Table 3
Morphological examinations in fetuses of rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0(control)	15	45	135
External examination				
No. fetuses (litters) examined	225(16)	228(16)	204(16)	91(13)
No. fetuses (litters) with malformations	0	0	0	0
Skeletal examination				
No. fetuses (litters) examined	116(16)	117(16)	105(16)	49(13)
No. fetuses (litters) with malformations	0	0	0	1(1)
Sternoschisis	0	0	0	1(1)
No. of fetuses (litters) with variations	11(7)	8(4)	10(7)	21(11)**
Cervical rib	4(2)	3(1)	3(2)	1(1)
Splitting of thoracic vertebral bodies	0	1(1)	0	0
14th ribs				
Extra	0	0	0	4(3)
Rudimentary	2(1)	1(1)	2(2)	9(7)**
Bipartite sternebrae	1(1)	2(1)	1(1)	9(7)**
Asymmetry of sternebrae	5(5)	1(1)	4(3)	3(3)
Degree of ossification[a]				
No. of ossification centers of caudal vertebrae	3.3±0.4	3.1±0.4	3.2±0.4	2.8±0.3**
No. of sternebrae	4.9±0.4	4.9±0.6	4.8±0.5	3.9±0.7**
Internal examination				
No. fetuses (litters) examined	109(16)	111(16)	99(16)	42(12)
No. fetuses (litters) with malformations	0	0	0	2(1)
Hypoplasia of spleen	0	0	0	2(1)
No. of fetuses (litters) with variations	5(3)	3(3)	2(2)	2(2)
Thymic remnant in neck	4(3)	1(1)	2(2)	2(2)
Left umbilical artery	1(1)	2(2)	0	0

[a] Values are given as mean±SD.
* Significantly different from the control, $P<0.05$.

during the administration period was found at 45 mg/kg and higher. Although pregnant rats in the 45 mg/kg group recovered with respect to body weight after cessation of administration of TP, such recovery did not occur in the high dose group. This may be due to a lack of conceptuses at 135 mg/kg. However, a significantly low adjusted weight gain at 45 mg/kg and higher may suggest maternal toxicity. These findings indicate that TP exerts maternal toxicity at 45 mg/kg and higher when administered during organogenesis in rats.

Developmental endpoints should include the number and percent of pre-and postimplantation loss, morphological alterations in fetuses, and decreased fetal weight (Kimmel and Price, 1990; Schardein, 2000; OECD, 2001). Schardein (2000) stated that fetal size is an important in the assessment of potential teratogen as an indicator of developmental toxicity, and reduction in size or growth retardation commonly occurs among fetuses of dams given dosages that are toxic to the dam, to the offspring, or both. In the present study, a significant increase in the incidence of postimplantation loss was found at 135 mg/kg and a significantly decreased weight of female fetuses was found at 45 mg/kg and higher. These findings indicated that TP is embryolethal at 135 mg/kg and toxic to fetal growth at 45 mg/kg and higher when administered during the period of organogenesis.

As for morphological examinations in the fetuses of exposed mother, a few fetuses with skeletal or internal malformations were found in the 135 mg/kg group. The malformations observed in the present study are not thought to be due to the administration of TP, because they occurred at a very low incidence and are of types that occur sporadically among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). Several types of skeletal and internal variations were also found in both the control group and TP-treated groups. These variations are frequently observed in fetuses of rats at term (Kimmel and Wilson, 1973; Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). In the 135 mg/kg group, a significant increase in the incidence of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs, but no extra ribs, and a significant decrease in the degree of ossification were accompanied by a significant decrease in the fetal weight. These findings show a correlation between these morphological alterations and growth retardation in fetuses. Although a skeletal variation, i.e. super-

numerary extra 14th ribs, is a warning sign of possible teratogenicity, the rudimentary 14th ribs, sternebral variations, and bilobed centra of the vertebral column are a normal variation (Kimmel and Wilson, 1973). Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and this might result from a delay in growth or morphologenesis that has otherwise followed a normal pattern of development. Consideration of these findings together suggests that the morphological changes observed in the present study do not indicate a teratogenic response and that TP possesses no teratogenic potential in rats.

In a developmental toxicity study in mice in which a single administration of TP was given at 420, 560, 750, or 1000 mg/kg by gastric intubation on day 9 of pregnancy, maternal deaths, dams with litter totally resorbed, and a significant increase in embryolethality were found at 750 mg/kg and higher (Ogata et al., 1999). A significant increase in the incidence of fetuses with malformations was accompanied by a significant decrease in fetal weight at 560 mg/kg and higher. Two highest doses, 750 and 1000 mg/kg, were maternally lethal, and the dose level of 560 mg/kg was very close to the maternally lethal dose. Thus, fetal malformations occurred after a single administration of TP at high doses in a single species. In other words, TP may be capable to produce fetal malformations under extreme experimental conditions in mice. Studies in additional species would be of great value in evaluating developmental toxicity of TP in conventional experimental conditions. We demonstrated here that TP possesses no adverse effects on morphological development in rat fetuses when administered during the whole period of organogenesis at doses which caused a decreased fetal weight, increased incidence of postimplantation loss, and maternal toxicity.

In conclusion, the administration of TP to pregnant rats throughout organogenesis had adverse effects on maternal rats and embryonic/fetal survival and growth but had no adverse effects on morphological development of fetuses even at maternally toxic and embryolethal doses. The data indicate that TP adversely affected the embryonic/fetal survival and growth only at maternally toxic doses in rats. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.

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Unexpected nephrotoxicity induced by tetrabromobisphenol A in newborn rats

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Abstract

The repeated dose toxicity of tetrabromobisphenol A (TBBPA), a flame retardant, was examined in male and female newborn rats given TBBPA orally at 0, 40, 200, or 600 mg/kg per day for 18 days from 4 days of age until weaning at 21 days of age. Half the rats in each dose group were sacrificed for a full gross necropsy and a histopathology on the organs and the tissues at 22 days of age and the remaining rats were reared without any treatment from post-weaning until 84 days of age to examine the recovery and the delayed occurrence of toxic effects. Treatment with 200 or 600 mg/kg TBBPA-induced nephrotoxicity characterized by the formation of polycystic lesions, and some deaths occurred in the 600 mg/kg group. There was no gender difference of nephrotoxicity and there were no other critical toxicities. At 85 days of age, nephrotoxic lesions were still present in the 200 and 600 mg/kg groups, but no abnormalities indicating delayed occurrence of toxic effects were found in the treated groups. In order to investigate the specificity of the nephrotoxicity induced by TBBPA in newborn rats, TBBPA was given to male and female young rats (5 weeks old) by oral administration at 0, 2000, or 6000 mg/kg per day for 18 days. The kidneys showed no histopathological changes even at the high dose. These results clearly indicate that the nephrotoxicity of TBBPA is specific for newborn rats although the toxic dose level was relatively high. To gain insight into the possible effects on human infants, the mechanism of this unexpected nephrotoxicity of TBBPA in newborn rats should be examined.

Keywords: Tetrabromobisphenol A; 4,4′-Isopropylidene bis(2,6-dibromophenol); Unexpected nephrotoxicity; Polycystic kidney; Newborn rats

1. Introduction

Recently, there is growing concern about the effects of environmental chemicals on children,

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particularly infants, who may be more sensitive on a body weight basis than adults to a given toxicant exposure (Scheuplein et al., 2002). To address this issue, we have conducted repeated toxicity studies of 18 chemicals in newborn rats as a Japanese National Project. So far, comparative evaluation of the toxicity in newborn and young rats has been conducted for four chemicals, 4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, and 3-methylphenol (Koizumi et al., 2001, 2002, 2003). The results showed that the susceptibility of newborn rats to these chemicals was approximately two to four times higher than that of young rats, although the toxicological profiles were almost the same at both ages.

Tetrabromobisphenol A (TBBPA), the fifth chemical subjected to the comparative analysis, has been widely used as a flame retardant. Its toxicity was previously investigated using young or young adult animals as follows: in 28- and 90-day feeding studies using rats, no toxic effects were observed up to 50 and 100 mg/kg per day, respectively (Goldenthal and Geil, 1972; Quast et al., 1975). In mice given TBBPA in their food for 90 days, all animals at 7100 mg/kg per day died while suffering from malnutrition and anemia (Tobe et al., 1986). Inhibition of body weight gain and anemia, but not death, were observed at 2200 mg/kg per day, and the non-toxic level was 700 mg/kg per day. There were no signs of maternal or developmental toxicity when rats were given this chemical during pregnancy up to 3000 mg/kg per day (Goldenthal et al., 1978; Noda et al., 1985). Recently, a 28-day repeated dose toxicity study of this chemical was conducted in rats using the Japanese test guidelines (equivalent to OECD guideline for testing of chemicals for repeated dose 28-day toxicity study in rodents (407)) under the Principles of Good Laboratory Practice, and showed no chemical-related effects up to 1000 mg/kg per day (MHLW, 2001).

In the present study, we performed a 18-day repeated dose oral toxicity study using newborn rats from 4 days of age under the same experimental conditions reported previously (Koizumi et al., 2001), and unexpectedly found severe nephrotoxicity. Therefore, a young rat study was also conducted at a dose up to 6000 mg/kg per day to confirm the specificity of the nephrotoxicity in newborn rats.

2. Materials and methods

2.1. Materials

Tetrabromobisphenol A: TBBPA (4,4′-isopropylidene bis(2,6-dibromophenol), molecular weight 543.88, CAS No.79-94-7, 99.5% purity) was obtained from Toso Co. Ltd. (Yamaguchi, Japan) and suspended in 0.5% (w/v) carboxymethylcellulose-Na (Kanto Chemicals Co. Ltd., Tokyo, Japan) solution with 0.1% (w/v) Tween 80 (Difco Laboratories, Detroit, Michigan, USA). The suspension was prepared at least once a week and stored hermetically in a cool and dark place (4 °C) until dosing. The stability of TBBPA under these conditions was confirmed to be at least 8 days by an analysis of dosing suspensions.

2.2. Animals

Sprague-Dawley SPF rats (Crj:CD(SD)IGS) were purchased from Charles River Japan Inc. (Atsugi, Japan) and maintained in an environmentally controlled room at 22 ± 3 °C with a relative humidity of 55 ± 10%, an air exchange rate of more than 10 times per hour, and a 12:12 h light/dark cycle. All animals were allowed free access to commercial solid diet (Labo MR Stock, Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan) and tap water. The animals used in the present study were reared, treated, and sacrificed in accordance with "The Provisions for Animal Welfare" of the Research Institute for Animal Science in Biochemistry and Toxicology, which follow the guidelines for animal experimentation issued by Japanese Association for Laboratory Animal Science.

2.3. Newborn rat study

For the study of newborn rats, 20 pregnant rats (gestation day 15) were purchased and were allowed to deliver spontaneously. Among all newborns separated from each dam at the age of 3 days, 48 males and 48 females were randomly selected and assigned to four dose groups, including controls. Twelve foster mothers suckled four males and four females assigned to each group up to weaning on day 21 after birth. After weaning, the animals of the recovery-maintenance group were individually maintained for 9 weeks.

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 (*$P < 0.05$; **$P < 0.01$).

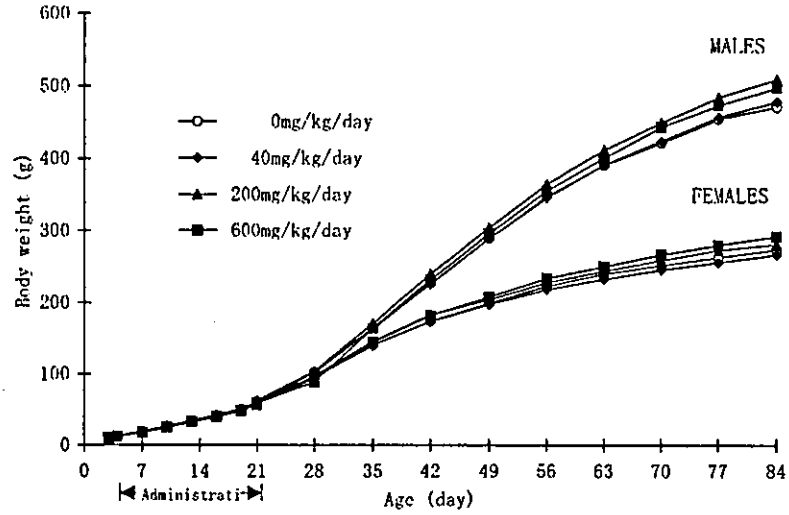

Fig. 1. Body weight changes of rats treated orally with TBBPA for 18 days from 4 days of age until weaning.

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,

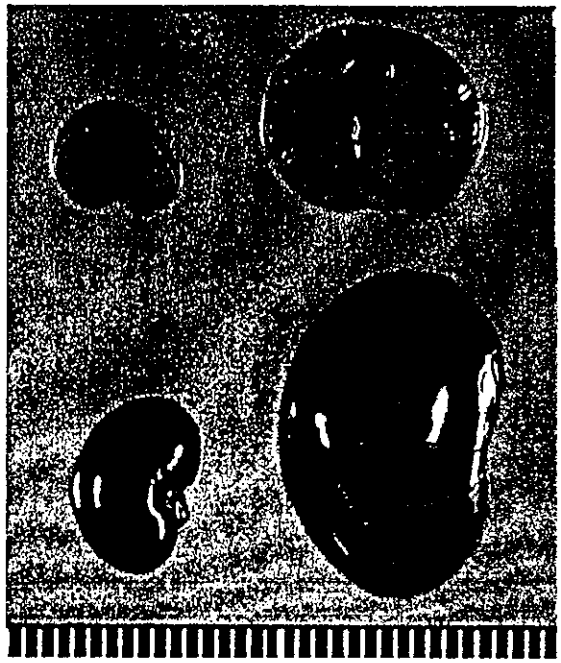

Fig. 2. Gross appearance of kidney (lower right) and its cross-section (upper right) in a 22-day-old rat treated with TBBPA (600 mg/kg body weight daily, orally) for 18 days. The kidney is markedly larger than that of a non-treated rat (left).

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.

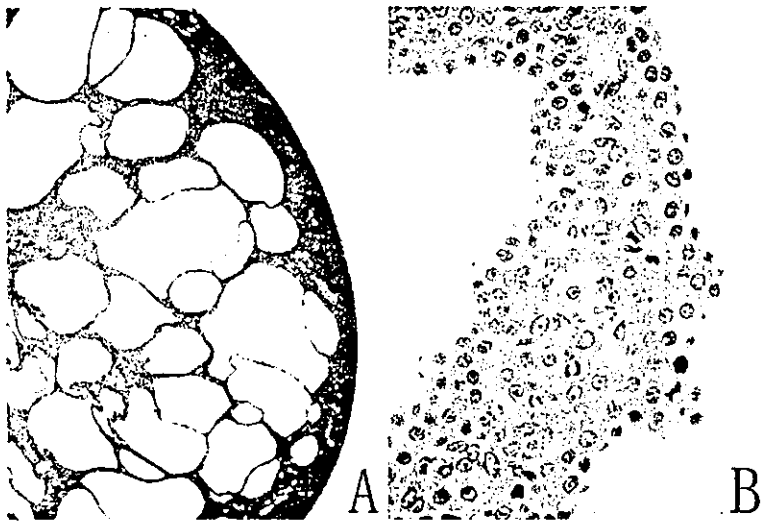

Fig. 3. Polyeystic renal lesion observed in a 22-day-old rat treated with TBBPA (600 mg/kg body weight daily, orally) for 18 days. H-E stain. (A) Dilatation of the tubules from the cortico-mudullary junction to the inner cortex, 40×; (B) hyperplasia of the tubular epithelium, 125×.

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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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.

29. Paragraph 43
The phrase '(tectum, tegmentum, and cerebral peduncles)' should be deleted.

30. Paragraph 44
The phrase 'typical of the adult brain' is not understandable. Are some words are missing?

31. Paragraph 46
The sentence 'While the use . . .' in lines 7–9 can be rewritten more simply. For instance, 'It is preferable that a pathologist who is unaware of the treatment information scores the slides to substantiate the dose–response relationship'.

32. Paragraph 48
Delete 'perinatal' in line 1. The name of this guideline is simply developmental neurotoxicity study.

The phrase 'human studies, case reports', is to be changed to 'human epidemiological studies or case reports', since case report is one of the categories of human studies.

33. Paragraph 47 after Test report
47 should be 51.

Insert water after diet in the 4th item of Test animals.

The phrase 'reflex ontogeny' in the 9th item of Results must be 'behavioral ontogeny'.

34. Literature
Try to unify the style of the reference presentation. In particular, the writing of journal titles should be uniform (e.g. compare 5 and 7 for Environ Health Perspect and italic presentations such as 28 and 32). It is recommended that the abbreviation of journal titles follows the PubMed, NLM style.

The presentation of the authors' names is also confusing (e.g. 5 vs. 9).

The placement of the published year is also variable (e.g. 3, 5 and 12).

Put a space between 18 and 19. Delete one space after 67.

Some good references as background information can be found in Massaro EJ. (2002) Handbook of neurotoxicology. Vols I and II. Humana Press, Totowa. The four papers in vol II (Henck JW, Rice SA, Cappon GD and Stump DG, and Tilson HA) are very valuable.

35. Appendix A
Totally redraw Fig. 1 according to the description in Tables 2 and 3, and also clarify in the figure legend that this scheme is based on Tables 2 and 3. A suggestion is attached.

REFERENCES

Organisation for Economic Co-operation and Development (OECD) (1995) *Draft Report of the OECD Ad Hoc Working Group on Reproduction and Developmental Toxicity.* Copenhagen, Denmark, 13–14 June 1995.

Organisation for Economic Co-operation and Development (OECD) (1996) *Final Report of the Consultation Meeting on Developmental Neurotoxicity.* Copenhagen, Denmark, 17–18 June 1996.

Organisation for Economic Co-operation and Development (OECD) (2003) *Report of the Expert Consultation Meeting in Developmental Neurotoxicity Testing.* Washington, US, 23–25 October 2000.

APPENDIX A

Fig. 1 Example of the testing scheme for assignment of animals for functional/behavioral tests, neuropathology evaluation, and brain weights, as described in paragraphs 13, 14, and 15. This diagram is based on the description in Tables 2 and 3. (PND = postnatal day).