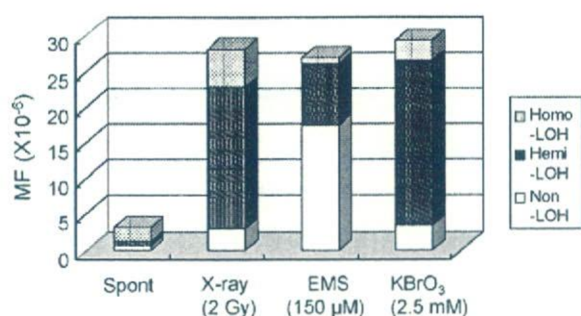

Fig. 2. *TK* mutation spectra in untreated, X-ray-treated (2 Gy), EMS-treated (150 μM, 4 h), and $KBrO_3$-treated (2.5 mM, 4 h) TK6 cells. The fraction of each mutational event was calculated by considering the ratio of NG to SG mutants and the results of molecular analysis (Table 1). The data for all but the $KBrO_3$ treatments were taken from our previous paper [20].

LOH mutants. $KBrO_3$ predominantly induced hemi-LOH, the result of large interstitial and terminal deletions, which we also frequently observed in the X-ray-induced LOH mutants. These results indicate that the genetic changes induced by $KBrO_3$ were similar to those induced by X-rays.

3.3. Gene expression analysis

Table 2 lists the genes that significantly increased expression following exposure to 2.5 mM $KBrO_3$. These genes are involved in stress response (6 genes), cell growth and DNA repair (19 genes), immune response (3 genes), apoptosis (3 genes), signal transduction (10 genes), transcription regulation (10 genes), chromosome organization (2 genes), protein modification (7 genes), energy metabolism (6 genes), lipid metabolism (2 genes), purine biosynthesis (3 genes), and unclassified functions (42 genes). Table 3 shows the genes whose expression was suppressed by the treatment. The number of up-regulated genes was greater that the number of down-regulated genes.

4. Discussion

$KBrO_3$ is a complete carcinogen, possessing both initiating and promoting activities in rodents [1]. While it shows clear positive responses in the COM assay, MN test, and chromosome aberration test using mammalian cells [4,14,17], the mutagenic potential of $KBrO_3$ in bacteria and the *Hprt* assay in Chinese hamster cells is weak or negative [1,14,17,30]. In our present study, $KBrO_3$ treatment strongly induced *TK* gene mutations. The reason we observed the induction of gene mutations and others did not is that $KBrO_3$ induces detectable mutagenecity in the *TK* gene but are only weakly mutagenic or non-mutagenic in the *Hprt* gene and in microbial assays [20]. The lower mutation frequency in the *Hprt* gene is due to the low recovery of large deletions, which are not detected because they are lethal. $KBrO_3$ is positive in mouse lymphoma cell assays that target the *Tk* gene [5]. In in vivo genotoxicity tests, $KBrO_3$ strongly induces MN in male ddY mice but is only weakly mutagenic in the *gpt* mutation assay in transgenic mice, which mainly detects point mutations and small deletions [31]. These results indicate that the property of genotoxicity

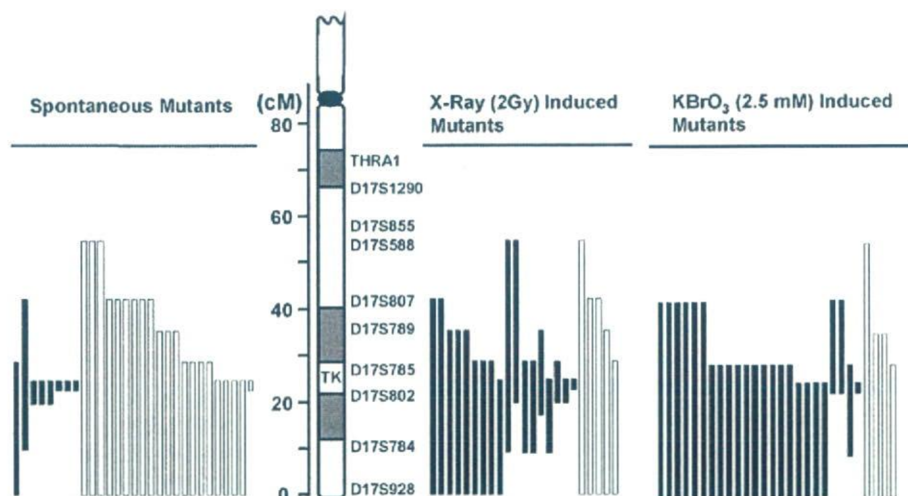

Fig. 3. The extent of LOH at the *TK* locus of TK6 cells that were untreated, X-ray-irradiated (2 Gy), or exposed to $KBrO_3$ (2.5 mM, 4 h). We examined 10 microsatellite loci on chromosome 17q that are heterozygous in TK6 cells. The human *TK* locus maps to 17q23.2. Open and closed bars represent homozygous LOH and hemizygous LOH, respectively. The length of the bar indicates the extent of the LOH. We analyzed 28 LOH mutants (4 NG and 24 SG). The data on spontaneous and X-ray-induced mutants were taken from our previous paper [20].

Table 2
Genes whose expression was up-regulated by $KBrO_3$ (2.5 mM, 4 h)

	Gene symbol	Ratio	Gene title
Stress response	CAT	2.77	Catalase
	DNAJC7	2.33	DnaJ (Hsp40) homolog, subfamily C, member 7
	FKBP5	2.87	FK506 binding protein 5
	HSPA8	3.02	Heat shock 70 kDa protein 8
	HSPCB	3.21	Heat shock 90 kDa protein 1, beta
	HSPD1	1.83	Heat shock 60 kDa protein 1
DNA repair, cell cycle, cell growth	BUB1	4.51	BUB1 budding uninhibited by benzimidazoles 1 homolog
	CCND2	5.08	Cyclin d2
	CCT2	3.33	Chaperonin containing TCP1, subunit 2 (beta)
	DKC1	2.37	Dyskeratosis congenita 1, dyskerin
	ENO1	2.10	Enolase 1 (alpha)
	HMGB1	2.16	High-mobility group box 1
	MAPRE1	2.32	Microtubule-associated protein, RP/EB family, member 1
	NME1	2.00	Non-metastatic cells 1, protein (NM23A) expressed in
	NOLC1	2.99	Nucleolar and coiled-body phosphoprotein 1
	NRAS	2.54	Neuroblastoma RAS viral (v-ras) oncogene homolog
	p21	3.22	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
	PPP2R1B	2.45	Protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform
	RAD21	2 34	RAD21 homolog
	RBBP4	2.00	Retinoblastoma binding protein 4
	RHOA	1.77	ras homolog gene family, member A
	SRPK1	2.75	SFRS protein kinase 1
	SSR1	2.66	Signal sequence receptor, alpha
Immune response	ARHGDIB	1.78	Rho GDP dissociation inhibitor (GDI) beta
	HLA-DRA	2.16	Major histocompatibility complex, class II, DR alpha
	IL2RG	2.43	Interleukin 2 receptor, gamma
Apoptosis	BCLAF1	6.42	BCL2-associated transcription factor 1
	FXR1	3.32	Fragile X mental retardation, autosomal homolog 1
	VDAC1	1.94	Voltage-dependent anion channel 1
Signal transduction	ANP32A	3.20	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
	OGT	2.74	O-linked *N*-acetylglucosamine (GlcNAc) transferase
	PIP5K1A	4.25	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha
	PLEK	2.95	Pleckstrin
	PTPN11	2.61	Protein tyrosine phosphatase, non-receptor type 11
	SPTLC1	2.62	Serine palmitoyltransferase, long chain base subunit 1
	SRPR	2.52	Signal recognition particle receptor
Transcription regulation	CDC5L	4.37	CDC5 cell division cycle 5-like
	HNRPC	4.40	Heterogeneous nuclear ribonucleoprotein C (C1/C2)
	MED6	2.45	Mediator of RNA polymerase II transcription, subunit 6 homolog
	MED6	2.45	Mediator of RNA polymerase II transcription, subunit 6 homolog
	NO NO	2.68	Non-POU domain containing, octamer-binding
	POLR1C	2.67	Polymerase (RNA) I polypeptide C, 30 kDa
	PRPF4	2.51	PRP4 pre-mRNA processing factor 4 homolog
Chromosome organization	CBX5	2.68	Chromobox homolog 5 (HP1 alpha homolog, Drosophila)
Protein modification	CANX	2.56	Calnexin
	COPA	6.55	Coatomer protein complex, subunit alpha
	EIF2S3	2.40	Eukaryotic translation initiation factor 2, subunit 3 gamma
	EIF4B	2.86	Eukaryotic translation initiation factor 4B
	RANBP2	3.96	RAN binding protein 2
	SEC23IP	2.67	SEC23 interacting protein

Table 2 (*Continued*)

	Gene symbol	Ratio	Gene title
Energy pathway	AFURS1	2.83	ATPase family homolog up-regulated in senescence cells
	CYB5-M	2.54	Cytochrome *b*5 outer mitochondrial membrane precursor
	TOMM22	3.07	Translocase of outer mitochondrial membrane 22 homolog
Lipid metabolism	HMGCS1	2.58	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1
	SCD	2.56	Stearovl-CoA desaturase
Purine biosynthesis	ENTPD1	2.36	Ectonucleoside triphosphate diphosphohydrolase 1
	GART	2.64	Phosphoribosylglycinamide formyltransferase
	PAICS	1.79	Phosphoribosylaminoimidazole carboxylase
Unclassified	BANF1	2.77	Barrier to autointegration factor 1
	BAT1	1.95	HLA-B associated transcript 1///HLA-B associated transcript 1
	C1orf16	2.37	Chromosome 1 open reading frame 16
	CALU	2.40	Calumenin
	DAZAP2	2.57	DAZ associated protein 2
	DDX18	2.34	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18
	DHX9	9.37	DEAH (Asp-Glu-Ala-His) box polypeptide 9
	EXOSC2	3.03	Exosome component 2
	FLJ10534	2.07	Hypothetical protein FLJ10534
	FLJ10719	2.42	Hypothetical protein FLJ10719
	FLJ12973	2.76	Hypothetical protein FLJ12973
	GANAB	2.07	Glucosidase, alpha; neutral AB
	HEM1	2.37	Hematopoietic protein 1
	IGHM	2.76	Anti-HIV-1 gp120 V3 loop antibody DO142-10 light chain variable region
	IGKC	3.15	Anti-rabies virus immunoglobulin rearranged kappa chain V-region
	LIN7C	3.51	lin-7 homolog C (*C. elegans*)
	LOC54499	2.31	Putative membrane protein
	M6PR	3.59	Mannose-6-phosphate receptor
	MGC8902	2.27	Hypothetical protein MGC8902/
	MOBK1B	2.67	MOB1, Mps one binder kinase activator-like 1B (yeast)
	NS	2.15	Nucleostemin
	NUSAP1	3.25	Nucleolar and spindle associated protein 1
	OK/SW-cl.56	1.85	Beta 5-tubulin
	OPRS1	2.76	Opioid receptor, sigma 1
	PEG 10	2.50	Paternally expressed 10
	PEX19	2.34	Peroxisomal biogenesis factor 19
	PGK1	2.11	Phosphoglycerate kinase 1
	RPE	2.35	Ribulose-5-phosphate-3-epimerase
	SDBCAG84	3.16	Serologically defined breast cancer antigen 84
	SMU1	2.70	smu-1 suppressor of mec-8 and unc-52 homolog (*C. elegans*)
	TAGLN2	2.03	Transgelin 2
	UBC	2.65	Ubiquitin C
	XPNPEP1	2.84	X-prolyl aminopeptidase
	YWHAE	6.39	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
	YWHAZ	2.50	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

of $KBrO_3$ predominantly causes gross structural changes rather than small genetic changes such as point mutations.

$KBrO_3$ generates high yields of 8OHdG DNA adducts, which is a marker of oxidative DNA damage widely used as a predictor of carcinogenesis [10]. 8OHdG has been reported to be highly mutagenic in some experiments. In cell-free system, 8OHdG induced mutation by misincorporating adenine instead of cytosine [12]. Artificially incorporated 8OHdG at specific codons in a shuttle vector system efficiently induced GC > TA transversions in mammalian cells and *E. coli* [8,32,33]. In mammalian gene mutation assays in vitro and in vivo, however, the relationship between the accumulation of 8OHdG and the induction of GC > TA transversion has not been clear. Takeuchi et al.

Table 3
Genes whose expression was down-regulated by $KBrO_3$ (2.5 mM, 4 h)

	Gene symbol	Ratio	Gene title
Cell cycle, cell growth	FH	0.51	Fumarate hydratase
	MYC	0.55	v-myc myelocytomatosis viral oncogene homolog
Signal transduction	DUSP2	0.37	Dual specificityphosphatase 2
	RRBP1	0.39	Ribosome binding protein 1 homolog 180 kDa
	TBL3	0 43	Transducin (beta)-like3
Transcription regulation	CITED2	0.45	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
	KIAA1196	0.43	KIAA1196 protein
	TZFP	0 39	Testis zinc finger protein
Chromosome organization	H1FX	0.14	H1 histone family member X
Protein modification	CLTB	0.43	Clathrin, light polypeptide (Lcb)
Energy pathway	FDX1	0.45	Ferredoxin 1
	QPRT	0.41	Quinolinate phosphoribosyltransferase
	SLC39A4	0.43	Solute carrier family 39 (zinc transporter), member 4
Unclassified	BTBD2	0.35	BTB (POZ) domain containing 2
	LOC339229	0.44	Hypothetical protein LOC339229
	MGRN1	0.44	Mahogunin, ring finger 1
	MRP63	0.41	Mitochondrial ribosomal protein 63
	PHLDA1	0.43	Pleckstrin homology-like domain, family A, member 1
	PTPLA	0.37	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a
	SPATA2	0.45	Spermatogenesis associated 2

examined the mutagenicity of a hydroxyl radical generator, *N,N′*-bis (2-hydroxyperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetra-carboxylic diimide (NP-III). Although NP-III highly produced 8OHdG upon irradiation with UV in V79 cells, the frequency of *Hprt* gene mutation was not significantly induced [34]. Molecular analysis demonstrated the no association of induction of 8OHdG with GC > TA transversion in the *Hprt* mutants [35]. 8OHdG is mainly removed by Ogg1 protein in a manor of the base excision repair (BER) pathway. Arai et al. investigated the relationship between the accumulation of oxidative DNA damage and the induction of gene mutation using *Ogg*1 deficient transgenic mice [36]. Although the 8OHdG level in kidneys of the *Ogg*1 deficient mice increase 200 times of the control level after 4 weeks' $KBrO_3$ treatment, the mutation frequency in the transgenic *gpt* gene was induced by less than 10 times of the control level. The molecular analysis revealed that the fraction of GC > TA transversions did not specifically increased. These results suggest that 8OHdG-mediated base substitutions do not mainly contribute to the mutagenic process involved in $KBrO_3$-induced carcinogenesis. Other genotoxic events must be involved in the carcinogenic process.

Our present studies strongly support this hypothesis. We demonstrated that $KBrO_3$ treatment clearly induced DNA damage in both the alkaline and neutral COM assay (Fig. 1). The alkaline COM assay is capable of detecting any DNA damages including DSB, single strand breaks (SSB), alkali-labile sites, DNA–DNA/DNA–protein cross-linking, and SSB associated with incomplete excision repair sites, while the neutral COM assay allows the detection of DSB, considered to be "biologically relevant" lesion of radiation damage [24]. $KBrO_3$ may have radio-mimic genotoxicity that yields oxidative DNA damage as well as DSB. $KBrO_3$ also induced MN formation and *TK* gene mutation significantly in TK6 cells. In the *TK* gene mutation assay, $KBrO_3$ predominantly produced SG mutants, but not NG mutants (Fig. 1c), implying that gross structural changes such as deletion and recombination are associated with the mutations. Molecular analysis of the *TK* mutants confirmed the assumption. Most of *TK* mutants showed LOH mutations, not non-LOH mutations, which are mainly point mutations. Harrington-Brock et al. also demonstrated that bromate compounds significantly induced *Tk* mutations in mouse lymphoma L5178Y cells, and almost all were LOH mutations [5]. LOH can be caused by deletions,

mitotic recombination between homologous alleles, or whole chromosome loss [20]. Molecular analysis can distinguish between them and reveal the mechanism and the characteristics of the mutants. In this study, $KBrO_3$ predominantly induced large deletions that resulted in hemizygous LOH (Table 1). The large deletions were mainly terminal deletions in the proximal region of chromosome 17q, which were rarely observed in spontaneously arising *TK* mutants (Fig. 3). The mutational spectrum and LOH pattern induced by $KBrO_3$ were similar to those induced by X-irradiation (Figs. 2 and 3) [20,21]. DSBs induced X-rays cause large deletions [19,20]. When the DSBs are repaired by the non-homologous end-joining pathway, interstitial deletions result. The broken chromosome ends can be also stabilized by the addition of new telomere sequences. Because TK6 cells have high telomerase activity [20], the result is terminal deletions. Thus, the major genotoxicity of $KBrO_3$ may be due to DSBs, but not to 8OHdG converting GC > TA transversion.

Some 8OHdG lesions can convert DSBs through the BER pathway [37]. In the initial step of BER, Ogg1 removes 8OHdG by DNA glycosylase activity and nicks the DNA backbone because of its associated lyase activity. The resulting SSB is processed by an apurinic endonuclease, which generates a single nucleotide gap. The gap is filled in by a DNA polymerase and sealed by a DNA ligase [38]. Clustered 8OHdG lesions induced by $KBrO_3$ may not be appropriately repaired by BER and cause DSB, however, because it is possible that two closely opposed 8OHdGs convert two closely opposed SSBs by BER resulting DSB [39,40]. Yang et al. developed Ogg1 over-expressing TK6 cell (TK6-hOGG1) and examined cytotoxic and mutagenic responses to gamma-irradiation [41]. They demonstrated that TK6-hOGG1 cells are more sensitive than the parental TK6 cells to cytotoxicity and mutagenicity by gamma-irradiation, and most of the induced *TK* mutants in TK6-OGG1 exhibited SG phenotype, which were probably large deletion mutants resulted by DSBs. This result clearly indicates that BER pathway contributes to convert oxidative damages to DSBs. Some clustered 8OHdG induced by $KBrO_3$ may covert to DSBs in TK6 cells, because TK6 is Ogg1 proficient cells [37].

To clarify the genotoxic characteristics of $KBrO_3$, we investigated the gene expression profile using Affymetrix GeneChip® Expression analysis. Many genes were up- or down-regulated by exposure to 2.5 mM $KBrO_3$ (Tables 2 and 3). Akerman et al. investigated the alterations of gene expression profiles in ionizing radiation-exposed TK6 cells [42]. They reported a >50% increase in expression of ATF-3 (stress response), Cyclin G (cell cycle), FAS antigen (apoptosis), GADD45 (repair and apoptosis), PCNA (repair), Rad51 (repair), and p21 (cell cycle) and a 40% decrease in expression of c-Myc (transcription factor), interferon stimulatory gene factor-3 (cell signaling), and p55CDC (cell cycle). We also observed up-regulation of p21 and down-regulation of c-Myc. Up-regulation of p21, however, is observed in TK6 cells exposed to any DNA-damaging chemical [43]. Islaih et al. also demonstrated the relationship between the gene expression profiles and the DNA damaging agents using TK6 cells [43]. They examined six chemicals including H_2O_2 and bleomycin which induce oxidative DNA damage. Although 10 genes were commonly up-regulated between H_2O_2 and bleomycin treatments, these genes except for p21 were not observed in our experiment. Thus, we could not find the similarity of gene expression profile by the treatment with $KBrO_3$ to by the treatment with ionizing radiation as well as oxidative damage inducers. Comparing gene expression profiles across platforms, laboratories, and experiments must be difficult [44]. Although it is difficult to judge from the expression analysis of the single chemical, information on genes which altered their expression gives a clue to understand the mechanism of action. Firstly, predominance of DNA repair and cell cycle related genes in up-regulated genes supports the genotoxic action of $KBrO_3$. Up-regulation of stress genes and apoptosis related genes suggests an involvement of oxidative stress. Up-regulation of catalase may be responsible for the oxidative damage by $KBrO_3$ (Table 2). Unclassified genes for alteration may have a functional relationship with genotoxic mechanism.

In conclusion, $KBrO_3$ predominantly induced large deletions at chromosomal level in human TK6 cells. The major genotoxicity leading to carcinogenesis of $KBrO_3$ may be due to DSBs rather than to 8OHdG adducts that lead to GC > TA transversions, as is commonly believed.

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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 I 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

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 (Kameyama 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 ($\sim$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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