kg bw/day and above of dinoseb in feed (Linder et al., 1982). This result is in concordance with a recent study by Takahashi et al. (2004) in which reduced sperm motility and increased incidence of tailless sperm were found.

Exposure to dinoseb may occur by direct contact, ingestion, and inhalation for users and producers, but potential indirect exposure to dinoseb via the environment for consumers is also anticipated. Dinoseb is not strongly adsorbed on most agricultural soils. Microbial breakdown of dinoseb is demonstrated on soils, but dinoseb persists for about 2–4 weeks after application. Dinoseb was reported to be detected in water supplies in Canada and the United States (Health Canada, 1991). The US FDA examined 70 food items in 1985 and 1986 for dinoseb residues. Although no residues were detected in most of crops treated with dinoseb, a positive result was obtained in one cotton meal sample (Health Canada, 1991). Dinoseb is listed in the most recent OECD List of High Production Volume (HPV) chemicals to be investigated for environment and human health effects (OECD, 2004). Although many studies had been conducted for developmental toxicity of dinoseb, these reports could be determined to be inadequate for the initial assessment of the chemical in the OECD HPV Chemicals Programme, because these studies were non-Good Laboratory Practice (GLP) studies or did not totally comply with a specific testing guideline (Klimisch et al., 1997; OECD, 2005). No studies on female reproductive performances were evaluated. Therefore, dinoseb was selected as a target substance for the Safety Examination of Existing Chemicals in Japan (MHLW, 2005) to obtain reliable information on the possible effects on reproduction and development in compliance with the OECD Test Guideline and in accordance with the principles for GLP. The present article reports the result of combined repeated dose and reproductive/developmental toxicity screening test of dinoseb in rats.

MATERIALS AND METHODS

This study was performed in 2003–2004 at the Nihon Bioresearch (Hashima, Japan) in compliance with OECD Guideline 422 Combined Repeated Dose Toxicity Study with Reproduction/Developmental Toxicity Screening Test (OECD, 1996) and in accordance with the OECD Principles for GLP (OECD, 1998) and Law Concerning the Examination and Regulation of Manufacture, etc., of Chemical Substances (EA, 1984). All animals were treated in accordance with the law governing the protection and management of animals (MOE, 1973), the guidelines for experiments using animals (JALAS, 1987), and the Regulations of the Committee for the Ethical Treatment of Animals (Hashima Laboratory, Nihon Bioresearch).

Animals

International Genetic Standard (Crj: CD(SD)IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and

historical control data are available. Males and females at 7 weeks of age were purchased from Hino Breeding Center, Charles River Japan, (Yokohama, Japan). The rats were quarantined for 5 days and acclimatized to the laboratory for 16 or 17 days before the start of the experiment. Male and female rats found to be in good health were selected for use. The vaginal smears of each female were recorded, and only females showing a 4-day or 5-day estrous cycle were used in the experiment. Male and female rats were distributed into four groups on a random basis. Each group consisted of six males and 12 females as main groups and six males and six females as recovery groups. Rats were housed individually except during the mating period. From day 18 of pregnancy to day 3 of lactation, individual dams and litters were reared using wooden chips as bedding (Sunflake®, Charles River Japan).

Animals were reared on a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and water *ad libitum* and maintained in an air-conditioned room at a room temperature of 20–26°C, a relative humidity of 40–70%, a 12-h light/dark cycle, and 12 air changes per hour.

Chemicals and Dosing

Dinoseb, yellowish crystals with pungent odour, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The dinoseb (Lot no. RWN9641) used in this study was 96% pure, and was stored under refrigeration before use. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with dinoseb at a dose of 0 (control), 0.78, 2.33, or 7.0 mg/kg bw. The dosage levels were determined based on a previous dose-finding study in which no effects were seen at 5 mg/kg bw/day, but deaths and lower body weights were found at 10 and 20 mg/kg bw/day in rats given dinoseb by gavage at 0, 2.5, 5, 10, or 20 mg/kg bw/day for 14 days. The dinoseb was suspended in corn oil, and the control rats were given only corn oil. Twelve males per group were dosed for a total of 42 days beginning 14 days before mating. After the administration period, 6 of 12 males per group were reared for 14 days without administration of dinoseb as the recovery groups. The main group females were dosed for a total of 44–48 days beginning 14 days before mating to day 6 of lactation throughout the mating and gestation period. The recovery group females were given dinoseb for a total of 42 days, followed by a recovery period of 14 days. The first day of dosing was designated as day 0 of administration and the day after the final dose was designated as day 0 of the recovery period. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight. The stability of formulations was confirmed after storage under refrigeration in the dark for 7 days. During use, the formulations were maintained under such conditions for up to 7 days, and the concentration of each preparation was within the acceptable range (91.3–96.4%).

Observations

All rats were observed daily for clinical signs of toxicity. Body weight was recorded twice a week in males and females of the recovery groups, and twice a week during the premating period, on days 0, 7, 14, and 21 of pregnancy and on days 0, 4, 6, and 7 of lactation in females of the main groups. Food consumption was recorded twice a week in males and in the recovery group females, and twice a week during the premating period, on days 2, 9, 16, and 20 of pregnancy and on day 2 of lactation in the main group females. Functional observation battery (FOB) in all animals was recorded once a week during the administration period for 1 h following administration, as follows: (i) posture, biting behaviour, eyelid closure, and convulsion, (ii) ease of removal from cage and handling, muscle tone, fur condition, lacrimation, salivation and respiration, and (iii) rearing frequency, grooming frequency, ambulation, palpebral closure, arousal, behavioural abnormality, and righting reflex.

Six animals in each group, with the exception of one surviving female in the 7.0 mg/kg bw/day main group, were subjected to the following observations and examinations unless noted otherwise. Sensory reactions for pupillary reflex, approximation reflex, behavioural abnormality, tactile reflex, auditory reflex and pain reflex, and grip strength of fore and hind limbs were tested in the main group males on day 40 of administration and in the main group females on day 3 of lactation. Spontaneous motor activity, ambulation, and rearing were recorded (Activity Monitor, Med Associate, Vermont) after administration for 1 h at intervals of 10 min on day 39 of administration in the main group males and on day 4 of lactation in the main group females. Fresh urine was sampled from animals using a urine-collecting cage under fasting and watering conditions and collected for 24 h.

The main group rats were euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 7 of lactation in females, and the recovery group rats were euthanized on the day after completion of the recovery period. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta. The brain (cerebrum, cerebellum, and medula oblongata), pituitary gland, thyroid, thymus, heart, liver, spleen, kidney, adrenal glands, testis, epididymis, tail of the epididymis, ovary, and uterus were isolated and weighed. The lung, trachea, pancreas, salivary gland (sublingual gland and submandibular gland), esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, lymph nodes (mandibular lymph nodes and

mesenteric lymph node), bladder, seminal vesicle, prostate, vagina, parathyroid, spinal cord, sciatic nerve, eye ball, Hardarian gland, sternum, and femur were removed. The numbers of corpora lutea and implantation sites were counted in all the main group females. The testes and epididymides were fixed with Bouin's solution and 90% alcohol. The eye ball was fixed with glutaraldehyde formalin and 20% neutral buffered formalin. Other organs were stored in 20% neutral buffered formalin. Histopathological evaluations were performed on these organs. The caudal epididymis was used for determination of the sperm motility, sperm viability, sperm morphology, and sperm count.

The right epididymal cauda was minced and stored for 5 min in Medium 199 containing 0.5% bovine serum albumin (BSA) at 37°C. This original sperm suspension was used for tests of sperm motility, viability, and morphology. The original sperm suspension was diluted with Medium 199 containing 0.5% BSA and incubated for about 30 min in 5% CO_2 in air at 37°C. After the incubation, the sperm samples were loaded into a chamber (Microslides #HTR1099; VitroCom, Mountain Lakes, NJ), and sperm motility was analyzed using a TOX IVOS (Hamilton Thorne Research, Beverly, MA). The original sperm sample was stained with calcein acetoxy methyl ester (CAM) and ethidium homodimer-1 (EthD-1) and incubated for about 60 min in 5% CO_2 in air. The population of viable and dead spermatozoa was identified by using a fluorescence microscopy according to Kato et al. (2002), and sperm viability and survivability rates were calculated. The original sperm suspension was mounted on a slide glass, fixed with the 10% neutral-buffered formalin and stained with 1% eosin. Sperm morphology was observed by using a microscope. The left epididymal cauda was stored at −80°C and homogenized with 0.1% Triton X-100. Number of sperm was determined with the TOX IVOS (Hamilton Thorne Research).

Urine samples were tested for colour, pH, protein, glucose, ketone body, bilirubin, occult blood, and urobilinogen. Urinary sediment was stained and examined microscopically. Urine volume was calculated from the specific gravity and weight. The collected blood samples were measured for the red blood cell (RBC) count, hemoglobin, platelet count, and white blood cell count. In addition, mean cell volume (MCV), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration, reticulocyte rate, and differential leukocyte rates were calculated. Prothrombin time (PT), activated partial thromboplastin time, and fibrinogen were determined. Blood chemistry was tested for aspartate aminotransferase, alkaline phosphatase (ALP), γ-glutamyl transpeptidase, total protein, albumin, albumin/globulin (A/G) ratio, total bilirubin, blood urea nitrogen (BUN), creatinine, glucose, total cholesterol, triglycerides, Na, K, Cl, Ca, and inorganic phosphate.

Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the premating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the 2-week mating period had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence for successful mating. Once insemination was confirmed, the females were checked for signs of parturition before 10:00 from day 21 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 6. The day on which parturition was completed by 10:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and live pups were sexed, and individually weighed on PNDs 0 and 4. Pups were inspected for external malformations and malformations within the oral cavity on PND 0. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross internal examinations were performed.

Data Analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. Mean and standard deviation in each dose group were calculated for the body weight gain, food consumption, grip strength, spontaneous motor activity, urine volume, urinary specific gravity, hematological test results, blood biochemical test results, absolute and relative organ weights, sperm test results, numbers of estrous cases and days till copulation after pairing, length of gestation, numbers of corpora lutea and implantations, implantation index, total number of pups born, number of live and dead pups, sex ratio, delivery index, birth index, live birth index, live pups and viability index on day 4 of lactation, and body weight of pups. These were analyzed with Bartlett's test (Bartlett, 1937) for homogeneity of variance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test (Dunnett, 1955) to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the Dunnett's rank test (Miller, 1981) was applied. The copulation index, fertility index, and gestation index were analyzed with Fisher's exact test (Fisher, 1973). The 5% levels of probability were used as the criterion for significance.

RESULTS

General Findings

Table 1 shows the general findings in male rats given dinoseb. No deaths were observed in any of the groups. Transient salivation was observed immediately after administration in one male in the 0.78 mg/kg bw/day dose group on day 14, in 11 males on day 8 or later in the 2.33 mg/kg bw/day dose group and in all 12 males on day 2 or later in the

TABLE I. General findings in male rats given dinoseb

Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0
No. of male rats	12	12	12	12
No. of deaths	0	0	0	0
Initial body weight (g)[a]	362 ± 13	363 ± 11	361 ± 13	360 ± 16
Body weight gain (g)				
The administration period				
Days 0–7	18 ± 9	23 ± 6	17 ± 8	7 ± 7**
Days 7–14	18 ± 7	18 ± 4	16 ± 6	14 ± 6
Days 14–21	18 ± 7	17 ± 5	17 ± 8	9 ± 10*
Days 21–28	22 ± 8	18 ± 6	21 ± 6	16 ± 7
Days 28–35	20 ± 4	22 ± 5	19 ± 5	19 ± 5
Days 35–41	10 ± 8	11 ± 6	11 ± 6	9 ± 8
The recovery period				
Days 0–7	13 ± 6	18 ± 4	14 ± 10	26 ± 7**
Days 7–14	6 ± 9	11 ± 3	13 ± 7	18 ± 3*
Food consumption (g/day/rat)				
The administration period				
Days 0–1	20 ± 2	20 ± 2	17 ± 3*	17 ± 2*
Days 3–4	21 ± 3	21 ± 2	20 ± 2	23 ± 2
Days 7–8	21 ± 3	21 ± 2	21 ± 3	23 ± 4
Days 10–11	20 ± 3	21 ± 2	21 ± 2	24 ± 2**
Days 28–29	21 ± 2	20 ± 2	21 ± 2	23 ± 3
Days 31–32	21 ± 2	21 ± 3	21 ± 2	24 ± 3**
Days 35–36	20 ± 2	21 ± 3	19 ± 2	23 ± 2*
Days 38–39	22 ± 2	21 ± 2	22 ± 3	24 ± 2*
The recovery period				
Days 0–1	22 ± 2 (6)	22 ± 2 (6)	21 ± 2 (6)	25 ± 2* (6)
Days 3–4	24 ± 2 (6)	24 ± 2 (6)	21 ± 2 (6)	25 ± 2* (6)
Day 7–8	26 ± 3 (6)	24 ± 3 (6)	25 ± 4 (6)	27 ± 2 (6)
Days 10–11	22 ± 3 (6)	25 ± 3 (6)	27 ± 2** (6)	27 ± 1** (6)

Figures in parentheses indicate number of males.
[a] Values are given as the mean ± SD.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

7.0 mg/kg bw/day dose group. Body weight gain was significantly decreased on days 0–7 and 14–21 at 7.0 mg/kg bw/day during the administration period, and it was significantly increased at 7.0 mg/kg bw/day during the recovery period. During the administration period, food consumption was significantly low on days 0–1 at 2.33 and 7.0 mg/kg bw/day, and it was significantly higher than controls on days 10–11, 31–32, 35–36, and 38–39 at 7.0 mg/kg bw/day. During the recovery period, no significant changes were observed in general condition and body weight gain at 0.78 and 2.33 mg/kg bw/day. Food consumption was significantly higher than controls on recovery days 10–11 at 2.33 and 7.0 mg/kg bw/day and on recovery days 0–1 and 3–4 at 7.0 mg/kg bw/day.

Table II presents the general findings in the main group female rats given dinoseb. In the 7.0 mg/kg bw/day main group, seven animals died on gestation day 19 and one on gestation day 21, and one animal was moribund on each of gestation days 19 and 20. Transient salivation was observed immediately after administration in five females on day 10 or later in the 2.33 mg/kg bw/day dose group and in 11 females on day 3 or later in the 7.0 mg/kg bw/day dose group. During the gestation period, transient salivation was observed immediately after administration in three animals at 2.33 mg/kg bw/day and in all females at 7.0 mg/kg bw/day. In addition, females in the highest dose group that died or were moribund showed a decrease in spontaneous motor activity, prone position, low body temperature, dyspnea, cyanosis, and bradypnea. In the 7.0 mg/kg bw/day main group, body weight gain was significantly lowered on administration days 0–7 compared with controls. During the gestation and lactation, no significant change was observed in body weight gain in any of the dinoseb-treated groups. There was significantly higher food consumption compared with controls on days 7–8 of the premating period and on gestation days 1–2, 8–9, and 15–16 at 7.0 mg/kg bw/day in the main groups.

In females of the recovery groups, no deaths were observed. Food consumption was significantly increased during the administration period at 7.0 mg/kg bw/day and

TABLE II. General findings in female rats given dinoseb

Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0
No. of female rats	12	12	12	12
No. of deaths during premating period	0	0	0	0
No. of deaths during pregnancy	0	0	0	10
Initial body weight (g)[a]	251 ± 9	250 ± 8	250 ± 9	251 ± 9
Body weight gain (g)				
Days 0–7	12 ± 9	8 ± 11	10 ± 6	2 ± 5**
Days 7–14	12 ± 7	9 ± 6	11 ± 4	8 ± 7
Days 0–7 of gestation	35 ± 9 (11)	35 ± 6 (11)	34 ± 6	37 ± 6
Days 7–14 of gestation	34 ± 4 (11)	34 ± 2 (11)	37 ± 4	38 ± 5
Days 14–21 of gestation	91 ± 12 (11)	79 ± 32 (11)	85 ± 13	65 (2)
Days 0–6 of lactation	6 ± 11 (11)	10 ± 9 (10)	14 ± 11	9 (1)
Food consumption (g/day/rat)				
Days 0–1	13 ± 2	15 ± 3	15 ± 1	14 ± 2
Days 3–4	15 ± 3	16 ± 4	15 ± 2	16 ± 3
Days 7–8	13 ± 2	14 ± 3	13 ± 3	17 ± 3**
Days 10–11	16 ± 4	16 ± 3	17 ± 3	18 ± 3
Days 1–2 of gestation	18 ± 2	18 ± 2	18 ± 1	22 ± 3**
Days 8–9 of gestation	19 ± 2	19 ± 2	19 ± 2	23 ± 2**
Days 15–16 of gestation	19 ± 2	20 ± 2	21 ± 2	23 ± 3*
Days 15–16 of gestation	19 ± 2	17 ± 5	20 ± 3	19 ± 5 (4)
Days 1–2 of lactation	23 ± 3	22 ± 4	21 ± 4	13 (1)

Figures in parentheses indicate number of dams.
[a] Values are given as the mean ± SD.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

during the recovery period at 2.33 mg/kg bw/day and above (data not shown).

Functional Observation

No treatment-related effects on the detailed clinical observations. FOB, sensory reactivity such as pupillary reflex, approximation reflex, behavioural abnormality, tactile reflex, auditory reflex and pain reflex, grip strength, and spontaneous motor activity were observed in males and females in the test and recovery groups (data not shown).

Urinalysis

Urinalysis revealed no significant differences in any parameters between the control and dinoseb-treated groups in males and females in the test and recovery groups (data not shown).

Hematology

Table III shows hematological findings in male and female rats. In males of the main groups, RBC count was significantly higher at 0.78 mg/kg bw/day and hematocrit was significantly higher at 0.78 mg/kg bw/day and above. Hemoglobin and PT were significantly higher than controls at 2.33 and 7.0 mg/kg bw/day. In the 7.0 mg/kg bw/day dose group, MCV was significantly higher than controls. As for females in the main groups, RBC count was significantly higher, and MCV and MCH were significantly lower at 0.78 mg/kg bw/day. Fibrinogen was significantly lower at 2.33 mg/kg bw/day. RBC count was significantly lower, and MCV was significantly higher in the 7.0 mg/kg bw/day recovery dose group males.

Blood Biochemistry

Blood biochemical findings are shown in Table IV. Total cholesterol was significantly lower at 2.33 mg/kg bw/day, and creatinine was significantly higher at 7.0 mg/kg bw/day in the main group males. Albumin and A/G ratio were significantly higher in females of the main groups at 2.33 mg/kg bw/day. In the recovery group males, A/G ratio and Cl were significantly lower at 0.78 mg/kg bw/day. A/G ratio was significantly lower, and total cholesterol was significantly higher at 2.33 mg/kg bw/day. In the 7.0 mg/kg bw/day group, ALP and albumin were significantly lower and BUN was significantly higher.

Organ Weight

Table V shows organ weight of male and female rats given dinoseb. At 7.0 mg/kg bw/day, relative brain weight was significantly higher in the main group males. At the end of

TABLE III. Hematological findings in male and female rats given dinoseb

	Main Group				Recovery Group			
Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0	0 (Control)	0.78	2.33	7.0
No. of male rats	6	6	6	6	6	6	6	6
RBC ($10^4/\mu L$)[a]	801 ± 13	844 ± 30*	833 ± 31	834 ± 29	858 ± 21	831 ± 22	852 ± 38	817 ± 22*
Hemoglobin (g/dL)	14.9 ± 0.5	15.5 ± 0.7	15.7 ± 0.5*	16.2 ± 0.5**	15.9 ± 0.4	15.3 ± 0.4	15.6 ± 1.1	15.8 ± 0.3
Hematocrit (%)	43.9 ± 0.8	46.4 ± 1.9*	46.6 ± 1.4**	47.7 ± 1.2**	46.8 ± 0.9	45.2 ± 1.2	46.6 ± 3.4	46.0 ± 1.0
MCV (fL)	54.8 ± 1.3	55.0 ± 1.1	56.0 ± 1.6	57.2 ± 1.5*	54.5 ± 0.8	54.4 ± 1.5	54.6 ± 1.8	56.3 ± 1.0
MCH (pg)	18.6 ± 0.7	18.4 ± 0.6	18.8 ± 0.6	19.4 ± 0.7	18.6 ± 0.5	18.4 ± 0.6	18.3 ± 0.7	19.3 ± 0.4*
MCHC (g/dL)	33.8 ± 0.5	33.5 ± 0.7	33.6 ± 0.4	33.9 ± 0.7	34.0 ± 0.4	33.9 ± 0.6	33.6 ± 0.4	34.3 ± 0.4
Platelet count ($10^4/\mu L$)	91.2 ± 10.2	95.0 ± 15.4	88.1 ± 11.9	78.2 ± 7.5	91.2 ± 10.2	95.0 ± 15.4	88.1 ± 11.9	78.2 ± 7.5
Reticulocyte (‰)	28 ± 3	24 ± 3	25 ± 4	24 ± 3	24 ± 6	23 ± 4	24 ± 6	22 ± 3
PT (s)	13.2 ± 0.3	13.6 ± 0.5	13.8 ± 0.3*	14.1 ± 0.4**	19.1 ± 3.1	19.5 ± 3.5	19.9 ± 3.6	16.3 ± 1.5
APTT (s)	27.6 ± 1.1	29.8 ± 1.6	28.3 ± 3.2	28.2 ± 1.8	37.1 ± 4.0	37.0 ± 2.2	38.2 ± 1.8	34.2 ± 3.2
Fibrinogen (mg/dL)	220 ± 10	221 ± 23	224 ± 16	207 ± 10	220 ± 10	221 ± 23	224 ± 16	207 ± 10
WBC ($10^2/\mu L$)	78 ± 22	61 ± 14	66 ± 23	55 ± 16	61 ± 16	54 ± 22	76 ± 20	49 ± 10
Differential leukocyte (%)								
Lymphocyte	94.7 ± 2.3	91.2 ± 2.9	91.7 ± 2.1	90.8 ± 5.9	90.2 ± 2.8	90.8 ± 4.2	92.2 ± 4.2	88.7 ± 7.2
Neutrophil	4.5 ± 1.8	7.8 ± 2.1	7.7 ± 1.5	8.5 ± 5.5	9.0 ± 3.0	8.2 ± 4.7	6.8 ± 3.9	10.0 ± 6.8
Eosinophil	0.5 ± 0.5	0.5 ± 0.8	0.3 ± 0.5	0.5 ± 0.5	0.3 ± 0.5	0.5 ± 0.8	0.5 ± 0.5	0.7 ± 0.5
Basophil	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Monocyte	0.3 ± 0.5	0.5 ± 0.5	0.3 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.5	0.7 ± 0.5
No. of female rats	6	6	6	1	6	6	6	6
RBC ($10^4/\mu L$)	702 ± 13	749 ± 25*	735 ± 41	725	739 ± 21	751 ± 45	758 ± 30	753 ± 26
Hemoglobin (g/dL)	14.4 ± 0.4	14.7 ± 0.5	14.8 ± 0.7	15.2	14.6 ± 0.4	14.8 ± 0.8	14.8 ± 0.5	14.9 ± 0.4
Hematocrit (%)	41.8 ± 1.1	42.8 ± 1.1	42.8 ± 1.9	44.6	41.4 ± 1.1	42.1 ± 2.1	42.3 ± 1.6	42.4 ± 1.5
MCV (fL)	59.5 ± 2.1	57.1 ± 1.1*	58.2 ± 1.2	61.5	56.0 ± 0.9	56.1 ± 1.4	55.8 ± 1.6	56.3 ± 0.6
MCH (pg)	20.6 ± 0.7	19.7 ± 0.2*	20.1 ± 0.6	21.0	19.7 ± 0.5	19.7 ± 0.6	19.4 ± 0.7	19.8 ± 0.4
MCHC (g/dL)	34.6 ± 0.6	34.4 ± 0.4	34.5 ± 0.5	34.1	35.2 ± 0.6	35.2 ± 0.3	34.8 ± 0.3	35.1 ± 0.5
Platelet count ($10^4/\mu L$)	105.2 ± 8.4	115.9 ± 9.9	112.6 ± 11.9	109.5	96.1 ± 13.0	94.9 ± 5.4	94.2 ± 11.4	90.3 ± 2.7
Reticulocyte (‰)	77 ± 16	64± 15	71 ± 9	52	27 ± 3	25 ± 5	24 ± 5	23 ± 3
PT (s)	13.5 ± 0.4	13.7 ± 0.5	13.3 ± 0.3	13.3	13.5 ± 0.6	13.1 ± 0.2	13.4 ± 0.4	13.2 ± 0.3
APTT (s)	25.1 ± 2.0	23.9 ± 2.1	23.6 ± 2.6	25.8	23.9 ± 1.6	22.9 ± 1.4	23.2 ± 1.2	24.4 ± 1.4
Fibrinogen (mg/dL)	257 ± 21	255 ± 28	217 ± 19*	210	177 ± 20	179 ± 13	180 ± 7	188 ± 29
WBC ($10^2/\mu L$)	49 ± 14	57 ± 10	54 ± 16	74	32 ± 15	39 ± 11	38 ± 10	37 ± 14
Differential leukocyte (%)								
Lymphocyte	86.7 ± 8.0	87.2 ± 5.6	89.0 ± 5.2	75.0	90.3 ± 3.7	89.8 ± 4.3	87.5 ± 5.5	88.7 ± 4.4
Neutrophil	12.0 ± 7.6	11.8 ± 5.5	9.5 ± 4.4	23.0	9.0 ± 3.8	9.2 ± 3.9	11.2 ± 5.3	10.3 ± 4.7
Eosinophil	0.5 ± 0.5	0.2 ± 0.4	0.5 ± 0.8	0.0	0.3 ± 0.5	0.7 ± 0.8	0.7 ± 0.8	0.7 ± 0.5
Basophil	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Monocyte	0.8 ± 0.8	0.8 ± 0.8	1.0 ± 0.6	2.0	0.3 ± 0.5	0.3 ± 0.5	0.7 ± 0.8	0.3 ± 0.5

[a] Values are given as the mean ± SD.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

the recovery period, only dose independent changes were found as follows. Relative brain weight was significantly lower and absolute liver weight was significantly higher at 0.78 mg/kg bw/day in males. In females, relative heart weight was significantly lower at 0.78 mg/kg bw/day. In addition, relative brain weight was significantly lower than controls at 2.33 mg/kg bw/day, and absolute heart weight was significantly higher than controls at 7.0 mg/kg bw/day. Absolute and relative weights of the testes, epididymides, ovaries, and uterus showed no significant difference from controls in any dinoseb-treated groups.

Necropsy and Histopathological Findings

Extramedullary hematopoiesis in the spleen was observed in six animals in the control female group with severity ranging from slight to moderate. Reduction in extramedullary hematopoiesis in the spleen was significant at 2.33 mg/kg bw/day in the main group females with severity ranging from slight to mild. At 7.0 mg/kg bw/day, one surviving female showed slight extramedullary hematopoiesis in the spleen, but the other surviving female did not show splenic extramedullary hematopoiesis. No histopathological

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TABLE IV. Blood biochemical findings in male and female rats given dinoseb

	Main Group				Recovery Group			
Dose (mg/kg bw/day)	0 (control)	0.78	2.33	7.0	0 (control)	0.78	2.33	7.0
No. of male rats	6	6	6	6	6	6	6	6
AST (IU/L)[a]	85.3 ± 19.0	85.9 ± 17.7	93.5 ± 20.1	91.3 ± 7.3	120.7 ± 44.1	115.1 ± 29.7	103.2 ± 24.4	102.7 ± 16.8
ALT (IU/L)	37.6 ± 6.7	34.3 ± 3.3	37.8 ± 5.5	40.1 ± 4.6	39.0 ± 13.8	35.9 ± 9.8	34.9 ± 12.1	29.9 ± 4.9
ALP (IU/L)	584.0 ± 91.4	641.4 ± 106.4	651.0 ± 166.2	950.3 ± 338.4	373.8 ± 56.4	310.4 ± 53.7	304.9 ± 77.7	256.2 ± 56.9**
γ-GTP (IU/L)	0.34 ± 0.15	0.46 ± 0.19	0.53 ± 0.20	0.53 ± 0.11	0.41 ± 0.22	0.29 ± 0.21	0.27 ± 0.12	0.39 ± 0.08
Total protein (g/dL)	5.8 ± 0.2	5.9 ± 0.2	5.8 ± 0.2	5.6 ± 0.2	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.4	5.4 ± 0.1
Albumin (g/dL)	2.95 ± 0.16	2.92 ± 0.12	2.91 ± 0.07	2.98 ± 0.15	2.93 ± 0.19	2.75 ± 0.12	2.84 ± 0.16	2.71 ± 0.07*
A/G ratio	1.04 ± 0.09	0.99 ± 0.03	1.00 ± 0.05	1.14 ± 0.10	1.04 ± 0.07	0.91 ± 0.05**	0.96 ± 0.05*	1.00 ± 0.04
Total bilirubin (mg/dL)	0.07 ± 0.02	0.06 ± 0.03	0.07 ± 0.04	0.05 ± 0.03	0.13 ± 0.03	0.12 ± 0.04	0.14 ± 0.02	0.11 ± 0.02
BUN (mg/dL)	17.7 ± 3.7	16.6 ± 1.3	16.4 ± 1.8	20.2 ± 1.3	15.3 ± 1.6	16.9 ± 2.2	16.7 ± 2.3	18.5 ± 2.1*
Creatinine (mg/dL)	0.27 ± 0.04	0.28 ± 0.02	0.28 ± 0.04	0.34 ± 0.04*	0.26 ± 0.03	0.30 ± 0.06	0.29 ± 0.04	0.29 ± 0.01
Glucose (mg/dL)	118.4 ± 7.3	119.6 ± 10.3	120.1 ± 9.2	112.7 ± 9.1	115.6 ± 15.2	121.4 ± 17.2	114.0 ± 22.5	102.7 ± 7.1
Total cholesterol (mg/dL)	68.0 ± 5.4	61.0 ± 4.4	56.7 ± 9.0*	60.6 ± 6.4	45.9 ± 10.0	60.6 ± 8.8	61.3 ± 10.6*	58.8 ± 11.2
Triglyceride (mg/dL)	63.4 ± 9.7	55.9 ± 21.3	62.8 ± 13.3	56.8 ± 15.4	37.5 ± 25.6	35.7 ± 17.7	36.9 ± 13.5	29.2 ± 10.7
Na (mEq/L)	144.5 ± 1.1	144.0 ± 1.3	144.3 ± 1.2	145.0 ± 0.7	144.8 ± 0.7	144.4 ± 0.7	145.2 ± 1.3	145.5 ± 0.9
K (mEq/L)	4.34 ± 0.27	4.35 ± 0.33	4.46 ± 0.29	4.12 ± 0.18	4.02 ± 0.34	4.23 ± 0.25	4.28 ± 0.22	4.16 ± 0.19
Cl (mEq/L)	105.8 ± 1.6	105.4 ± 1.0	105.1 ± 1.6	026.1 ± 1.1	106.8 ± 0.7	105.1 ± 1.4*	105.8 ± 0.9	106.9 ± 1.1
Ca (mg/dL)	9.9 ± 0.3	9.7 ± 0.3	9.6 ± 0.3	9.5 ± 0.3	9.4 ± 0.3	9.4 ± 0.3	9.5 ± 0.1	9.3 ± 0.2
Inorganic phosphate (mg/dL)	6.2 ± 1.3	6.2 ± 0.9	7.0 ± 0.6	5.7 ± 0.9	7.0 ± 0.5	7.3 ± 0.9	7.3 ± 0.7	7.4 ± 0.6
No. of female rats	6	6	6	1	6	6	6	6
AST (IU/L)	90.0 ± 16.4	98.7 ± 29.3	94.4 ± 21.4	78.4	86.0 ± 17.0	91.7 ± 11.7	85.8 ± 11.1	92.5 ± 11.2
ALT (IU/L)	23.8 ± 2.2	25.3 ± 3.9	26.6 ± 5.1	32.4	23.2 ± 3.9	26.5 ± 6.0	22.8 ± 1.6	26.2 ± 5.9
ALP (IU/L)	192.6 ± 46.8	178.8 ± 48.0	202.2 ± 23.1	194.7	129.3 ± 39.4	122.4 ± 17.5	135.5 ± 27.6	169.8 ± 49.1
γ-GTP (IU/L)	0.45 ± 0.12	0.65 ± 0.23	0.49 ± 0.17	0.55	0.46 ± 0.25	0.58 ± 0.25	0.59 ± 0.21	0.66 ± 0.26
Total protein (g/dL)	6.5 ± 0.2	6.4 ± 0.3	6.7 ± 0.5	6.1	6.5 ± 0.5	6.5 ± 0.3	6.2 ± 0.2	6.2 ± 0.4
Albumin (g/dL)	3.19 ± 0.15	3.16 ± 0.20	3.48 ± 0.25*	3.29	3.61 ± 0.36	3.59 ± 0.26	3.40 ± 0.16	3.32 ± 0.30
A/G ratio	0.98 ± 0.05	0.98 ± 0.07	1.10 ± 0.02*	1.17	1.24 ± 0.08	1.25 ± 0.11	1.23 ± 0.08	1.18 ± 0.09
Total bilirubin (mg/dL)	0.11 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.05	0.10 ± 0.03	0.11 ± 0.03	0.10 ± 0.03	0.12 ± 0.03
BUN (mg/dL)	20.5 ± 2.5	18.9 ± 4.4	23.5 ± 1.9	18.4	18.2 ± 1.4	16.1 ± 1.7	18.1 ± 2.5	18.0 ± 1.6
Creatinine (mg/dL)	0.39 ± 0.01	0.38 ± 0.05	0.40 ± 0.01	0.44	0.37 ± 0.02	0.34 ± 0.04	0.39 ± 0.02	0.36 ± 0.05
Glucose (mg/dL)	127.2 ± 13.2	129.0 ± 14.3	123.3 ± 13.7	130.7	120.7 ± 20.5	123.2 ± 9.8	126.3 ± 11.4	119.2 ± 15.1
Total cholesterol (mg/dL)	72.2 ± 5.5	81.9 ± 20.7	70.3 ± 9.3	86.4	81.5 ± 8.4	87.0 ± 10.8	75.7 ± 11.2	84.4 ± 17.4
Triglyceride (mg/dL)	41.8 ± 13.7	56.9 ± 24.5	54.3 ± 16.7	82.4	25.7 ± 5.4	38.9 ± 22.7	42.7 ± 29.2	22.7 ± 6.4
Na (mEq/L)	141.1 ± 1.0	140.7 ± 0.8	140.5 ± 0.9	142.2	143.6 ± 0.9	142.9 ± 1.4	142.7 ± 1.3	144.2 ± 0.5
K (mEq/L)	4.23 ± 0.32	4.14 ± 0.34	4.08 ± 0.31	3.38	4.04 ± 0.27	4.16 ± 0.17	3.98 ± 0.25	3.83 ± 0.26
Cl (mEq/L)	104.6 ± 1.8	104.3 ± 0.9	104.3 ± 2.1	104.9	108.0 ± 1.3	107.1 ± 1.5	107.0 ± 1.0	107.2 ± 1.7
Ca (mg/dL)	10.9 ± 0.3	10.8 ± 0.3	10.7 ± 0.5	10.4	9.6 ± 0.3	9.7 ± 0.2	9.5 ± 0.2	9.5 ± 0.3
Inorganic phosphate (mg/dL)	7.8 ± 1.2	7.6 ± 0.8	6.8 ± 1.1	5.6	4.3 ± 0.7	4.4 ± 0.7	4.3 ± 0.6	4.7 ± 0.4

[a] Values are given as the mean ± SD.

* Significantly different from the control group ($p < 0.05$).

** Significantly different from the control group ($p < 0.01$)

TABLE V. Organ weight of male and female rats given dinoseb

Dose (mg/kg bw/day)		Main Group				Recovery Group			
		0 (Control)	0.78	2.33	7.0	0 (Control)	0.78	2.33	7.0
No. of male rats		6	6	6	6	6	6	6	6
Body weight	(g)[a]	451 ± 19	449 ± 27	438 ± 42	409 ± 29	458 ± 22	474 ± 19	461 ± 29	461 ± 20
Brain	(g)[b]	2.04 ± 0.05	2.02 ± 0.04	2.07 ± 0.04	2.04 ± 0.06	2.12± 0.08	2.02 ± 0.10	2.09 ± 0.09	2.03 ± 0.13
	(g%)[c]	0.45 ± 0.02	0.45 ± 0.02	0.48 ± 0.05	0.50 ± 0.03*	0.46 ± 0.02	0.43 ± 0.02*	0.46 ± 0.03	0.44 ± 0.02
Pituitary	(mg)	12.7 ± 1.1	13.3 ± 1.9	13.2 ± 1.0	11.7 ± 1.1	14.3 ± 1.2	15.1 ± 1.5	13.6 ± 0.8	15.4 ± 1.0
	(mg%)	2.8 ± 0.2	3.0 ± 0.5	3.0 ± 0.3	2.9 ± 0.4	3.1 ± 0.2	3.2 ± 0.2	3.0 ± 0.2	3.4 ± 0.2
Thyroids	(mg)	20.7 ± 5.6	19.1 ± 6.4	23.0 ± 3.1	20.3 ± 3.5	18.5 ± 2.8	22.1 ± 4.2	20.6 ± 2.0	21.1 ± 4.5
	(mg%)	4.6 ± 1.3	4.3 ± 1.6	5.3 ± 0.6	5.0 ± 1.0	4.0 ± 0.6	4.7 ± 0.7	4.5 ± 0.4	4.6 ± 1.0
Thymus	(mg)	367 ± 126	260 ± 76	296 ± 120	245 ± 54	268 ± 41	316 ± 90	265 ± 86	296 ± 64
	(mg%)	82 ± 30	58 ± 16	67 ± 22	60 ± 14	59 ± 10	66 ± 17	58 ± 18	65 ± 15
Heart	(g)	1.41 ± 0.14	1.40 ± 0.07	1.37 ± 0.14	1.35 ± 0.11	1.52 ± 0.19	1.56 ± 0.09	1.62 ± 0.28	1.55 ± 0.25
	(g%)	0.31 ± 0.02	0.31 ± 0.01	0.31 ± 0.02	0.33 ± 0.02	0.33 ± 0.05	0.33 ± 0.03	0.35 ± 0.05	0.33 ± 0.04
Liver	(g)	15.98 ± 1.00	15.65 ± 1.04	15.49 ± 1.93	15.57 ± 1.02	10.44 ± 1.36	11.87 ± 0.72*	11.48 ± 1.09	11.17 ± 0.49
	(g%)	3.55 ± 0.21	3.49 ± 0.18	3.54 ± 0.28	3.81 ± 0.18	2.88 ± 0.24	2.51 ± 0.17	2.50 ± 0.27	2.42 ± 0.05
Spleen	(mg)	763 ± 110	791 ± 77	704 ± 61	682 ± 111	774 ± 70	696 ± 93	785 ± 208	780 ± 80
	(mg%)	170 ± 30	177 ± 17	162 ± 20	166 ± 16	170 ± 21	147 ± 20	173 ± 58	169 ± 13
Kidneys	(g)	2.91 ± 0.19	2.97 ± 0.15	2.96 ± 0.19	2.82 ± 0.19	3.10 ± 0.51	2.86± 0.07	2.87 ± 0.24	2.92 ± 0.15
	(g%)	0.65 ± 0.05	0.66 ± 0.05	0.68 ± 0.07	0.69 ± 0.05	0.68 ± 0.11	0.60 ± 0.03	0.62 ± 0.04	0.64 ± 0.02
Adrenals	(mg)	50.7 ± 8.3	57.4 ± 8.6	54.8 ± 6.3	58.8 ± 11.2	55.6 ± 7.8	52.4 ± 6.8	53.2 ± 9.9	61.4 ± 7.3
	(mg%)	11.3 ± 2.1	12.8 ± 2.1	12.5 ± 1.2	14.4 ± 2.8	12.2 ± 1.8	11.0 ± 1.2	11.5 ± 1.8	13.3 ± 1.5
Testes	(g)	3.11 ± 0.35	3.18 ± 0.42	3.36 ± 0.18	3.33 ± 0.30	3.29 ± 0.34	3.30 ± 0.40	3.25 ± 0.28	3.36 ± 0.27
	(g%)	0.69 ± 0.10	0.71 ± 0.11	0.78 ± 0.10	0.82 ± 0.06	0.72 ± 0.08	0.70 ± 0.09	0.71 ± 0.05	0.73 ± 0.06
Epididymides	(mg)	1120 ± 71	1170 ± 100	1158 ± 74	1150 ± 88	1250 ± 135	1255 ± 101	1222 ± 80	1224 ± 101
	(mg%)	249 ± 23	262 ± 28	267 ± 29	281 ± 14	274 ± 31	265 ± 25	265 ± 8	265 ± 17
No. of female rats		6	6	6	2	6	6	6	6
Body weight	(g)	301 ± 12	298 ± 14	301 ± 11	295	297 ± 26	306 ± 8	327 ± 13**	310 ± 9
Brain	(g)	1.97 ± 0.08	1.95 ± 0.05	2.01 ± 0.08	1.98	1.99 ± 0.09	1.91 ± 0.09	1.95 ± 0.08	1.99 ± 0.02
	(g%)	0.66 ± 0.03	0.66 ± 0.03	0.67 ± 0.03	0.68	0.67 ± 0.08	0.63 ± 0.03	0.60 ± 0.03*	0.64 ± 0.02
Pituitary	(mg)	16.7 ± 2.8	16.2 ± 2.1	16.7 ± 2.3	15.8	18.9 ± 3.18	20.1 ± 3.0	18.9 ± 3.7	18.3 ± 2.6
	(mg%)	5.5 ± 0.8	5.5 ± 0.7	5.6 ± 0.9	5.3	6.5 ± 1.5	6.6 ± 1.1	5.8 ± 1.3	5.9 ± 0.9
Thyroids	(mg)	16.8 ± 2.4	16.5 ± 3.6	17.7 ± 4.0	17.4	20.0 ± 3.9	20.2 ± 1.9	18.0 ± 3.0	22.7 ± 4.4
	(mg%)	5.6 ± 0.8	5.6 ± 1.2	5.9 ± 1.3	5.9	6.8 ± 1.6	6.6 ± 0.7	5.5 ± 1.1	7.4 ± 1.5
Thymus	(mg)	234 ± 48	266 ± 73	292 ± 77	225	284 ± 59	273 ± 29	282 ± 75	303 ± 65
	(mg%)	78 ± 17	90 ± 25	97 ± 26	76	96 ± 19	89 ± 8	86 ± 22	98 ± 21
Heart	(g)	1.03 ± 0.08	0.99 ± 0.08	1.00 ± 0.05	1.03	0.95 ± 0.06	0.90 ± 0.03	1.00 ± 0.05	1.04 ± 0.07**
	(g%)	0.34 ± 0.03	0.33 ± 0.02	0.33 ± 0.02	0.35	0.32 ± 0.03	0.29 ± 0.01*	0.31 ± 0.01	0.34 ± 0.02
Liver	(g)	9.92 ± 0.90	9.66 ± 0.92	9.84 ± 0.55	10.70	7.33 ± 0.67	7.62 ± 0.59	8.03 ± 0.66	7.95 ± 0.59
	(g%)	3.29 ± 0.26	3.24 ± 0.21	3.27 ± 0.15	3.64	2.47 ± 0.15	2.49 ± 0.14	2.46 ± 0.24	2.57 ± 0.13
Spleen	(mg)	690 ± 79	679 ± 145	719 ± 100	606	537 ± 86	522 ± 93	560 ± 69	593 ± 28
	(mg%)	229 ± 26	227 ± 42	239 ± 31	203	182 ± 31	170 ± 28	172 ± 28	192 ± 8
Kidneys	(g)	2.14 ± 0.15	2.05 ± 0.20	2.04 ± 0.11	1.99	1.94 ± 0.09	1.98 ± 0.07	1.95 ± 0.16	2.01 ± 0.19
	(g%)	0.71 ± 0.06	0.69 ± 0.05	0.68 ± 0.04	0.67	0.66 ± 0.06	0.65 ± 0.02	0.60 ± 0.05	0.65 ± 0.05
Adrenals	(mg)	74.6 ± 7.6	70.0 ± 10.4	69.9 ± 7.2	83.5	72.9 ± 6.1	62.1 ± 12.2	71.1 ± 6.2	70.5 ± 11.1
	(mg%)	24.9 ± 3.1	23.5 ± 2.9	23.2 ± 2.1	28.6	24.7 ± 3.1	20.3 ± 3.9	21.8 ± 2.5	22.7 ± 3.1
Ovaries	(mg)	110.0 ± 15.3	100.9 ± 8.3	102.2 ± 8.9	120.1	82.9 ± 20.3	79.9 ± 10.0	91.9 ± 14.9	96.3 ± 4.2
	(mg%)	36.6 ± 4.8	34.0 ± 3.2	34.0 ± 2.9	40.9	27.7 ± 4.8	26.1 ± 3.0	28.2 ± 4.7	31.1 ± 0.9
Uterus	(mg)	605 ± 165	570 ± 123	631 ± 135	2133	672 ± 139	622 ± 174	706 ± 168	576 ± 161
	(mg%)	202 ± 58	193 ± 46	210 ± 47	742	229 ± 59	203 ± 55	216 ± 49	186 ± 48

[a] Values are given as the mean ± SD.
[b] Absolute organ weight.
[c] Relative organ weight.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

TABLE VI. Sperm analysis of male rats at completion of the administration period

Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0
No. of animals	6	66	6	
Sperm motility[a]				
Motile sperm rate (%)	71.3 ± 2.8	72.8 ± 5.3	75.0 ± 7.4	59.2 ± 6.0**
Progressive sperm rate (%)	34.4 ± 3.7	37.3 ± 7.5	38.2 ± 8.4	22.9 ± 4.5*
Path velocity (μm/s)	150.7 ± 4.8	152.3 ± 6.1	153.6 ± 6.9	143.8 ± 6.0
Straight line velocity (μm/s)	110.4 ± 4.2	112.6 ± 5.5	111.7 ± 6.4	97.7 ± 8.2**
Curvilinear velocity (μm/s)	339.4 ± 11.4	335.9 ± 17.0	344.9 ± 12.8	319.4 ± 15.9
Amplitude of lateral head displacement (μm)	19.9 ± 0.3	19.5 ± 0.7	19.9 ± 1.0	20.9 ± 0.4*
Beat cross frequency (Hz)	29.5 ± 0.8	28.3 ± 1.6	29.6 ± 1.4	28.5 ± 1.7
Viability rate (%)[b]	99.8 ± 0.2	99.4 ± 0.5	99.4 ± 0.9	97.9 ± 2.2*
Survivability rate (%)[c]	76.7 ± 8.2	83.4 ± 6.4	80.9 ± 6.8	66.9 ± 10.1
Sperm morphology[d]				
Abnormal sperm rate (%)	2.5 ± 1.8	1.9 ± 1.0	2.0 ± 1.0	6.9 ± 4.6*
Abnormal head rate (%)	2.5 ± 1.8	1.9 ± 0.9	1.8 ± 0.8	5.6 ± 4.3
Abnormal tail rate (%)	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.3	1.3 ± 1.0**
No. of sperm in left cauda epididymis ($\times 10^6$)	268.8 ± 25.1	342.9 ± 50.1	334.8 ± 49.2	308.4 ± 114.2
No. of sperm/g weight of left cauda epididymis ($\times 10^6$)	1070.0 ± 94.9	281.1 ± 109.5	1238.2 ± 114.3	1227.4 ± 279.5

[a] Values are given as the mean ± SD.
[b] (Number of live sperm + number of sperm that died during incubation/number of sperm examined) × 100.
[c] (Number of live sperm/number of sperm examined) × 100.
[d] (Number of abnormal sperm/number of sperm examined) × 100.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

changes were found in the epididymides, ovaries, and uterus in the dinoseb-treated groups (data not shown).

Sperm Analysis

Table VI shows results of sperm analysis at the end of the administration period in male rats given dinoseb. Sperm analysis showed that there were no significant differences between the 0.78 and 2.33 mg/kg bw/day groups and controls in any of the sperm tests. The motile sperm rate, progressive sperm rate, straight line velocity, and viability rate in the 7.0 mg/kg bw/day dose group were significantly lower than controls, and the amplitude of lateral head displacement, abnormal sperm rate, and abnormal tail rate were significantly higher than controls.

Table VII shows results of sperm analysis at the end of the recovery period in male rats given dinoseb. At completion of the recovery period, sperm analysis revealed no significant changes at 0.78 and 2.33 mg/kg bw/day. At 7.0 mg/kg bw/day, a significantly lower viability rate and survivability rate, and higher abnormal sperm rate and abnormal head rate were found.

Reproductive and Developmental Findings

Table VIII shows the reproductive and developmental findings in rats given dinoseb. No change attributable to the chemical was noted in the number of estrous cases, copulation index, number of days till copulation after pairing, number of pregnant females, fertility index, gestation length, number of corpora lutea, number of implantation sites, implantation index or delivery, and nursing conditions. A number of dams delivered their pups and of dams with live pups at delivery was significantly lowered in the 7.0 mg/kg bw/day group. Slight focal atrophy of the seminiferous tubule was observed in one male at 7.0 mg/kg bw/day. No changes attributable to the chemical were noted in the total number of births, number of stillbirths, number of live pups born, sex ratio, delivery index, birth index, live birth index, general condition, number of live pups on day 4 of lactation, viability index, body weight of offspring, appearance, and necropsy findings in the 0.78 and 2.33 mg/kg bw/day dose groups. Acaudate or short tail was observed in each one pup at 0.78 mg/kg bw/day.

DISCUSSION

The present study was conducted to obtain initial information on the possible repeated dose toxicity and reproductive and developmental toxicity of dinoseb in rats. The data show that dinoseb exerts general toxicity and reproductive and developmental toxicity, but is unlikely to possess teratogenic potential under this test condition.

The dosage used in the present study was sufficiently high such that it should be expected to induce general toxic effects such as body weight and food consumption changes. A significant decrease in body weight gain was found at

TABLE VII. Sperm analysis of male rats at completion of the recovery period

Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0
Number of animals	6	6	6	6
Sperm motility[a]				
Motile sperm rate (%)	78.5 ± 6.5	76.6 ± 10.3	72.7 ± 3.3	71.5 ± 4.4
Progressive sperm rate (%)	29.3 ± 9.5	30.4 ± 12.2	33.3 ± 11.2	26.5 ± 6.7
Path velocity (μm/s)	144.2 ± 8.0	147.9 ± 8.0	147.8 ± 8.6	141.3 ± 5.5
Straight line velocity (μm/s)	101.9 ± 6.2	104.1 ± 6.8	107.0 ± 10.3	97.7 ± 6.4
Curvilinear velocity (μm/s)	327.4 ± 22.2	335.4 ± 26.4	324.6 ± 22.6	324.2 ± 13.9
Amplitude of lateral head displacement (μm)	19.9 ± 0.3	19.5 ± 0.7	19.9 ± 1.0	20.9 ± 0.4*
Beat cross frequency (Hz)	20.2 ± 0.9	20.4 ± 0.8	19.8 ± 1.0	20.3 ± 0.8
Viability rate (%)[b]	99.9 ± 0.2	99.8 ± 0.3	99.7 ± 0.3	98.8 ± 1.1*
Survivability rate (%)[c]	84.1 ± 4.1	83.1 ± 3.3	81.0 ± 4.6	78.2 ± 3.1*
Sperm morphology[d]				
Abnormal sperm rate (%)	1.5 ± 1.0	2.4 ± 2.2	2.3 ± 1.4	4.6 ± 2.8*
Abnormal head rate (%)	1.5 ± 1.0	2.3 ± 2.2	2.2 ± 1.4	4.2 ± 2.5*
Abnormal tail rate (%)	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.2	0.3 ± 0.4
No. of sperm in left cauda epididymis ($\times 10^6$)	358.4 ± 17.4	385.5 ± 70.5	347.8 ± 48.8	332.1 ± 28.8
No. of sperm/g weight of left cauda epididymis ($\times 10^6$)	1134.5 ± 72.6	1201.3 ± 1191.9	1118.9 ± 97.2	1087.3 ± 90.0

[a] Values are given as the mean ± SD.
[b] (Number of live sperm + number of sperm that died during incubation/number of sperm examined) × 100.
[c] (Number of live sperm/number of sperm examined) × 100.
[d] (Number of abnormal sperm/number of sperm examined) × 100.
* Significantly different from the control group ($p < 0.05$).

7.0 mg/kg bw/day in both sexes as expected. On the other hand, a significant increase in food consumption was observed from the mid stage of the administration period at 7.0 mg/kg bw/day in both sexes, and this effect was continued during the recovery period. In the 1930s, 2,4-dinitrophenol, a compound very similar to dinoseb, was prescribed as a weight-reducing agent. It is estimated that about 100 000 people in the United States used this drug while it was available. Because the energy is released as heat by uncoupling of electron transport from ATP synthesis, weight loss was remarkable (ATSDR, 1995; DOH, 2005). The decreased body weight gain with increased food consumption observed in this study is consistent with dinoseb's action as a metabolic activator. Because body weight gain was significantly increased during the recovery period at 7.0 mg/kg bw/day in males, the metabolic activation due to dinoseb seems temporary. At the end of the administration period, relative brain weight was significantly higher than controls with no significant difference in absolute weight in males at 7.0 mg/kg bw/day, suggesting that the difference in body weight in comparison with controls was responsible.

Transient salivation was observed immediately after administration at 0.78 mg/kg bw/day and higher, but it was considered as a result of stimulation by dinoseb because neither morphological changes in the salivary gland nor other neurological changes were observed. Females that died or were moribund showed a decrease in spontaneous motor activity, prone position, low body temperature, dyspnea, cyanosis, and bradypnea. In the acute toxicity, dinoseb can cause death because of respiratory and circulatory disorders (Rotterdam Convention, 2003). In this study, females that died or were moribund also showed adverse effects in the respiratory and circulatory systems with congestion in the lungs and livers.

Significant increases in RBC count, hematocrit, haemoglobin, or MCV found in the treated group animals indicate that dinoseb has a potential to induce polycythemia in rats. These changes were considered to be a result of regulation to low oxygen levels of cells. Dinoseb increases the rate of oxygen consumption in cells (Palmeira et al., 1994), and RBC production can be regulated by erythropoietin for need of oxygen (MedicineNet, 2001). Splenic extramedullary hematopoiesis, which was substantial in control pregnant rats, was significantly decreased in the 2.33 mg/kg bw/day females. This effect was also observed in one surviving female at 7.0 mg/kg bw/day. Reduction in splenic extramedullary hematopoiesis was considered to be a physiologic response to the decreased need for hematopoiesis because of polycythemia condition. In the blood chemistry, creatinine was significantly higher at 7.0 mg/kg bw/day in the main group males. However, no histopathological changes were found in the kidney, and other related parameters were not affected, suggesting that this change seems unlikely to be due to kidney failure but rather due to changes in muscle metabolism by dinoseb as an uncoupling agent. PT was significantly higher at 2.33 mg/kg bw/day (13.8 ± 0.3 s) and 7.0 mg/kg bw/day (14.1 ± 0.4 s), but

TABLE VIII. Reproductive and developmental findings in rats given dinoseb

Dose (mg/kg bw/day)	0 (control)	0.78	2.33	7.0
No. of females	12	12	12	12
No. of estrous cases before pairing (14 days)[a]	3.5 ± 0.5	3.3 ± 0.5	3.4 ± 0.5	3.3 ± 0.5
No. of pairs with successful copulation	12	12	12	12
Copulation index (%)[b]	100	100	100	100
No. of days till copulation after pairing[a]	2.3 ± 1.1	2.5 ± 1.2	2.4 ± 1.2	2.5 ± 1.3
No. of pregnant females	11	11	12	12
Fertility index (%)[c]	91.7	91.7	100.0	100.0
Length of gestation (days)[a]	22.2 ± 0.4	22.4 ± 0.9	22.2 ± 0.6	22.0
No. of corpora lutea[a]	16.6 ± 2.2	16.3 ± 2.1	16.1 ± 1.4	15.3 ± 1.2
No. of implantations[a]	14.6 ± 2.4	14.8 ± 1.8	14.6 ± 1.6	13.9 ± 1.1
Implantation index (%)[a,d]	87.6 ± 7.4	91.3 ± 5.5	90.6 ± 4.5	91.4 ± 6.6
No. of dead or moribund pregnant females during pregnancy	0	0	0	10
No. of dams delivered pups	11	11	12	2**
No. of dams without live pups	0	0	0	1
No. of dams with live pups	11	10	12	1**
No. of pups born[a]	13.9 ± 1.9	13.2 ± 3.4	13.2 ± 2.6	11.0
No. of stillbirths[a]	0.2 ± 0.4	1.2 ± 1.6	0.2 ± 0.4	6.0
No. of live pups born[a]	13.7 ± 1.8	12.0 ± 4.3	13.0 ± 2.5	5.0
Sex ratio at birth (male/female)[a,e]	1.09 ± 0.65 (74/77)	1.12 ± 0.44 (67/65)	1.28 ± 0.94 (80/76)	1.00 (5/5)
Delivery index (%)[a,f]	95.5 ± 5.5	88.2 ± 20.3	89.9 ± 14.1	84.5
Birth index (%)[a,g]	94.4 ± 5.7	79.4 ± 28.4	88.8 ± 13.4	41.5
Live birth index (%)[a,h]	98.8 ± 2.6	85.4 ± 29.7	98.8 ± 2.7	50
No. of live pups on day 4 of lactation[a]	13.6 ± 1.7	12.5 ± 1.6	12.8 ± 2.6	8.0
Viability index (%)[a,i]	99.4 ± 2.1	95.4 ± 10.3	98.1 ± 4.7	80
Body weight of pups[a]				
Male				
Day 0	6.8 ± 0.5	6.5 ± 0.4	6.7 ± 0.5	6.8
Day 4	11.1 ± 1.4	10.8 ± 1.1	10.6 ± 1.2	8.2
Female				
Day 0	6.4 ± 0.6	6.2 ± 0.5	6.3 ± 0.6	6.8
Day 4	10.2 ± 1.5	10.5 ± 1.3	9.9 ± 1.5	8.4
No. of fetuses (litters) with external malformation	0	2 (2)	0	0
Acaudate	0	1 (1)	0	0
Short tail	0	1 (1)	0	0

[a] Values are given as the mean ± SD.
[b] (Number of pairs with successful copulation/number of pairs) × 100.
[c] (Number of pregnant females/number of pairs with successful copulation) × 100.
[d] (Number of implantation scars/number of corpora lutea) × 100.
[e] Number of male pups/number of female pups.
[f] (Number of pups born/number of implantation scars) × 100.
[g] (Number of live pups born/number of implantation scars) × 100.
[h] (Number of live pups born/number of pups born) × 100.
[i] (Number of live pups on day 4/number of live pups born) × 100.
** Significantly different from the control group ($p < 0.01$).

these were within the range of historical background data for the laboratory that performed this study (16.2 ± 1.8 s). In the 7.0 mg/kg bw/day group, ALP and albumin were significantly lower and BUN was significantly higher. All of these differences were not observed at completion of the administration period and suggested not to be attributable to administration of the test substance. Other changes in hematology or blood chemistry were dose independent.

It seems unlikely that dinoseb exerts reproductive toxicity to female rats when administered during the premating and early pregnancy period because no adverse effects on the estrous cyclicity, copulation, or fertility were caused by the administration of dinoseb in females. However, dinoseb is suggested to be reproductively toxic during mid and late pregnancy because death was found during late pregnancy in the main group females but not in the recovery group

females, more specifically in nonpregnant females, at 7.0 mg/kg bw/day.

In the 7.0 mg/kg bw/day dose group males, motile sperm rate, progressive sperm rate, straight line velocity, and viability rate were significantly lower than controls, and the amplitude of lateral head displacement, abnormal sperm rate, and abnormal tail rate were significantly higher than controls at the end of the administration period. The viability rate and survivability rate were also significantly lower than controls and the abnormal sperm rate and abnormal head rate were significantly higher than controls at the end of the recovery period. These effects are thought to be caused by the uncoupling effects of dinoseb rather than due to a body weight loss and a body temperature increase (Linder et al., 1982). On the histopathological findings of this study, focal atrophy of the seminiferous tubule in the testis was observed in one male at 7.0 mg/kg bw/day. However, this change was considered to be spontaneous because the degree of histopathological change was slight, and the incidence at this dose was low and not significantly different from the control group. There were no dose-related effects on spermatogenesis at stages of spermatogonia and spermatocyte in this study. However, in a feeding study of dinoseb (0–22.2 mg/kg bw/day) to Sherman rats, histopathological changes to spermatogonia, spermatocyte, spermatid, or sperm in the testes were observed after 20 or 30 days of administration at 22.2 mg/kg bw/day, and a critical effect to the spermatogonia was observed after 50 days of treatment at 22.2 mg/kg bw/day. After 11-week administration, sperm counts were significantly decreased at 9.1 mg/kg bw/day and above. In addition, spermatozoa were not found in sections of the epididymides at 22.2 mg/kg bw/day (Linder et al., 1982). Another spermatotoxicity study, in which males rats were treated with dinoseb for 5 days, showed no effect of treatment on sperm parameters in the cauda epididymis 3 days after the final dose but after 14 days, reduced sperm motility and increased incidence of tailless sperm were noted (Takahashi et al., 2004). These findings suggest that dinoseb affects sperm indirectly by disturbing spermatogenesis or maturation process of sperm in the epididymis and that prolonged exposure to dinoseb in feed affects the early stage of spermatogenesis.

As for the developmental parameters, no changes attributable to the chemical were noted in the 0.78 and 2.33 mg/kg bw/day dose groups. Developmental toxicity of dinoseb was not completely estimated in the present study, because only one dam with live pups was obtained at the highest dose. At 0.78 mg/kg bw/day, one pup was observed to be acaudate and another to have a short tail. However, incidences of fetuses with an external malformation at this dose were very low and not significantly different from those in the control group. The external malformations observed in the present study are of the types that occur spontaneously among control rat fetuses (Kamcyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). Prenatal developmental toxicology studies by feeding administration of dinoseb showed teratogenic effects such as hypoplastic tails or microphthalmia in rats (Spencer and Sing, 1982; Giavini et al., 1986), but teratogenic effects were not induced by gavage dose (Giavini et al., 1986) or intraperitoneal administration (McCormack et al., 1980; Daston et al., 1988) in rats. Giavini et al. (1986) reported that gavage dose of dinoseb (0, 2.5, 5, 10, or 15 mg/kg bw/day) induced both maternal toxicity and developmental toxicity without teratogenic effects; however, administration of dinoseb in a diet induced microphthalmia at 200 ppm (~15 mg/kg bw/day in feed) with reduction in maternal body weight gain. Based on the results of the present study, dinoseb is considered to be unlikely to exert teratogenic effects by gavage dose, which is in agreement with the literature. There are no studies showing differences in toxicokinetics of dinoseb by gavage dose and feed administration, but there are some examples that gavage and feed administration show differences in toxicokinetics of chemicals (Yuan et al., 1994, 1995). Differences in absorption, distribution, metabolism, and/or elimination may explain the different teratogenic responses of dinoseb observed in these studies. Further studies are needed to clarify the differences in the toxicokinetics of dinoseb by gavage dose and feeding.

The present study was performed in compliance with the OECD guideline 422 (OECD, 1996), and this screening test does not provide complete information on all aspects of reproduction and development because of the relatively small numbers of animals in the dose groups. In the present study, only two females delivered their pups at the highest dose group; therefore, the developmental toxicity to pups was not fully assessed. The LOAEL for males and NOAEL for females were 0.78 mg/kg bw/day based on increases in hematocrit in males at 0.78 mg/kg bw/day and higher and decreased extramedullary hematopoiesis of the spleen in females at 2.33 mg/kg bw/day. The NOAEL for reproductive/developmental toxicity was considered to be 2.33 mg/kg bw/day based on sperm motility and morphology in males and decreased number of dams with live pups in females.

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Reproductive and developmental toxicity screening test of tetrahydrofurfuryl alcohol in rats

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Abstract

Twelve male and female rats per group were given tetrahydrofurfuryl alcohol (THFA) by gavage at 0, 15, 50, 150 or 500 mg/kg/day. Males were dosed for 47 days, beginning 14 days before mating, and females were dosed for 42–52 days beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. Changes in locomotor activity, inhibition of body weight gain, and/or histopathological changes in the thymus, spleen, testes and/or epididymides were observed in males and females at 150 mg/kg and above. No effects of THFA were found on the copulation index, fertility index, or the number of corpora lutea and implantations in pregnant females. At 500 mg/kg, no pregnant females delivered any pups. At 150 mg/kg, gestation length was prolonged, and the total number of pups born and the number of live pups on postnatal days 0 and 4 was markedly decreased. No effects of THFA were found on the sex ratio and body weight of live pups, or the incidence of pups with malformations or variations. Based on these findings, the NOAELs for parental and reproductive/developmental toxicity of THFA were concluded to be 50 mg/kg/day in rats.

Keywords: Tetrahydrofurfuryl alcohol; Reproductive and developmental toxicity; Postimplantation loss; Postnatal loss; Testicular toxicity; Rat

1. Introduction

Tetrahydrofurfuryl alcohol (THFA; CAS No. 97-99-4) is a colorless and flammable liquid with a slight ether odor [1]. In Japan, the annual production and import volume of THFA was reported to be from 100 to 1000 tonnes in 2004 [2], but there is no data available on that in other countries. The major uses of this chemical are as a solvent for various products (fats, waxes, resins, dyes and others) and as an intermediate in industrial applications [1]. While the extensive use of THFA by industry creates significant potential for occupational exposure, there is also the possibility of exposure of the general population to THFA because some of the applications include consumer uses, such as floor polish removers, graffiti removers and oven cleaners [3]. In particular, THFA application as a solvent for nail-cleaning agents [1] and absorption enhancer in various lotions and transdermal medications [4] would cause relatively high levels of exposure due to direct use on the skin. Such occupational and consumer exposure could occur through inhalation and dermal routes. On the other hand, THFA is directly added to food as a flavoring agent in Japan [5], and its use as a food additive for flavoring is also permitted in the US [6] and EU [7]. Furthermore, this chemical is known as the "solvent of choice" for a variety of agricultural applications, including pest control, weed control and growth regulation [3]. These uses suggest possible exposure of the general population to THFA via food. For each application, there are no data available on the actual use volume and exposure levels at this time. The possibility of human exposure to THFA has aroused concern regarding its toxicological potential.

Only limited information is available about the toxicity of THFA. It was reported that oral LD_{50} was 1.6–3.2 g/kg in rats and 0.8–1.6 g/kg in guinea pigs, and inhalation exposure for 6 h caused 2/3 deaths of rats at 12,650 ppm [8]. THFA showed eye irritation in rabbits [9] but did not irritate mouse skin [10].

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Unpublished repeated dose toxicity data are briefly summarized in OECD SIDS (Screening Information Data Set) documents [11]. In a 90-day feeding study using rats, body weight gain was depressed at 1000 ppm and above, the relative weight of epididymides decreased at 5000 ppm and above, and relative testis weight decreased with moderate testicular degeneration accompanied with complete loss of spermatogenic activity observed at 10,000 ppm. Adverse effects on body weight gain and male reproductive organs were also found in a 90-day inhalation and dermal study of THFA using rats. As for reproductive and developmental toxicity, only a dose range-finding developmental toxicity study is available [11]. In rats given THFA by gavage on days 6–15 of pregnancy, total embryonic loss occurred in all females at 500 mg/kg and above, at which inhibition of maternal body weight gain was also observed. Fetuses with a filamentous tail (5/124 fetuses) and lowering of fetal weight were found at 100 mg/kg without maternal toxicity.

Since there is insufficient information on toxicity, this chemical was selected as an object substance in an existing chemical testing program by the Japanese government [12]. In this program, a reproduction/developmental toxicity screening test was performed according to OECD test guideline 421 [13], because the evaluation of reproductive and developmental toxicity is essential in the risk assessment of chemicals. The results are summarized in OECD SIDS documents [11] and an assessment report prepared by US EPA, "Hazard assessment for the tolerance reassessment of tetrahydrofurfuryl alcohol (THFA)" [14]; however, detailed data have not been published in scientific journals. In this paper, therefore, we reported the data of a reproduction/developmental toxicity screening test of THFA.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 "Reproduction/Developmental Toxicity Screening Test" [13], and in accordance with the principles for Good Laboratory Practice [15,16] at the Research Institute for Animal Science in Biochemistry & Toxicology (Sagamihara, Japan). The experiment was approved by the Animal Care and Use Committee of the Research Institute for Animal Science in Biochemistry & Toxicology, and was performed in accordance with the ethics criteria contained in the bylaws of the Committee.

2.1. Animals and housing conditions

Crj:CD(SD)IGS rats (SPF, 8 weeks old) were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimatized to the laboratory for 13 days and subjected to treatment at 10 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. In addition, vaginal smears of each female were recorded, and only females showing a 4- to 5-day estrous cycle were used in the experiment. On the day before initial treatment, the rats were distributed into 5 groups of 12 males and 12 females each by stratified random sampling based on body weight.

Throughout the study, animals were maintained in an air-conditioned room at 21.9–22.4 °C, with a relative humidity of 49–57%, a 12-h light/dark cycle, and ventilation with more than 10 air changes/h. A basal diet (Labo MR Stock; Nosan Corporation, Yokohama, Japan) and sterile water were provided *ad libitum*. They were housed individually, except for mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and/or litters were reared using wood chips as bedding (White Flake; Charles River Japan, Inc., Yokohama, Japan).

2.2. Chemicals and doses

THFA was obtained from Koatsu Chemical Industries, Ltd. (Osaka, Japan) and kept in a cool (4 °C) and dark place. The THFA (Lot no. 2002-4) used in this study was 99.5% pure, and stability during the study was verified by gas chromatography. The test article was dissolved in purified water (Kyouei Pharmaceutical Co. Ltd., Takaoka, Japan), and administered to the animals by gastric intubation. Control rats received the vehicle alone. Dosing solutions were prepared at least once a week and kept in a cool (4 °C) and dark place until dosing, as stability under these conditions has been confirmed for up to 7 days. The concentrations of THFA in the formulations were confirmed to be 97.7–103.0% of the target by gas chromatography analysis.

Prior to the present reproductive and developmental toxicity screening study, a 14-day dose-finding study was performed. In the dose-finding study, male and female rats were given THFA by gavage at 50, 100, 200, 500 or 1000 mg/kg/day for 14 days. Changes in locomotor activity were observed at 100 mg/kg and above, decreases in absolute and relative weight of the pituitary and thymus were detected at 200 mg/kg and above, and piloerection, decrease in food consumption and dilatation of the cecum were found at 500 mg/kg and above (data not shown). Taking into account the results of this dose-finding study, the dose levels of THFA in the present study were set as 15, 50, 150 or 500 mg/kg/day. The daily application volume (5 ml/kg body weight) was calculated according to the latest body weight.

2.3. Study design

Male rats were dosed once daily for 47 days, beginning 14 days before mating and throughout the mating period. Female rats were also dosed once daily from 14 days prior to mating, and throughout the mating and gestation periods, to day 4 of lactation. The total administration period was 42–52 days. The day of the first dosing was designated as day 0 of the administration/premating period.

During the first 14-day administration period (premating period), vaginal lavage samples of each female were evaluated daily for estrous cyclicity. After this premating period, female rats were transferred to the home cage of a male of the same group, and cohabited on a 1:1 basis until successful copulation occurred or the mating period of 2 weeks had elapsed. During the mating period, vaginal smears were examined daily for the presence of sperm, and the presence of sperm in the vaginal smear and/or a vaginal plug were considered as evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. Pregnant females were allowed to deliver spontaneously and nurse their pups, and the day on which parturition was completed by 9:30 was designated as day 0 of lactation or postnatal day (PND) 0.

Throughout the study, all parental animals were observed for clinical signs of toxicity at least twice a day. The body weight was recorded on days 0, 7, 14, 21, 28, 35, 42 and 46 of the dosing period in males, and on days 0, 7 and 14 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 4 of the lactation period in females. Food consumption was recorded on days 0, 7, 21, 28, 35, 42 and 45 of the dosing period in males, and on days 0 and 7 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 3 of the lactation period in females.

All surviving male rats were euthanized by exsanguination under ether anesthesia on the day after the last administration. All female rats showing successful reproductive performance were euthanized in a similar way on day 5 of lactation. Females that did not copulate were euthanized on the day after the 52nd administration. Females that had not completed parturition were euthanized 5 days after the expected day of parturition (day 22 of gestation). When total litter loss was observed, the dams were euthanized within 4 days. For all parental animals, the external surfaces were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. For females, the numbers of corpora lutea and implantation sites were recorded. In males, the testes and epididymides were removed and weighed. The pituitary, thymus and kidneys were also weighed in both sexes.

Histopathological evaluations were performed on the pituitary, thymus, testes, epididymides and ovaries of all animals in the control and highest dose groups. In addition, the spleen of five animals in the control group and of all animals in the highest dose group was examined as test substance-related changes were macroscopically found in this organ. As a result of histopathological examination, test substance-related changes were found in the thymus,