In the dose finding study, newborn rats (five/sex/group) were administered TBBPA by gastric intubation at 0, 40, 200 or 1000 mg/kg per day from days 4–21 after birth. They were examined daily for general behavior and measured twice a week for body weight, and sacrificed at postnatal day 22, after overnight starvation, for assessment of hematology, blood biochemistry, macroscopic findings and weight of organs.

In the main study, newborn rats were administered TBBPA at 0 (vehicle as a control), 40, 200 or 600 mg/kg per day, based on the results of the dose finding study, by gastric intubation daily from 4 to 21 days after birth, and sacrificed under ether anesthesia after overnight starvation following the last treatment (scheduled-sacrifice group). Recovery-maintenance groups at the same dosages were maintained for 9 weeks without chemical treatment and sacrificed at 12 weeks of age. The number of animals at each sex/dose was six for both the scheduled-sacrifice and recovery-maintenance groups.

General behavior was observed daily. Body weights were measured twice a week during the dosing period and once a week during the recovery-maintenance period. Food consumption during 24 h was measured once a week during the recovery-maintenance period. At day 20 after birth for males and day 21 for females, gait condition, pupillary reflex, auricular reflex, corneal reflex, visual placing reflex, surface and mid-air righting reflexes, and ipsilateral flexor reflex were examined (Moser et al., 1991). Furthermore, fur appearance, incisor eruption and eye opening were examined in all animals from postnatal days 7, 9 and 11, respectively, and testes descent or vaginal opening was observed only in the recovery-maintenance group from postnatal day 17 or 29, respectively. During the period from days 78–82 after birth (only in the recovery-maintenance group), urine samples were obtained for the determination of pH, protein, glucose, ketone bodies, bilirubin, urobilinogen and occult blood using Multistix (Biel-Sankyo, Tokyo, Japan). Color, sediment, specific gravity and volume of the urine were also examined. For hematology and blood biochemistry, blood was collected from the abdominal aorta under ether anesthesia at sacrifice after overnight starvation for both the scheduled-sacrifice and recovery-maintenance groups. One part of the blood was examined for hematological parameters such as the red blood cell count, hemoglobin, hematocrit, white blood cell count, platelet count using a automatic blood cell analyzer (Sysmex E-4000,Toa Medical Electronics Co. Ltd., Kobe, Japan). The reticulocyte count and the differential leukocyte count were obtained by examining brilliant-cresyl-blue-stained and May-Giemsa-stained blood smears, respectively. In addition, blood clotting parameters such as prothrombin time (PT) and activated thromboplastin time (APTT) were measured using a coagulometer (Amelung-Coagulometer KC-10, Baxter Co. Ltd., Tokyo, Japan). Plasma obtained from the other portion of the blood was analyzed for blood biochemical parameters such as total protein, albumin, albumin–globulin ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin urea nitrogen (BUN), creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), γ-glutamyl transpeptidase, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), choline esterase, calcium, inorganic phosphorus using a clinical biochemistry analyzer (JCA-BM8, JEOL. Ltd., Tokyo, Japan). In addition, serum levels of sodium, potassium and chloride were determined using an auto electrolyte analyzer (NAKL 132, TOA Electronics Ltd., Tokyo, Japan). After recording the macro findings for all organs of animals sacrificed under ether anesthesia, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenal glands, thyroids, lungs, testes, epididymides, prostate, ovaries, and uterus were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The trachea, stomach, intestine, pancreas, lymph node, urinary bladder, spinal cord, sciatic nerve, seminal vesicles, bone, and bone marrow as well as the above organs were fixed with 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were prepared using routine methods and stained with hematoxylin-eosin for microscopic examination. For other groups, the organs in which dose-related effects were evident on microscopic examination for the highest dose group were examined.

2.4. Young rat study

In the study of young rats, 4-week-old male and female rats were obtained and used when they were 5-week-old, after 1 week acclimation. Five male and

female SD young rats for each group were administered TBBPA at 0, 2000 or 6000 mg/kg per day by gavage for 18 days. General behavior was observed daily and body weight was measured twice a week. At the termination of the treatment, animals were sacrificed under ether anesthesia and macroscopic findings of the major organs were recorded. The kidneys were removed and weighed, and histopathological examination was performed.

2.5. Statistical analysis

Continuous data were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett, 1964) ($P < 0.01$ or 0.05) was conducted for comparison between control and individual treatment groups after one-way layout analysis of variance (Yoshimura, 1997). If the data were not homogenous, they were analyzed using the Kruskal–Wallis test (Kruskal and Wallis, 1952) following a mean rank test of the Dunnett type (Hollander and Wolfe, 1973) ($P < 0.01$ or 0.05). Quantitative data were analyzed by Fisher's exact test (Fisher, 1973) ($P < 0.01$ or 0.05).

3. Results

3.1. Newborn rat study

In the dose finding study, various abnormalities were observed at 1000 mg/kg as follows: diarrhea, lowering of body weight, decreases in prothrombin time, activated thromboplastin time and hemoglobin, increase in platelet count, LDH, GOT, BUN, total bilirubin and creatinine, remarkable enlargement of kidneys, slight dilation of the cecum, and increases in the absolute and relative weights of the liver and kidneys (Table 1). Unexpectedly, the relative weights of the kidneys for both sexes reached approximately six times higher than those in controls. No histopathological information on the kidneys was obtained because of the lack of an examination schedule in the protocol. In the 200 mg/kg group, there were no significant changes except for a decrease in prothrombin time in females. Based on these results, 600 mg/kg, at which toxic effects should be clearly observed, was selected as the high dose, 40 mg/kg as the low (non-toxic) dose, and 200 mg/kg as the medium dose in the main study.

Table 1
Relative weights of the major organs at the termination of treatment in dose finding and main newborn studies

	mg/kg per day	Number of rats	Body weight (g)	Brain	Liver	Kidney	Testis	Ovary
Dose finding								
Males	0	5	56 ± 4	2.71 ± 0.08	2.94 ± 0.10	1.15 ± 0.04	0.53 ± 0.02	
	40	5	57 ± 3	2.74 ± 0.11	2.86 ± 0.04	1.16 ± 0.06	0.53 ± 0.03	
	200	5	55 ± 5	2.79 ± 0.24	2.92 ± 0.14	1.17 ± 0.06	0.54 ± 0.04	
	1000	5	53 ± 2	2.79 ± 0.10	3.42 ± 0.13**	6.96 ± 2.21	0.51 ± 0.04	
Females	0	5	56 ± 4	2.84 ± 0.12	2.92 ± 0.06	1.24 ± 0.05		0.036 ± 0.015
	40	5	57 ± 3	2.83 ± 0.16	2.96 ± 0.09	1.26 ± 0.08		0.031 ± 0.006
	200	5	55 ± 5	2.78 ± 0.17	3.01 ± 0.12	1.15 ± 0.08		0.032 ± 0.009
	1000	5	53 ± 2	2.84 ± 0.11	3.47 ± 0.23**	7.61 ± 3.05		0.024 ± 0.010
Main								
Males	0	6	51 ± 3	2.93 ± 0.23	3.25 ± 0.14	1.26 ± 0.04	0.57 ± 0.07	
	40	6	52 ± 3	2.97 ± 0.14	3.27 ± 0.11	1.28 ± 0.04	0.60 ± 0.04	
	200	6	52 ± 3	3.01 ± 0.14	3.37 ± 0.09	1.22 ± 0.03	0.60 ± 0.04	
	600	6	51 ± 2	3.02 ± 0.14	3.60 ± 0.17**	3.57 ± 0.77*	0.58 ± 0.04	
Females	0	6	48 ± 4	3.18 ± 0.21	3.21 ± 0.21	1.33 ± 0.07		0.028 ± 0.004
	40	6	48 ± 2	3.04 ± 0.10	3.24 ± 0.05	1.33 ± 0.06		0.033 ± 0.008
	200	6	48 ± 2	3.06 ± 0.17	3.32 ± 0.11	1.37 ± 0.10		0.031 ± 0.008
	600	6	48 ± 4	3.01 ± 0.22	3.44 ± 0.26	4.86 ± 4.47**		0.029 ± 0.007

Values are given as mean ± S.D.
Significantly different from control (*$P < 0.05$; **$P < 0.01$).

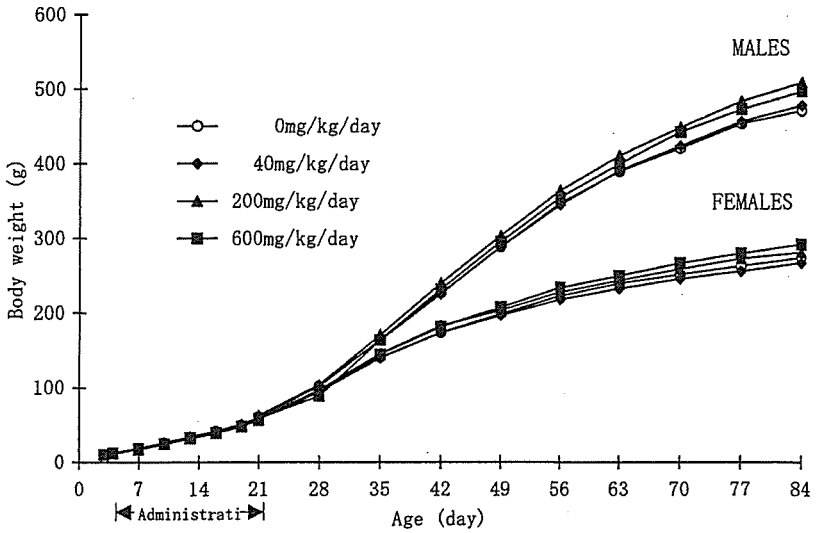

Fig. 1. Body weight changes of rats treated orally with TBBPA for 18 days from 4 days of age until weaning.

In the main study, diarrhea occurred sporadically during the treatment period in some males and females in the 200 and 600 mg/kg groups. There were no differences in body weight gain between the control and TBBPA-treated groups (Fig. 1). No definitive changes in physical development or reflex ontogeny were detected in any dose group. At the scheduled-sacrifice, the hematological and blood biochemical examinations showed decreases in hemoglobin in females and activated thromboplastin time in males, and increase of 600 mg/kg in total bilirubin in both the sexes (Table 2). The absolute and relative kidney weights dramatically increased in both sexes and the relative liver weight increased slightly in males (Table 1). The relative kidney weights were 2.8 times higher in males and 3.7 times higher in females than those in the control groups. The macroscopic appearance of the kidneys is shown in Fig. 2.

Histopathological findings of the kidneys are shown in Table 3. In the kidneys of two of six males in the 200 mg/kg group and all six males and six females in the 600 mg/kg group, polycystic lesions associated with the dilation of the tubules were noticed bilaterally from the cortico-medullary junction to the inner cortex (Fig. 3A). The changes of the lesions in the 600 mg/kg group were so severe that the tissue specimen looked like a sponge in gross examinations. In addition, hyperplasia of the renal tubular epithelium was observed from the cortico-medullary junction to the inner cortex (Fig. 3B), and the outer cortex was contracted due to the pressure produced by the cysts. Some rats also had marked hyaline casts within tubules and/or regenerating basophilic tubules or suppurative inflammatory reactions. Regarding other histopathological changes,

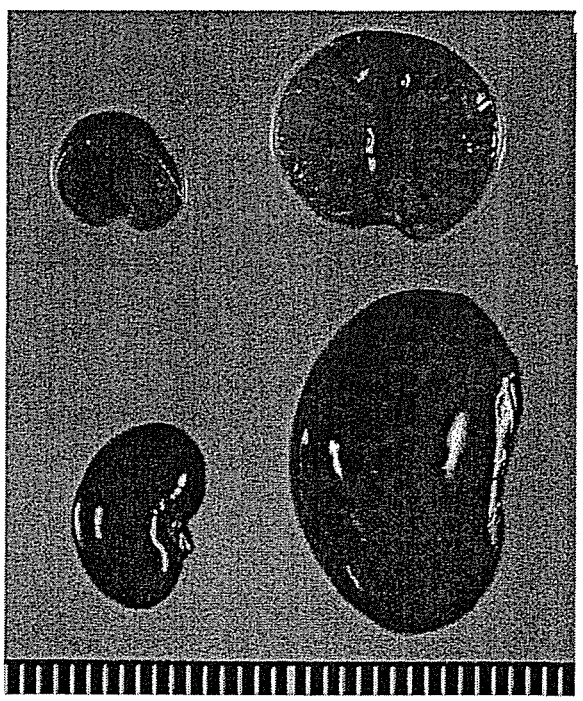

Fig. 2. Gross appearance of kidney (lower right) and its cross-section (upper right) in a 22-day-old rat treated with TBBPA (600 mg/kg body weight daily, orally) for 18 days. The kidney is markedly larger than that of a non-treated rat (left).

Table 2
Hematological and blood biochemical findings at 22 days of age of rats treated orally with TBBPA for 18 days from 4 days of age until weaning

Item	Dose (mg/kg per day)			
	0	40	200	600
Males				
Number of animals	6	6	6	6
Erythrocyte ($10^4/\mu l$)	480 ± 9	479 ± 14	483 ± 24	483 ± 19
Hemoglobin (g/dl)	9.7 ± 0.4	9.6 ± 0.5	9.3 ± 0.9	8.9 ± 0.7
Hematocrit (%)	30.9 ± 1.2	30.7 ± 1.7	30.4 ± 2.3	29.1 ± 1.6
Leukocyte ($10^2/\mu l$)	16 ± 5	18 ± 5	16 ± 7	18 ± 7
Platelet ($10^4/\mu l$)	145 ± 14	139 ± 11	141 ± 9	153 ± 15
PT (s)	13.6 ± 0.3	13.7 ± 0.3	13.7 ± 0.3	13.3 ± 0.4
APTT (s)	15.2 ± 0.4	14.4 ± 0.7	14.4 ± 0.8	14.2 ± 0.3*
LDH (IU/l)	521 ± 120	462 ± 198	557 ± 143	536 ± 143
GOT (IU/l)	127 ± 12	129 ± 11	132 ± 10	139 ± 17
GPT (IU/l)	25 ± 1	28 ± 5	28 ± 4	31 ± 5
ALP (IU/l)	995 ± 184	1079 ± 138	1075 ± 96	1224 ± 146
Total bilirubin (mg/dl)	0.41 ± 0.02	0.40 ± 0.03	0.43 ± 0.03	0.50 ± 0.05**
Total protein (g/dl)	4.93 ± 0.12	4.71 ± 0.24	4.69 ± 0.23	4.70 ± 0.16
Albumin (g/dl)	3.13 ± 0.07	2.98 ± 0.21	2.95 ± 0.18	2.99 ± 0.14
Total cholesterol (mg/dl)	80 ± 15	82 ± 7	74 ± 13	80 ± 12
BUN (mg/dl)	15.2 ± 3.4	15.4 ± 3.0	16.0 ± 4.0	14.8 ± 4.2
Creatinine (mg/dl)	0.45 ± 0.03	0.43 ± 0.05	0.45 ± 0.02	0.45 ± 0.02
Na (meq./l)	143 ± 1	142 ± 1	142 ± 1	142 ± 1
K (meq./l)	6.93 ± 0.65	7.07 ± 0.31	7.19 ± 0.60	6.80 ± 0.54
Cl (meq./l)	107 ± 2	108 ± 1	107 ± 1	106 ± 2
Females				
Number of animals	6	6	6	6
Erythrocyte ($10^4/\mu l$)	507 ± 26	512 ± 12	507 ± 23	503 ± 12
Hemoglobin (g/dl)	10.0 ± 0.8	10.2 ± 0.4	9.9 ± 0.5	9.0 ± 0.4**
Hematocrit (%)	31.5 ± 2.0	32.7 ± 1.3	32.0 ± 1.8	29.5 ± 1.0
Leukocyte ($10^2/\mu l$)	23 ± 7	23 ± 6	26 ± 14	25 ± 4
Platelet ($10^4/\mu l$)	142 ± 17	155 ± 15	152 ± 22	160 ± 23
PT (s)	13.9 ± 0.2	14.0 ± 0.6	13.6 ± 0.4	13.6 ± 0.4
APTT (s)	14.4 ± 0.7	15.6 ± 0.9*	14.2 ± 0.6	13.5 ± 0.9
LDH (IU/l)	598 ± 249	613 ± 48	479 ± 88	615 ± 158
GOT (IU/l)	135 ± 18	137 ± 16	119 ± 11	148 ± 23
GPT (IU/l)	19 ± 2	21 ± 4	20 ± 4	23 ± 4
ALP (IU/l)	925 ± 189	1007 ± 99	983 ± 150	1109 ± 94
Total bilirubin (mg/dl)	0.38 ± 0.03	0.39 ± 0.03	0.41 ± 0.02	0.50 ± 0.13**
Total Protein (g/dl)	5.01 ± 0.25	4.94 ± 0.07	4.77 ± 0.17	4.82 ± 0.39
Albumin (g/dl)	3.25 ± 0.20	3.15 ± 0.11	3.03 ± 0.18	3.03 ± 0.08
Total cholesterol (mg/dl)	69 ± 13	74 ± 23	72 ± 11	93 ± 31
BUN (mg/dl)	18.5 ± 4.1	16.6 ± 2.6	14.6 ± 2.0	21.6 ± 13.4
Creatinine (mg/dl)	0.46 ± 0.04	0.47 ± 0.03	0.43 ± 0.03	0.48 ± 0.11
Na (meq./l)	142 ± 1	142 ± 1	142 ± 1	142 ± 1
K (meq./l)	7.18 ± 0.69	7.29 ± 0.43	4.24 ± 0.20	7.01 ± 0.53
Cl (meq./l)	108 ± 2	108 ± 1	107 ± 1	106 ± 3

Each value is expressed as mean ± S.D.
Significantly different from control (*$P < 0.05$; **$P < 0.01$).

Table 3
Incidence of renal histopathological findings of rats treated orally with TBBPA for 18 days from 4 days of age until weaning

Findings	Grade	22 Days of age								85 Days of age								FD/KE	
		0[a]		40[a]		200[a]		600[a]		0[a]		40[a]		200[a]		600[a]		600[a]	
		M 6[b]	F 6[b]	M 6[b]	F 6[b]	M 6[b]	F 6[b]	M 6[b]	F 6[b]	M 6[b]	F 6[b]	M 6[b]	F 6[b]	M 6[b]	F 6[b]	M 4[b]	F 5[b]	M 2[b]	F 1[b]
Cyst, multiple	+	0	0	0	0	2	0	0	0	0	0	0	0	1	1	0	1	0	0
	++	0	0	0	0	0	0	0	6	0	0	0	0	0	0	3	4	0	0
	+++	0	0	0	0	0	0	6	0	0	0	0	0	0	0	1	0	2	1
Cast, hyaline	+	0	0	0	0	0	0	2	3	0	0	0	0	0	0	1	2	0	0
	++/+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	1
Cast, granular	+/++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	1
Necrosis, tublar epithelium	+/++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1
Basophilic tubules	+	4	6	5	5	5	5	4	4	2	0	3	2	3	1	1	3	0	0
	++/+++	0	0	0	0	0	0	2	1	0	0	0	0	0	0	2	1	2	0
Cellular infiltration, lymphocytes	+	0	0	1	0	0	0	0	0	0	1	1	0	1	0	2	1	0	0
	++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0
Inflammation, suppurative	+++	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Hyperplasia, tublar epithelium	+	0	0	0	0	2	0	6	3	0	0	0	0	0	0	2	2	1	0
	++	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1
Atrophy, cortical	+	0	0	0	0	0	0	5	5	0	0	0	0	0	0	1	0	0	0
	++/+++	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	2	1
Fibrosis, interstitial	+	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
	++/+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	0	0

M: male; F: female; (+): slight; (++): moderate; (+++): severe; FD/KE: found dead or killed in extremis.
[a] Doses in milligram per kilogram per day.
[b] Number of animals.

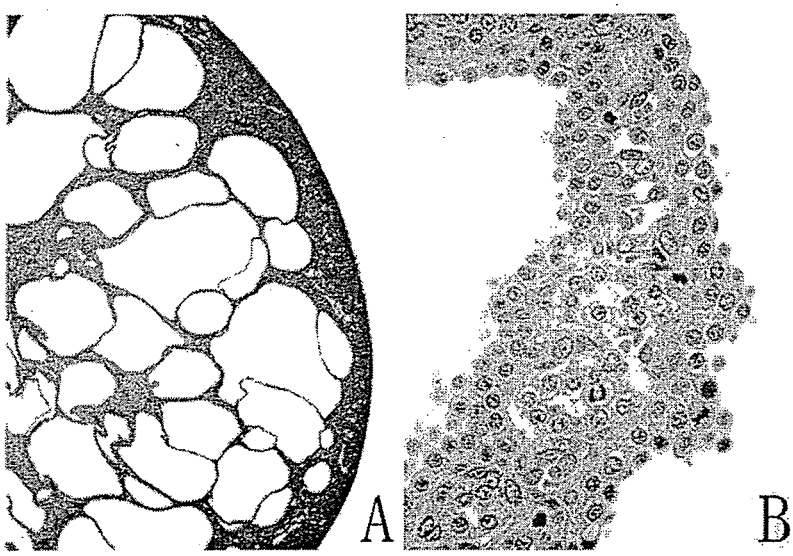

Fig. 3. Polyeystic renal lesion observed in a 22-day-old rat treated with TBBPA (600 mg/kg body weight daily, orally) for 18 days. H-E stain. (A) Dilatation of the tubules from the cortico-mudullary junction to the inner cortex, 40×; (B) hyperplasia of the tubular epithelium, 125×.

only a slight change of the liver (centrilobular hypertrophy of the hepatocytes in 3/6 males) of 600 mg/kg group was observed.

During the recovery-maintenance period, clinical signs such as emaciation, decrease in spontaneous activity and pale skin were observed only in two males and one female of the 600 mg/kg group from 4 days after the termination of the treatment. On day seven after termination of the treatment, one male and one female were found dead and one male was killed in moribund condition in this group. The kidneys of these three rats had necrosis of the tubular epithelium and formation of granular casts in addition to multiple cystic lesions. No dose-related changes in body weight, food consumption, parameters of sexual maturation or urinalysis were detected.

At the end of the recovery-maintenance period, the absolute kidney weights of males and females in the 600 mg/kg group were still 1.3 times higher than those in the control group. Histopathological examinations revealed multiple cysts of the kidneys in one male and one female of the 200 mg/kg group and in all males and females of the 600 mg/kg group (Table 3). However, these kidneys contained reparative changes with interstitial fibrosis, in contrast to the kidneys at the scheduled-sacrifice.

3.2. *Young rat study*

In order to compare the nephrotoxic effects of TBBPA in newborn rats with those in young rats, young rats were administered TBBPA by gavage at 2000 or 6000 mg/kg per day for 18 days. There were no TBBPA-induced changes in general behavior, body weight or kidney weight. The histopathological examination of the kidneys showed no abnormalities in either sex in any group.

4. Discussion

It has been generally accepted that TBBPA has no critical toxicity for major organs, including the kidneys, in young and adult rats or mice (IPCS/WHO, 1995). The marked nephrotoxicity characterized by the formation of polycystic lesions (polycystic kidney) observed at 200 and 600 mg/kg in our newborn rat study was completely unexpected based on the general repeated dose toxicity studies and teratogenicity studies in young and adult animals. This nephrotoxicity is likely to be reproducible because the dose finding study in newborn rats showed a six-fold increase of the relative kidney weight at 1000 mg/kg. Since it was not observed in our young rat study after 18 days of TBBPA treatment even at the extremely high dose of 6000 mg/kg, the nephrotoxicity of TBBPA was considered to be specific for newborn rats versus young rats.

Lau and Kavlock (1994) have reviewed publications on the breadth of critical periods for renal toxicity of therapeutic agents, hormonal manipulations and environmental agents. Chlorambucil is highly effective in inducing renal hypoplasia and altered function when exposure occurs at the time of induction of the metanephric blstema (Kavlock et al., 1987). 2,3,7,8-Tetrachloro-1,4-dibenzodioxine (TCDD) and some other chemicals induce hydronephrosis specifically in fetal/newborn animals after maternal exposure during pregnancy and/or the lactating period (Couture-Haws et al., 1991). Enalapril, an angiotensin-converting enzyme inhibitor (Minsker et al., 1990) and glucocorticoids (Slotkin et al., 1991, 1992) are renal developmental toxicants when exposure occurs during late gestation, and difluoromethylornithine induces persistent effects on the kidney when exposure occurs in the early postnatal period (Gray and Kavlock, 1991). On the other hand, it is well-known that mercuric chloride is a potent nephrotoxicant in adult rats, but has little effect on newborns (Daston et al., 1983, 1984). Clinically, it is known that antibacterial agent-induced kidney damage (especially that caused by amino glycosides or glycopeptides) is less frequent and severe in newborns than in adults (Fanos and Cataldi, 1999).

Some investigations on the mechanism of the context of morphologic events occurring during those periods have been reported. Angiotensin-converting enzyme inhibitors cause excessive disturbances in normal physiology in a system with immature feedback loops in late fetal development (Brent and Beckman, 1991; Hanssens et al., 1991). Mercuric chloride is thought to interact initially with the brush border of the proximal tubules (Daston et al., 1983), whereas dichlorovinylcysteine requires activation by renal β-lyase before achieving toxicity (Darnerud et al., 1991), thus suggesting a biochemical immaturity

of the neonatal kidney that may offer a degree of protection from the effects of some nephrotoxicants. On the other hand, chlorambucil is thought to cause renal hypoplasia by a direct action on rapidly proliferating cell populations during induction of the renal anlagen (Kavlock et al., 1987). The mechanism of the hydronephrosis caused by methylsalicylate was suggested to be differences in the growth rate between the papillae and the parenchyma in the developing kidney (Woo and Hoar, 1972).

These reports suggest that there does not appear to be a good concordance between agents that induce renal toxicities in the fetus, newborn or adult.

Polycystic kidneys, in which the renal parenchyma is occupied by innumerable cysts of various sizes, have been reported to be induced by diphenylamine (Gardner et al., 1976), nordihydroguaiaretic acid (Evan and Gardner, 1979), diphenylthiazole (Gardner and Evan, 1983), alloxan (Kovacs et al., 1998), ferric-nitrilotriacetate (Kovacs et al., 1998), streptozocin (Kovacs et al., 1998), and 2-amino-4,5-diphenylthiazole (Tsumatani et al., 1997) in young and adult animals. Polycystic kidney is also known as an inherited disease in humans and some other species.

As a pathogenesis of the cyst formation in human cases, it is considered that epithelial hyperplasia results in tubular enlargement and obstruction (Bernstein, 1992). Pathogenesis of chemical-induced polycystic kidneys is also considered that chemicals cause some changes in metabolism of the epithelium or basement membrane of the tubules, resulting in abnormal extracellular matrices and hyperplasia of the epithelium, leading to the occlusion of the tubules (Carone et al., 1992; Avner, 1988). Then, an increase in the pressure of the lumen of occluded tubules is considered to cause formation of multiple renal cysts. In the present study, hyperplasia of the renal tubular epithelium was observed. Although no initial changes of hyperplasia of the tubular epithelium were detected, it is assumed that TBBPA may also have a damaging effect on the tubular epithelium and cause reactive hyperplasia of the damaged epithelium, leading to occlusion of the tubules.

As the same nephrotoxicity as that induced by TBBPA, characterized by polycystic kidney, para-nonylphenol was reported in rat neonates exposed via the maternal placenta and breast milk, but was not so obvious in adults (Latendresse et al., 2001). Since this effect on the kidneys was affected by phytoestrogens in the diet, the authors discussed the possible role of the estrogenic activity in this nephrotoxicity. In the case of TBBPA, the possibility of an estrogenic mechanism appears to be unlikely because there was no evidence of estrogenic activity in our previous and present studies.

It was known that nephrons in the kidneys of rats are formed in the period from the advanced stage of pregnancy until 2 weeks after birth (Chevalier, 1998), and only 10% of nephrons are present at birth (Merlet-Benichou et al., 1994). This period is analogous to that of the midtrimester human fetus, during which the major features of obstructive nephropathy including cystic changes evolve (Daikha-Dahmane et al., 1997).

Compared to the adult, the rapidly growing neonatal rat kidney appears to be particularly susceptible to interference with cellular proliferation and stimulation of apoptosis (programmed cell death) as a result of chronic unilateral ureteral obstruction (Chevalier et al., 1998). The mechanisms underlying these effects are complex, involving the interaction of multiple growth factors and cytokines (Chevalier, 1996).

These observations suggest that developing renal tubules in neonatal rats may be easy to cause hyperplasia of the tubular epithelium by a cellular damage due to a toxic effect of the agents.

On the other hand, a recent study using bile duct-cannulated rats showed that approximately 70% of ^{14}C-TBBPA orally administered at 2.0 mg/kg was excreted to the bile (Hakk et al., 2000). As bile synthesis, conjugation, transport and secretion are known to be immature at birth and the maturation usually occurs after weaning in animals (Scheuplein et al., 2002; Chuang and Haber, 1998), it is possible that the kidneys of the newborn were exposed to higher levels of TBBPA than the young adults. However, the mechanism of vulnerability specific to TBBPA in newborn but not young rats remains to be elucidated.

The relative weight of the liver increased slightly in the males of the 600 mg/kg group in the newborn rat study. Although some animals showed a slight centrilobular hepatocellular hypertrophy, the results of the biochemistry examinations did not indicate any abnormality in the liver function. Although no hepatotoxicity was found in adult animals (IPCS/WHO,

1995), a recent study suggested the possibility that TBBPA may disturb the heme metabolism in the rat liver (Szymanska et al., 2000).

TBBPA is a commercial product used as a polymer in resins such as acrylonitrile, butadiene, styrene, epoxy, polycarbonates and polystyrene. In general, the intake of TBBPA at home is estimated not to be harmful or to pose any risk, because most of the general population is only indirectly exposed to TBBPA through products made from these polymers (IPCS/WHO, 1995). Additionally, as the nephrotoxicity occurred only at relatively high TBBPA doses in the newborn rats, the results of the present study do not indicate a warning of any risk of TBBPA to human infants. However, the reason why TBBPA-induced polycystic kidney is specific to newborn rats should be determined.

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Prenatal developmental toxicity study of the basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Abstract

Pregnant rats were given 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 10, 20 or 40 mg/kg bw/day on days 6–19 of pregnancy and the pregnancy outcome was determined on day 20 of pregnancy. At 40 mg/kg bw/day, deaths were observed in four out of 24 females. The incidences of females showing mydriasis at 20 and 40 mg/kg bw/day and showing decreased locomotor activity at 40 mg/kg bw/day were significantly increased. Alopecia, bradypnea, prone position and tremor were also observed at 40 mg/kg bw/day. The maternal body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were significantly reduced. A significantly decreased weight of the gravid uterus, increased incidence of postimplantation loss, decreased number of live fetuses, and lowered weights of fetuses and placentae were found at 40 mg/kg bw/day. The incidences of the total number of fetuses with external malformations at 40 mg/kg bw/day and with skeletal malformations at 20 and 40 mg/kg bw/day were significantly increased. Significantly higher incidences of fetuses with brachydactyly and short tail and defects of caudal vertebrae, phalanges and metacarpals were observed at 40 mg/kg bw/day. Delayed ossification was also noted at 40 mg/kg bw/day. The data indicate that DTG is teratogenic at maternal toxic doses and the NOAELs of DTG for maternal and developmental toxicity are 10 mg/kg bw/day in rats.

Keywords: Di-*o*-tolylguanidine; Rubber accelerator; Sigma ligand; Prenatal developmental toxicity; Teratogenicity; Malformation; Rat

1. Introduction

1,3-Di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the USA [1] and used as a basic rubber accelerator [2]. DTG is known to be a selective ligand receptor for the sigma site in the mammalian central nervous system [3]. Many findings have suggested that the sigma site plays a role in movement and posture through its association with brainstem and forebrain motor control circuits [4]. DTG has been reported to cause hypothermia after intraperitoneal injection in mice [5] and subcutaneous or intracerebroventricle injection in rats [6,7]. Intraperitoneal injection of DTG reduced the pain behavior in the acute phase, but increased pain behavior in the tonic phase in the formalin test in mice [8], and produced significant, but short-lived, increases in the withdrawal latencies in mice [5]. In rats, DTG also caused circling behavior after unilateral intranigral injection [4], decreased locomotor activity after intraperitoneal injection [9,10], increased bladder capacity after intravenous injection in the anaesthetized condition [11], and no change in immobility time in the forced swimming test after intraperitoneal injection [12].

It is generally assumed that the biological effects produced by chemicals should be studied in laboratory animals to investigate possible influences in human health, and the results of animal tests on chemical toxicity are relevant to humans [13]. Toxicological studies on DTG have given little information on acute animal toxicity [14]: intraperitoneal LD50 was 25 mg/kg bw in mice; the oral LD50 was 500 mg/kg bw in rats; the lowest published lethal dose of oral administration was 80 mg/kg bw in rabbits; and the lowest published lethal dose was 120 mg/kg bw after oral administration in mammals, species unspecified. We recently investigated the reproductive and developmental toxicity of DTG, according to the OECD guideline 421 reproduc-

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tion/developmental toxicity screening test in rats given DTG by gavage at 0, 8, 20 or 50 mg/kg bw/day [15], to obtain the preliminary information on the reproductive and developmental effects of DTG, because the testing for reproductive and developmental toxicity has become an important part of the overall toxicology. Males were given DTG for a total of 49 days beginning 14 days before mating, and females were given DTG for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. In this screening study, deaths in both sexes at 50 mg/kg bw/day, lowered body weight gain and food consumption in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day, and neurobehavioral changes such as mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation in both sexes at 20 and 50 mg/kg bw/day were found. Although no effects of DTG were detected on the estrous cyclicity, precoital interval, copulation, fertility and gestation indexes, numbers of corpora lutea and implantations, and gestation length, significant decreases in the number, body weight and viability of offspring and a significant increase in the incidence of fetuses with external malformations were noted at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were frequently observed at the highest dose. The total number of fetuses with external malformations, but not individual malformation, was significantly increased at 50 mg/kg, and the teratogenic effect of DTG was strongly suggested. However, this screening test does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. Only external examination in the newborn rats was performed, and no internal or skeletal examinations were carried out in this screening test. The prenatal developmental toxicity study was therefore conducted to accurately evaluate the developmental toxicity, including the teratogenicity of DTG in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 414 Prenatal Developmental Toxicity Study [16] and in accordance with the principles for Good Laboratory Practice [17], "Law for the Humane Treatment and Management of Animals" [Law No. 105, October 1, 1973, revised June 15, 2005] and "Standards Relating to the Care and Management, etc. of Experimental Animals" [Notification No. 6, March 27, 1980 of the Prime Minister's Office].

2.1. 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 toxic studies, including reproductive and developmental toxicity studies, and historical control data are available. Males at 11 weeks of age and females at 10 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for five days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered tap water ad libitum, and they were maintained in an air-conditioned room at 22 ± 3 °C, with a relative humidity of 50 ± 20%, a 12-h light/dark cycle, and ventilation of 10–15 air changes/hour. Virgin female rats were mated overnight with male rats. The day when the sperm in the vaginal smear and/or vaginal plug were detected was considered to be day 0 of pregnancy. The copulated females were distributed into four groups to equalize the female body weights among groups. The copulated females were housed individually.

2.2. Chemicals and dosing

DTG was obtained from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). DTG, a white powder, is slightly soluble in hot water and alcohol, soluble in chloroform, and very soluble in ether, and its melting point is 179 °C, specific gravity is 1.10 and molecular weight is 239.3 [2]. The DTG (Lot no. 34K21) used in this study was 99.5% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before and after the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 10, 20 or 40 mg/kg bw on days 6 through 19 of pregnancy. The dosage levels were determined based on the results of our reproduction/developmental toxicity screening test [15], in which deaths at 50 mg/kg bw/day and neurobehavioral changes and lowered body weight gain and food consumption at 20 and 50 mg/kg bw/day in females, and decreases in the number, body weight and viability of offspring and increased incidence of fetuses with malformations at 50 mg/kg bw/day were found. DTG was suspended in 0.5% (w/v) carboxymethylcellulose–Na solution with 0.1% (w/v) Tween 80. The volume of each dose was adjusted to 5 ml/kg body weight based on daily body weight. The control rats were given only 0.5% (w/v) carboxymethylcellulose–Na solution with 0.1% (w/v) Tween 80. The stability of formulations has been confirmed for up to 8 days. During use, the formulations were maintained under such conditions for less than 7 days, and each formulation was analyzed for concentration of DTG and the results revealed 90.3–99.5% of the intended concentration.

2.3. Observations

All females were observed daily during the pre-administration period and on the day of sacrifice, and twice a day (before and after administration) during the administration period for clinical signs of toxicity. Maternal body weight was recorded on days 0, 3 and 6–20 of pregnancy. Food consumption was recorded on days 0, 3, 6, 9, 12, 15, 18 and 20 of pregnancy. The pregnant rats were euthanized by exsanguination under ether anesthesia on day 20 of pregnancy. The peritoneal cavity was opened, and the uterus was removed from the maternal body and weighed. The numbers of corpora lutea, implantation sites, live and dead fetuses and resorptions were counted. The live fetuses were removed from the uterus and sexed, weighed and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected, fixed in alcohol, stained with alizarin red S and alician blue [18] and examined for skeletal anomalies. The remaining live fetuses in each litter were fixed in Bouin's solution. Their heads were subjected to free-hand razor-blade sectioning [19], and the thoracic areas were subjected to microdissecting [20] to reveal internal abnormalities.

2.4. Data analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, weight of the gravid uterus, food consumption, numbers of corpora lutea, implantations and live fetuses, fetal weight and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalences, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney *U*-test. The incidences of pre- and postimplantation embryonic loss and fetuses with malformations and variations and sex ratio of live fetuses were analyzed using Wilcoxon's rank sum test. The rates of pregnancy, non-pregnancy and females showing clinical signs of toxicity were analyzed with Fisher's exact test. The 0.05 level of probability was used as the criterion for significance.

Table 1
Maternal findings in rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
No. of rats	24	24	24	24
No. of pregnant rats	24	24	24	24
Initial body weight	256 ± 13	256 ± 13	256 ± 13	256 ± 13
No. of females showing clinical sign of toxicity				
Death	0	0	0	4
Alopecia	2	2	3	2
Bradypnea	0	0	0	2
Decreased locomotor activity	0	0	1	11**
Mydriasis	0	0	12**	24**
Prone position	0	0	0	3
Salivation	0	0	2	2
Soil of perigenital	0	0	1	4
Tremor	0	0	0	2
Body weight gain during pregnancy (g)[a]				
Days 0–6	40 ± 8	39 ± 8	40 ± 8	39 ± 8
Days 6–15	50 ± 7	49 ± 9	37 ± 11**	23 ± 10**
Days 15–20	77 ± 9	77 ± 9	71 ± 10	47 ± 16**
Days 0–20	167 ± 17	165 ± 21	148 ± 24**	109 ± 21**
Adjusted weight gain[b]	88 ± 15	87 ± 19	77 ± 15	49 ± 17**
Food consumption during pregnancy (g/day)[a]				
Days 0–6	23 ± 2	23 ± 2	23 ± 2	23 ± 2
Days 6–15	26 ± 2	26 ± 2	24 ± 3	20 ± 3**
Days 15–20	28 ± 2	28 ± 3	26 ± 2	22 ± 3**
Days 0–20	25 ± 2	26 ± 2	24 ± 2	21 ± 2**
Weight of gravid uterus (g)[a]	79 ± 10	78 ± 11	72 ± 15	59 ± 10**

[a] Values are given as the mean ± S.D.
[b] Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.
** Significantly different from the control ($p < 0.01$).

3. Results

Table 1 shows the maternal findings in rats given DTG on days 6–19 of pregnancy. At 40 mg/kg bw/day, death was found on day 8 of pregnancy in two females and on days 7 and 19 of pregnancy in one female each. Statistically significant increases in the incidence of mydriasis occurred at 20 and 40 mg/kg bw/day, and in decreased locomotor activity at 40 mg/kg bw/day. Additional findings that appeared to be treatment related, but not statistically significant were decreased locomotor activity at 20 mg/kg bw/day, salivation and soil of the perigenital area at 20 and 40 mg/kg bw/day, and bradypnea, prone position and tremors at 40 mg/kg bw/day. These signs were observed consistently throughout the dosing period and relatively higher incidences of these signs were noted during the early administration period. Maternal body weight gain was significantly decreased on days 6–15 and 0–20 of pregnancy at 20 mg/kg bw/day, and on days 6–15, 15–20 and 0–20 of pregnancy at 40 mg/kg bw/day. Adjusted weight gain, the net weight gain of maternal rats during pregnancy, and the weight of the gravid uterus were also significantly reduced at 40 mg/kg bw/day. At this dose, food consumption was significantly lowered on days 6–15, 15–20 and 0–20 of pregnancy.

Table 2 presents the reproductive findings in rats given DTG on days 6–19 of pregnancy. No dam with total litter loss was observed in any group. No effects of DTG were found on the numbers of corpora lutea and implantations, or the incidence of preimplantation loss. At 40 mg/kg bw/day, a significantly increased incidence of postimplantation loss, a decreased number of live fetuses and lowered weights of male and female fetuses and placentae were noted. The sex ratio of live fetuses was significantly reduced in the DTG-treated groups.

The summarized results of external and internal examinations in fetuses of rats given DTG on days 6–19 of pregnancy are shown in Table 3. No fetuses with external malformations were observed in the control group. One fetus with cleft palate was found at 10 mg/kg bw/day. Fetuses with external malformations were found in 13 out of the 328 fetuses (three out of the 24 litters) at 20 mg/kg bw/day and 33 out of the 251 fetuses (11 out of the 20 litters) at 40 mg/kg bw/day, and significantly increased incidence of the total number of fetuses with external malformations was noted at 40 mg/kg bw/day. Incidences of fetuses with brachydactyly and with short tail were increased at 20 and 40 mg/kg bw/day, and significantly increased incidences were found at 40 mg/kg bw/day. As for internal malformations, one fetus each with microphthalmia in the control and 20 mg/kg bw/day groups, one fetus with dilatation of the lateral ventricles in the control group and one fetus with undescended testes in the 40 mg/kg bw/day were observed. Variations in the internal organs were observed in 11–19 fetuses in all groups. However, no significant differences in the incidences of

Table 2
Reproductive findings in rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
No. of litters	24	24	24	20
No. of litters totally resorbed	0	0	0	0
No. of corpora lutea per litter[a]	15.7 ± 2.1	14.8 ± 1.6	14.9 ± 1.9	15.3 ± 1.5
No. of implantations per litter[a]	15.3 ± 1.9	14.7 ± 1.8	14.2 ± 2.7	15.2 ± 1.4
% Preimplantation loss per litter[b]	2.4	0.9	5.6	0.9
% Postimplantation loss per litter[c]	3.5	3.4	4.8	16.4**
No. of live fetuses per litter[a]	14.8 ± 1.9	14.2 ± 2.1	13.7 ± 2.9	12.6 ± 1.9**
Sex ratio of live fetuses (male/female)	0.56	0.49*	0.46*	0.46*
Body weight of live fetuses (g)[a]				
Male	3.64 ± 0.17	3.72 ± 0.18	3.59 ± 0.24	3.19 ± 0.31**
Female	3.42 ± 0.16	3.53 ± 0.25	3.41 ± 0.18	3.03 ± 0.26**
Placental weight (g)[a]	0.47 ± 0.04	0.47 ± 0.03	0.50 ± 0.16	0.40 ± 0.04**

[a] Values are given as the mean ± S.D.
[b] (No. of preimplantation embryonic loss/no. of corpora lutea) × 100.
[c] (No. of resorptions and dead fetuses/no. implantations) × 100.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

fetuses with internal malformations and variations were detected between the control and DTG-treated groups.

The summarized results of skeletal examinations in the fetuses of rats given DTG on days 6–19 of pregnancy are presented in Table 4. Fetuses with skeletal malformations were found in one out of the 184 fetuses (one out of the 24 litters) in the control group, one out of the 176 fetuses (one out of the 24 litters) at 10 mg/kg bw/day, 13 out of the 170 fetuses (six out of the 24 litters) at 20 mg/kg bw/day, and 26 out of the 130 fetuses (12 out of the 20 litters) at 40 mg/kg bw/day. Significantly higher incidences of the total number of fetuses with skeletal malformations were observed at 20 and 40 mg/kg bw/day. Incidences of fetuses with absence, fusion or malposition of the caudal vertebrae and with absence or fusion of phalanges were higher at 20 and 40 mg/kg bw/day, and significantly increased incidences of fetuses with these malformations and fetuses with the absence or fusion of metacarpals were found at 40 mg/kg bw/day. Although skeletal variations in the vertebral column, ribs and sternebrae were observed in all groups, no significant differences in the incidences of fetuses with skeletal variations were detected between the control and DTG-treated groups. A significantly delayed ossification, as evidenced by the numbers of sacral and caudal vertebrae, sternebrae, and metatarsi, was also noted at 40 mg/kg bw/day.

4. Discussion

In order to obtain further information on the reproductive and developmental toxicity of DTG, the present study was conducted in compliance with OECD guideline 414 Prenatal Developmental Toxicity Study [16]. DTG was given to pregnant rats during the time of implantation to the term of pregnancy to

Table 3
External and internal examinations in fetuses of rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
External examination				
Total no. of fetuses (litters) examined	354 (24)	341 (24)	328 (24)	251 (20)
Total no. of fetuses (litters) with malformations	0	1	13 (3)	33 (11)**
Cleft palate	0	1	0	0
Brachydactyly	0	0	8 (3)	31 (11)**
Short tail	0	0	7 (2)	10 (7)**
Internal examination				
Total no. of fetuses (litters) examined	170 (24)	165 (24)	158 (24)	121 (20)
Total no. of fetuses (litters) with malformations	1	0	1	1
Microphthalmia	1	0	1	0
Dilatation of lateral ventricles	1	0	0	0
Undescended testes	0	0	0	1
Total no. of fetuses (litters) with variations	16 (10)	11 (9)	13 (7)	19 (12)
Thymic remnants in neck	13 (10)	8 (7)	12 (7)	17 (11)
Dilated renal pelvis	2 (2)	2 (2)	0	0
Left umbilical artery	1	1	1	2 (2)

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

Table 4
Skeletal examinations in fetuses of rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
Total no. of fetuses (litters) examined	184 (24)	176 (24)	170 (24)	130 (20)
Total no. of fetuses (litters) with malformations	1	1	13 (6)*	26 (12)**
Split cartelige of thoracic centrum	0	0	1	1
Fused cartilage of cervical vertebral arches	0	1	1	1
Fused cartilage of ribs	1	0	0	0
Absence, fusion or malposition of caudal vertebrae	0	0	8 (3)	10 (8)**
Absence or fusion of phalanges	0	0	5 (3)	18 (9)**
Fusion of metacarpal/metatarsal and phalanx	0	0	0	2 (2)
Absence or fusion of metacarpals	0	0	0	4 (4)*
Shortening of tibia and fibula	0	0	0	1
Total no. of fetuses (litters) with variations	10 (7)	16 (9)	16 (11)	12 (8)
Bipartite ossification of thoracic centrum	0	2 (1)	1	0
Dumbbell ossification of thoracic centrum	0	1	0	0
Unossified thoracic centrum	1	1	0	1
Variation of number of lumbar vertebrae	1	0	0	2 (1)
Wavy ribs	0	1	1	0
Short supernumerary rib	9 (6)	12 (7)	14 (10)	4 (4)
Short 13th rib	0	0	0	2 (2)
Sacralization of lumbar vertebra	0	0	0	2 (1)
Bipartite ossification of sternebra	0	0	1	1
Asymmetry of sternebra	0	0	0	1
Degree of ossification[a]				
No. of sacral and caudal vertebrae	7.3 ± 0.5	7.5 ± 0.5	7.5 ± 0.5	7.0 ± 0.6*
No. of sternebrae	4.6 ± 0.4	4.8 ± 0.5	4.6 ± 0.4	4.2 ± 0.4*
No. of metatarsals	8.0 ± 0.0	7.9 ± 0.3	7.8 ± 0.4	6.7 ± 1.4*

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

characterize the effects of DTG on embryonic/fetal development. The findings of the present study confirmed the results of a previous screening study and extended the understanding of the reproductive and developmental toxicity of DTG. The present data showed that the prenatal oral administration of DTG produced maternal toxicity, as evidenced by deaths, neurobehavioral changes, decreased body weight gain and reduced food consumption, and developmental toxicity, as evidenced by a high incidence of postimplantation loss, a decreased number of live fetuses and lower weight of fetuses, and teratogenicity, as evidenced by a higher incidence of fetuses with external and skeletal malformations.

DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,21,22]. The systemic injection of DTG has been reported to cause neurobehavioral changes in rats [4,6,7,9,22]. The present study shows that the oral administration of DTG also induced neurobehavioral changes at 20 and 40 mg/kg bw/day in pregnant rats. Lowered body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were also observed in pregnant rats. These findings indicate that DTG is maternally toxic at 20 mg/kg bw/day and higher.

The sex ratio (males/females) was significantly lowered in all DTG-treated groups. The values for sex ratio were 0.429–0.521 in the background control data for the last 6 years in the laboratory performed present study. Statistically significant changes in the sex ratio observed in the present study were considered to be unrelated to the administration of DTG, because the values for sex ratio in the DTG-treated groups were within the range of the historical control data, no increased embryonic/fetal deaths were detected at 10 and 20 mg/kg bw/day and the control value for the sex ratio was very high in the present study. A decreased number of live fetuses, increased incidence of postimplantation loss, and reduced weights of fetuses and placentae were detected at 40 mg/kg bw/day. A decreased number of live fetuses and increased incidence of postimplantation loss indicate embryonic/fetal lethality, and reduced weights of fetuses and placentae indicate intrauterine growth retardation. These findings indicate that DTG is toxic to embryonic/fetal survival or fetal growth at 40 mg/kg bw/day when administered during the time of implantation to the term of pregnancy.

In our previous reproductive and developmental screening test [15], the total number of fetuses with external malformations, but not individual malformation, was significantly increased at 50 mg/kg. At this dose, oligodactyly and tail anomalies were frequently observed, and the teratogenic effect of DTG was strongly suggested. No malformed fetuses were found at 20 mg/kg bw/day in our previous study. In the present study, morphological examinations in the fetuses of exposed mothers revealed increased incidence of fetuses with external and skeletal malformations at 20 and 40 mg/kg bw/day.

Fetuses with external, internal and/or skeletal malformations and/or variations were found in all groups. The malformations and variations observed in the present study are of the types that occur spontaneously among the control rat fetuses [23–26]. At 40 mg/kg bw/day, significantly higher incidences of the total number of fetuses with external and skeletal malformations were detected, and significantly higher incidences of individual types of external and skeletal malformation were also noted. At 20 mg/kg bw/day, the incidence of the total number of fetuses with skeletal malformations was significantly higher than that of control group. Although the incidence of individual types of skeletal malformation was not significantly increased at 20 mg/kg bw/day, types of external and skeletal malformations observed at this dose were the same as those observed at 40 mg/kg bw/day. Consideration of the sum of these findings suggests that a conservative estimate of the LOAEL for the teratogenic dose of DTG is 20 mg/kg bw/day in rats when administered during the time of implantation to the term of pregnancy. DTG caused suppression of body weight gain and neurobehavioral changes in dams and abnormally morphological development and developmental delay in the offspring of rats at 20 and 40 mg/kg bw/day. Therefore, the teratogenic effects of DTG at doses without maternal toxicity, a selective teratogenicity of DTG, was not found in the current study. There are no available reports in which the developmental toxicity of DTG is assessed in any other animal species. Further studies are needed to confirm the reproductive and developmental toxicity of DTG in additional species. Developmental neurotoxicity and multigeneration studies are also required to support the conclusion of the prenatal hazard of DTG.

In conclusion, DTG caused maternal neurobehavioral changes and decreased body weight gain at 20 mg/kg bw/day and higher, embryonic/fetal deaths and lowered fetal weight at 40 mg/kg bw/day, and increased incidence of fetuses with malformations at 20 mg/kg bw/day and higher when administered during the time of implantation to the term of pregnancy in rats.

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COMMENTARY

Comments from the Behavioral Teratology Committee of the Japanese Teratology Society on OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426, Developmental Neurotoxicity Study, Draft Document (September 2003)

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ABSTRACT **In September 2003, a new revision of the draft guideline (Organization for Economic Co-operation and Development [OECD] Guideline for the Testing of Chemicals, Proposal for a New Guideline 426, Developmental Neurotoxicity Study) was distributed. The draft guideline consists of 51 paragraphs and an appendix. The National Coordinators were requested to arrange national expert reviews of the guideline proposal in their member countries. The member of the Behavioral Teratology (BT) Committee of the Japanese Teratology Society (JTS) reviewed, discussed and commented on the draft Test Guideline proposal. The BT Committee of the JTS also commented that the International Collaborative Study to validate this protocol should be definitely performed. These comments were sent to the OECD Secretariat. The BT Committee of the JTS expects that the comments are useful for further discussion.**

Key Words: behavior, developmental neurotoxicity, OECD, test guideline

INTRODUCTION

The Organization for Economic Co-operation and Development (OECD) Working Group on Reproduction and Developmental Toxicity at Copenhagen in June 1995 (OECD 1995) recommended that a guideline for developmental neurotoxicity should be written. In June 1996 at Copenhagen, an OECD Consultation Meeting on Developmental Neurotoxicity provided the Secretariat with the draft report on the outline of a new guideline (OECD 1996). The Behavioral Teratology (BT) Committee of the Japanese Teratology Society (JTS), in association with the Meeting of Neurobehavioral Toxicology of the Japanese Society of Toxicology, commented on this draft report. After this meeting, a draft

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proposal for Test Guideline 426, Developmental Neurotoxicity Study was developed, and was submitted to the Secretariat in February 1998. The draft guideline was distributed in December 1998. The BT Committee of the JTS commented again on this draft guideline. The draft guideline proposal was extensively revised and distributed in October 1999. General issues regarding the design of developmental neurotoxicity studies were discussed in an OECD Expert Consultation Meeting and International Life Sciences Institute (ILSI) Risk Science Institute Workshop in Washington, DC, USA, in October 2000 (OECD 2003). In September 2003, a new revision of the guideline was distributed. This revised draft Test Guideline proposal is posted on the OECD public web pages of the Test Guidelines Programme at: http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html. The draft guideline consists of 51 paragraphs and an appendix. National Coordinators were requested to arrange national expert reviews of the guideline proposal in their member countries. The deadline for the expert responses to this revised draft Test Guideline was January 16, 2004.

A meeting of the BT Committee (Chairman: Dr Y. Fukui, Professor, University of Tokushima School of Medicine) of the JTS was held on January 11, 2004, in Osaka, and the members of this committee reviewed, discussed and commented on the draft Test Guideline proposal. The BT Committee of the JTS also commented that the International Collaborative Study to validate this protocol as indicated in OECD ENV/EHS/HK/mc/2003.49 should be definitely performed. These comments were sent to the OECD Secretariat through the Japanese National Coordinator (Director of the Office of Chemical Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Japan) on January 16, 2004, but it is to be noted that they are not official comments from Ministry of Health, Labour and Welfare, Japan.

The BT Committee of the JTS expects that the comments are useful for further discussion.

The comments from the BT Committee of the JTS are as follows:

GENERAL COMMENTS

1 New terms such as behavioral ontogeny, instead of reflex ontogeny in the 1999 draft, are introduced in the 2003 draft, but unification of terms is insufficient in the various parts of the text.
2 The rationale for the weaning day should be stated. Day of weaning is recommended to be PND 22, but PND 21 from the previous draft still appears in some parts of the text. The description day of test performance should be unified throughout the text.
3 More flexibility of the study design must be stressed. The use of 'should' is seen too frequently.
4 Guidance for higher levels of the study, such as social behavior, pharmacologic challenge, and neurochemistry, is insufficient.
5 Examination of maternal toxicity is insufficient except for clinical signs. It is advised that dams are autopsied and examined at least macroscopically.
6 The description of use of species other than rats, such as non-human primates, is scanty.
7 Considerable recent references have been added, but there is more pertinent literature to be cited.
8 The front page (DRAFT DOCUMENT [September 2003]) should be page 1, and the present page 1 is to be changed to page 2, and so on. The final page, Appendix A, would be page 21.

SPECIFIC COMMENTS

1. Paragraph 2

The exposure period is expanded from 'lactation' to 'during early life'. This change is very welcomed, but the following explanation is limited to the exposure until weaning. Some description of administration of the test substances directly to offspring after weaning should be given, since human developmental neurotoxicity of chemicals in early childhood has become a great concern.

The phrase 'during pregnancy or' should be '*in utero* and'. Pregnancy primarily refers to dams, not to fetuses.

2. Paragraph 3

The phrase 'developmental toxicity and/or adult neurotoxicity study (e.g. Test Guidelines 415, 416, 424)' is to be changed to 'prenatal developmental toxicity, one- or two-generation study and/or adult neurotoxicity study (e.g. Test Guidelines 414, 415, 416, 424)'.

The phrase 'or as an add-on study' should be concretely explained, since the meaning is not clear.

Does 'other types of toxicity' include developmental (fetal) toxicity or is it limited to adult? It is necessary to specify this.

3. Paragraph 4

The phrase 'perinatal' in line 2 is to be 'prenatal', since the latter is the OECD term of Guideline 414.

4. Paragraph 5

The word 'and/or' in line 2 is to be 'and'.

The term 'reflex ontogeny' in line 5 is to be 'behavioral ontogeny'.

5. Paragraph 6

Since 'stand-alone' is a specific computer term, it is preferable to replace it with a more common word.

6. Paragraph 7

The usefulness of other species, especially non-human primates, for higher levels of learning and memory study, may be more circumstantially stated.

7. Paragraph 9

The third sentence should be changed to 'After evidence of copulation, individual housing of mated animals is recommended'. The sentence 'If mated animals are caged in small groups, animals should be caged separately in individual cages no later than day 15 of pregnancy' should be inserted following the third sentence.

8. Paragraph 10

It may be necessary to describe the males used for mating.

Usually, rats are obtained as a lot that may contain some brothers. Therefore, it is not practical for breeding males to be equalized across a group.

9. Paragraph 12

The numbers '8–12' in line three are to be changed to '8–10'. In cases of litter sizes of 12, many litters may be insufficient in number. When the number of pups in a litter is less than the designated number, it is not acceptable to add some pups from different dams for fostering.

Those litters with an insufficient number of pups should not be principally used for the study. These remarks are to be clearly described here.

Identification of individual pups is recommended to be performed at birth or soon after birth when the body weight is measured.

10. '*Assignment of . . .*' and paragraphs 13–15

It is recommended that this portion is placed after *Dosage* and *Administration of doses*, since dosage and administration are more directly related to dams than assignment of offspring.

11. Paragraph 14

The rationale is not clear why the same pair of male and female littermates is assigned for motor activity testing, while for all the other tests the same or separate pairs may be used.

12. Paragraph 15

'Behavioral/functional tests' in Tables 1 and 2 should be 'Functional/behavioral tests', concordant with the description in Table 3. Function is a broader category than behavior.

The contents of 'functional/behavioral test' in Tables 1 and 2 are not clear. In the text, 'functional tests' are listed in line 11. In Table 3, 'functional/behavioral endpoints' consist of three major items, motor activity, motor and sensory function, and learning and memory. Therefore, the major component of 'functional/behavioral tests' in Tables 1 and 2 would be motor and sensory function.

Note (c) to Table 1 is questionable unless the same pups are used to check the changes of findings in adolescent and young adult ages. Moreover, the number of animals tested is recommended to be 20 in Table 2. Therefore, it is generally preferable to adopt the procedures indicated in Table 2 since the offspring tested for cognitive function etc. are examined for neuropathology, and the correlation between behavioral abnormalities and neuropathological changes can be checked. Thus, Table 2 is recommended to be the first choice and treated as Table 1. The total sentences in this paragraph should be rewritten according to this consideration. Optional and Neuropathology in Tables 1 and 2 should be optional and neuropathology (small letters).

Pups no. of the female in the preweaning investigation in Table 2 is 5, not 2.

13. Paragraph 16

The phrase 'maternal or developmental toxicity or neurotoxicity' in line 10 is to be changed to 'maternal or developmental toxicity' or 'maternal or developmental toxicity including neurotoxicity', since neurotoxicity is a part of toxicity and is related to both dams and offspring.

In some cases, a high dose can not be chosen to induce maternal toxicity. Thus, it is highly recommended to add a sentence to explain the rationale in cases where no maternal toxic dose level is selected for the high dose.

A description regarding limit dose should be added.

14. Paragraph 17

The word 'should' should be changed to 'may' (lines 1 and 4).

The sentence 'However, an evaluation of direct dosing to pups has not been established yet.' should be inserted following the last sentence.

15. Paragraph 19

In case of dietary or via drinking water administration, due consideration should be taken that pups receive the test substances not only from milk but also considerably from diet or water in the later period of lactation.

The phrase 'except for the day of parturition' and the sentence 'The test substance should be administered after completion of parturition.' should be inserted following the end of the last sentence.

16. Paragraph 20

The first sentence should be deleted. In reproductive and developmental studies including teratological study and pre- and postnatal study, the dosage volume in each dam is practically calculated by two different methods: (a) based only on body weight on day 6 of gestation or (b) based on the

most recent body weight. Body weights on day 6 and day 20 of gestation are 300–320 g and 400–420 g, respectively, in SD rats. When the dosage volume is calculated based on the recent body weight, dams will be exposed to overdose (approximately 1.3 times) and excess toxicity to dams must be noted.

17. Paragraph 21
A marginal note * is to be incorporated into the text because this is an important item.

18. Paragraph 24
Delete 'secretion and' in line 3 (duplicated).

19. Paragraph 27
PND 21 is to be PND 22.

Measurement of food consumption is recommended at administration via other routes than diet since food consumption is an important indicator of maternal general toxicity.

20. Paragraph 31
The headline 'Developmental landmarks' is to be 'Physical and developmental landmarks' since body weight, described in paragraph 31, is certainly an indicator of physical development.

'Pinna reflex' is to be 'Pinna detachment'.

Add eye opening since it is an important index related to motor activity.

21. Paragraph 32
The following reference is to be cited in explanation of the usefulness of postcoital age: Tachibana T., Narita H., Ogawa T., Tanimura T. (1998) Using postnatal age to determine test dates leads to misinterpretation when treatments alter gestation length: Results from a collaborative behavioral teratology study in Japan. Neurotoxicol Teratol. 20: 449–457.

Table 3 should be carefully revised since neuropathological examination on PND 11 is no longer routinely recommended. 'Age Period' is to be 'Age period'. [Before PND 21] is to be [At and before PND 21] since PND 21 is the last day of the preweaning period. [PND 21–59(a)] is [PND 22–59(a)]. In the row of physical development, 'weekly' is to be at the level of Body weight (one line downward). In the row of Brain weight and Neuropathology, delete 'at PND 22' in the column of Preweaning since preweaning ends at PND 21. Only a remark (b) may remain in this place (for examination on PND 11). Delete 'optional' in the column of Adolescence. In Note (a), weaning (generally PND 21) is weaning (generally PND 22), and (PND 23–24) should be (PND 24–25).

22. Paragraph 33
Delete the heading 'Physical development'. The reason is given in comment 19.

It is suggested that this paragraph is moved before paragraphs 31 and 32, since the counting and sexing of live pups are the first steps for offspring observation.

23. Paragraph 34
Surface righting, cliff avoidance and swimming development should be added as examples. Also, give pertinent literature on these tests. Swimming is an especially good indicator of behavioral ontogeny.

24. Paragraph 35
The phrase 'preweaning and adult age' in line 1 should be 'preweaning, adolescence and young adult age', according to Table 3.

It is important to minimize maternal stress at the test of motor activity. Practically, the manipulation of separating the pups from the mother and returning them to the cage should be performed as gently as possible. This caution may be applied at other preweaning tests such as body weight measurement.

The description of 'Among the variables . . .' in lines 16–18 may be also applied to tests other than motor activity. Therefore, these statements should be placed in the appropriate earlier paragraphs as a general caution.

An explanation regarding the phrase '1–3 times' is needed (third line from the bottom, second column in Table 3).

25. Paragraph 36
Rotarod, open field and olfactory orientation tests are to be added as examples. As for a reference of olfactory orientation, Gregory EH, Pfaff DW. (1971) Development of olfactory guided behavior in infant rat. Physiol Behav. 6 : 573–576, is suggested.

References should be separately given for each test for the readers' convenience.

26. Paragraph 37
The headline 'Learning and memory tests' should be 'Learning and memory tests (Cognitive function tests)' or 'Cognitive function tests' (Refer to Tables 1–3).

The Biel maze (multiple T-water maze) should be added as an example. The shuttle box avoidance test (active avoidance) may be also added. Pertinent literature on these tests is also to be described.

Two or more different categories of learning and memory tests may be planned to reveal the nature of disturbances of learning and memory.

27. Paragraph 38
PND 21 is to be PND 22.

28. Paragraph 41
Some explanation of GFAP is necessary, together with references, or '(e.g. GFAP)' should be deleted.