

ORIGINAL ARTICLE

Elevated susceptibility of newborn as compared with young rats to 2-*tert*-butylphenol and 2,4-di-*tert*-butylphenol toxicity

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ABSTRACT In order to determine the susceptibility of newborn rats to 2-*tert*-butylphenol (2TBP) and 2,4-di-*tert*-butylphenol (DTBP) toxicity, studies were conducted with oral administration from postnatal days (PND) 4 to 21 and the findings were compared with results for young rats exposed from 5 or 6 weeks of age for 28 days. In the newborn rats, specific effects on physical and sexual development and reflex ontogeny were not observed. While there were no clear differences in toxicological profiles between newborn and young rats, the no-observed-adverse-effect levels (NOAELs) differed markedly. For 2TBP, clinical signs such as ataxic gait, decrease in locomotor activity and effects on liver, such as increase in organ weight, were observed and the NOAELs were concluded to be 20 and 100 mg/kg/day in newborn and young rats, respectively. Based on hepatic and renal toxicity (histopathological changes and increase in organ weight with blood biochemical changes), the respective NOAELs for DTBP were concluded to be 5 and 20 mg/kg/day. Therefore, the susceptibility of newborn rats to 2TBP and DTBP was found to be 4–5 times higher than that of young rats.

Key Words: 2, 4-di-*tert*-butylphenol, 2-*tert*-butylphenol, susceptibility of newborn rats

INTRODUCTION

Protection of humans against disease and injury caused by chemicals in the environment is the ultimate goal of risk assessment and risk management (Landrigan *et al.* 2004). However, the focus has long been solely on adult exposure and toxicity and the fetus via maternal transfer, with little consideration given to early childhood. In the past decade, stimulated especially by the 1993 US National Research Council (NRC) report *Pesticides in the Diets of Infants and Children* (NAS 1993), recognition that special consideration is required for children in risk assessment has grown. The NRC report noted that 'children are not little adults', because of their unique patterns of exposures to environmental hazards and their particular vulnerability.

For the susceptibility of children to environmental chemicals, the early postnatal period (the suckling period) is of particular note. During this period, the infant could be exposed to various chemicals not only through mothers' milk, but also directly, by having

chemical-contaminated baby food, mouthing toys or household materials, and so on; however, current risk assessment gives no consideration to toxic effects resulting from direct exposure to chemicals. An approach that adequately takes into account the susceptibility of infancy is urgently required. However, because there is no standard testing protocol intended for direct exposure of preweaning animals (newborn animals) to chemicals, and toxicity studies using newborn animals are complicated by practical difficulties regarding grouping, direct dosing, and general and functional observation, there is only limited information on susceptibility of the newborn at the present.

We therefore have established a new protocol for repeated dose toxicity studies using newborn rats (newborn rat studies) (Koizumi *et al.* 2001) for systematic application. Results have been compared with those of 28-day repeated dose toxicity studies using young rats (young rat studies) to provide a basis of analyzing susceptibility. Since young rat studies are routinely conducted as one of a battery of minimum toxicity tests and data are stored for many chemicals, comparative analyzes should provide important information for considering effects of direct exposure to chemicals during the suckling period.

We have already reported analytical results for eight chemicals (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, 1,3-dibromopropane, 1,1,2,2-tetrabromoethane, 2,4,6-trinitrophenol, and tetrabromobisphenol A) (Koizumi *et al.* 2001, 2002, 2003; Fukuda *et al.* 2004; Takahashi *et al.* 2004; Hirata-Koizumi *et al.* 2005). The susceptibility of newborn rats to the toxicity of the first four agents was four times higher than that of their young counterparts at a maximum. For 1,3-dibromopropane and 1,1,2,2-tetrabromoethane, while the doses causing clear toxicity were lower in newborn rats, doses at which toxic signs began to appear were paradoxically higher in the newborn case. These six chemicals had no impact on development in the newborn period and showed similar toxicity profiles in both age groups. For the other two chemicals, there were marked differences in toxicity profile between the newborn and young rats. Especially, in the case of tetrabromobisphenol A, a specific rather than enhanced renal toxicity was observed in newborn case.

In the present investigation, two *tert*-butylphenols, 2-*tert*-butylphenol (2TBP), and 2,4-di-*tert*-butylphenol (DTBP), were chosen for comparative toxicity analysis. 2TBP has been used in the production of agricultural chemicals, aroma chemicals, and resins (New Chemical Index 2001), and DTBP in the production of antioxidants and ultraviolet absorbers (Chemical Products' Handbook 2004). For either chemical, there is no available toxicity information on human. Regarding toxicity to experimental animals, results from young rat studies of both chemicals are available in

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Toxicity Testing Reports of Environmental Chemicals of the Japanese government (MHLW 2001a, 2001b), but no other data have been reported regarding repeated dose toxicity. Since the young rats were only evaluated for toxicity profiles and no-observed-effect levels, we re-evaluated the results for a more practical evaluation index, the no-observed-adverse-effect level (NOAEL), which could serve as the basis for determining tolerable daily intake (TDI) or acceptable daily intake (ADI) for risk assessment, and conducted comparative analyzes with newborn rats.

MATERIALS

2-*tert*-Butylphenol (2TBP, CAS no. 88-18-6, purity: 99.97%) and 2,4-di-*tert*-butylphenol (DTBP, CAS no. 96-76-4, purity: 99.67%), obtained from Dainippon Ink and Chemicals, Incorporated (Tokyo, Japan), were dissolved in olive oil and corn oil, respectively. The test solutions were prepared once a week as stability for eight days had been confirmed. All other reagents used in this study were specific purity grade.

METHODS

All studies were performed under Good Laboratory Practice conditions and in accordance with 'Guidance for Animal Care and Use' of Panapharm Laboratories Co., Ltd, Research Institute for Animal Science in Biochemistry and Toxicology, or Mitsubishi Chemical Safety Institute Ltd.

Animals

In the newborn rat studies of 2TBP and DTBP, pregnant SPF Sprague-Dawley rats [Crj:CD(SD)IGS] were purchased at gestation days 13–15 from Charles River Japan Inc. (Yokohama, Japan), and allowed to deliver spontaneously. All newborn were separated from dams at postnatal day (PND) 3 (the date of birth was defined as PND 0), and pooled according to sex. At the same time, 12 foster mothers were selected among dams, based on the nursing condition. Each foster mother suckled four male and four female newborn, assigned to each of the four dose groups, including the controls, up to weaning on PND 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group (see Study Design) were individually maintained for nine weeks.

In the young rat studies, 4–5 week-old males and females of the same strain were obtained from the same supplier as for the newborn rat studies, and used at ages of 5–6 weeks after acclimation.

All animals were maintained in an environmentally controlled room at 20–26°C with a relative humidity of 40–70%, a ventilation rate of more than ten times per hour, and a 12:12 h light/dark cycle. They were allowed free access to a basal diet (MF: Oriental Yeast Co. Ltd, Tokyo, Japan, or LABO MR Stock: Nihon Nosan Kogyo Inc., Yokohama, Japan) and water (sterile tap water or well water treated with sodium hypochlorite) throughout.

Study design

1. 18-day repeated dose toxicity study in newborn rats (newborn rat study)

Newborn rats (12/sex/dose) were administered the test substances by gastric intubation on PNDs 4–21. On PND 22, six males and six females in each treated group were sacrificed for autopsy (the scheduled-sacrifice group). The remaining animals in all groups (6 rats/sex/dose) were maintained for nine weeks without chemical treatment and then sacrificed at 12 weeks of age (the recovery-maintenance group).

Based on the results of dose-finding studies conducted prior to the main study, the dose, which would show clear toxicity, was selected as the top dose, that without potentially toxic effects as the lowest dose, and the medium dose was set between them. In the dose-finding study for 2TBP (oral administration from PNDs 4–21), some clinical signs and suppressed body weight gain were observed at 200 mg/kg and an increase in relative liver weight at 60 mg/kg and more. For DTBP (oral administration from PNDs 4–17), all of the four males and four females died at 500 mg/kg, and the death of one of the four males, an increase in serum total cholesterol and phospholipid, and increase in relative liver weight were noted in the 100 mg/kg group. Therefore, the doses were set at 0, 20, 60, or 200 mg/kg/day for 2TBP and at 0, 5, 40, or 300 mg/kg/day for DTBP.

During the study, the rats' general condition was observed at least once a day (details of clinical signs noted in this study are described in 'Glossary of terms for toxicity testing' [NIHS 1994]). Body weight and food consumption (only the recovery-maintenance period) was examined once or more a week. As developmental parameters, fur appearance, incisor eruption, pinna detachment and eye opening were assessed for physical development, and testes descent or preputial separation and vaginal opening for sexual development (OECD 2004). In addition, reflex ontogeny, such as visual placing reflex, and surface and mid-air righting reflexes, were also examined (Adams 1986; Jensch & Brent 1988). Urinalysis (color, occult blood, pH, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, specific gravity, and volume of the urine) was conducted in the last week of the recovery-maintenance period.

At PNDs 22 and 85, blood was collected from the abdominal aorta under ether anesthesia (for 2TBP) or from the postcaval vein under pentobarbital sodium anesthesia (for DTBP) after overnight starvation for the scheduled-sacrifice and recovery-maintenance groups, respectively. One portion was treated with EDTA-2K and examined for hematological parameters, such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count. In the recovery-maintenance group, part of the blood was treated with 3.8% sodium citrate, and blood clotting parameters such as prothrombin time (PT) and activated partial thromboplastin time (APTT) were examined. Serum from the remaining portions of blood for both the scheduled-sacrifice and recovery-maintenance groups were analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio [A/G ratio], glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen [BUN], creatinine, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, γ -glutamyl transpeptidase [γ -GTP], calcium, inorganic phosphorus, sodium, potassium, and chlorine). Following collection of blood, all animals were sacrificed by exsanguination, and all organs and tissues were macroscopically examined. Then, the brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The above-listed organs were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopy. For other groups, organs with macroscopically abnormal findings or in which chemical-related effects were evident on microscopic examination for the highest dose group, were similarly investigated.

2. 28-day repeated dose toxicity study in young rats (young rat study)

Five to six week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed following the last treatment (the scheduled-sacrifice group). Recovery groups were maintained for two weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. The number of animals was six for each sex/dose for both scheduled-sacrificed and recovery cases.

The doses were selected in the same way as the newborn rat studies. In the 12-day dose-finding study for 2TBP, ataxic gait was observed at 300 mg/kg and more, and increase in relative liver and kidney weight at 500 mg/kg. For DTBP, with 14-day administration, the death of one of the four females, various changes in some blood biochemical parameters, increase in relative liver weights and light gray macules on kidneys were found at 500 mg/kg. Increase in serum phospholipid and relative liver weights were also demonstrated in the 100 mg/kg group. Based on the results, the doses were determined at 0, 4, 20, 100, or 500 mg/kg/day for 2TBP and at 0, 5, 20, 75, or 300 mg/kg/day for DTBP. Recovery groups were set at 0, 100, 500 mg/kg/day for 2TBP and 0, 300 mg/kg/day for DTBP.

During the study, rats were examined for general condition, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights, and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances).

Statistical analysis

Data for body weights, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological, blood biochemical findings (except for differential leukocyte count), and organ weights were analyzed by the Bartlett's test (Bartlett 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett 1964) was conducted for comparison between control and individual treatment groups ($P < 0.01$ or 0.05). If not homogeneous or for qualitative urinalysis data and differential leukocyte count, the data were analyzed using Steel's multiple comparison tests (Steel 1959), or tests of the Dunnett type (Hollander & Wolfe 1973) ($P < 0.01$ or 0.05). For reflex ontogeny, and physical and sexual development parameters in the newborn rat studies, the χ^2 -test (Fisher 1922) was conducted ($P < 0.01$ or 0.05).

RESULTS

2-tert-butylphenol (2TBP)

Newborn rat study

Various clinical signs such as decrease in locomotor activity, ataxic gait, deep respiration, and muscle weakness were observed throughout the dosing period in the 200 mg/kg group, as shown in Table 1. With 60 mg/kg, transient decrease in locomotor activity was noted on the first dosing day limited to only one of 12 males. Body weights were lowered by 8–17% from dosing day 7 through to the end of the dosing period in males and to recovery-maintenance day 14 in females given 200 mg/kg. At the scheduled sacrifice, there were no hematological changes at any dose, but blood biochemical examination of the 200 mg/kg group showed increases in γ -GTP in both sexes and total protein in males. In addition, significant increase in relative liver weights was noted in 9% of the females in the 60 mg/kg group and in 21–23% of both males and females in the 200 mg/kg group. On histopathological examination, slight hypertrophy of centrilobular hepatocytes was found in one female of the 60 mg/kg group, and in four males and three females from the 200 mg/kg group. During the recovery-maintenance period, no clinical signs were observed and the lowered body weights showed a tendency for recovery. In parameters for physical and sexual development and reflex ontogeny, no definitive changes were detected. At the end of the recovery-maintenance period, no chemical-related changes, also in urinalysis data, were found in any dose group.

The results of the newborn rat study of 2TBP are summarized in Table 2. Since clinical signs and histopathological changes in the liver were observed in the 60 mg/kg group, the NOAEL was concluded to be 20 mg/kg/day.

Young rat study

Ataxic gait were observed sporadically during the dosing period in nine males and 12 females, and decrease in locomotor activity in two females from the 500 mg/kg group. During the dosing period, there were no changes in body weight, food consumption, and urinalysis data. At the scheduled sacrifice, hematological and blood biochemical examination also showed no changes. Eighteen to 19% increases were found in relative liver weights of both sexes receiving 500 mg/kg, but no histopathological changes in liver were observed at any dose. No chemical-related changes were noted during and at the end of the recovery period.

Table 1 Clinical signs observed during the dosing period in the newborn rat study of 2-tert-butylphenol

	Dose (mg/kg/day)			
	0	20	60	200
No. animals (Male/Female)	12/12	12/12	12/12	12/12
No. animals with clinical signs				
Decrease in locomotor activity	0/0	0/0	1†/0	12/12
Ataxic gait	0/0	0/0	0/0	4/6
Deep respiration	0/0	0/0	0/0	12/12
Tremors	0/0	0/0	0/0	2/4
Muscle weakness	0/0	0/0	0/0	12/12
Emaciation	0/0	0/0	0/0	2/2
Pale skin	0/0	0/0	0/0	4/2

†Observed only on the first dosing day.

Table 2 Summary of the results of the newborn and young rat study of 2-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	20	60	200	
Clinical signs	-	M: Decrease in locomotor activity	Various†	
Body weight changes	-	-	8-17%↓	
Blood biochemical changes	-	-	GTP↑, M: TP↑	
Changes in relative organ weights	-	F: Liver 9%↑	Liver 21-23%↑	
Histopathological findings in liver				
- Slight centrilobular hypertrophy of hepatocytes	-	M: 0/6, F: 1/6	M: 4/6, F: 3/6	
Young rat study				
Dose (mg/kg/day)	4	20	100	500
Clinical signs	-	-	-	Ataxic gait F: Decrease in locomotor activity
Body weight changes	-	-	-	-
Blood biochemical changes	-	-	-	-
Changes in relative organ weights	-	-	-	Liver 18-19%↑
Histopathological findings	n.d.	n.d.	n.d.	-

Statistically significant increases ($P < 0.05$) in body weights, blood biochemical parameters and relative organ weights are shown as ↑, while decreases are shown as ↓. Data on histopathological findings are given as no. of animals with the findings/no. of animals examined, according to sex. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. No chemical-related changes were observed in developmental parameters (conducted only in newborn rat study), urinalysis (only in young rat study), and hematological parameters. †Decrease in locomotor activity, ataxic gait, deep respiration, tremors, muscle weakness, emaciation, and pale skin were observed, as shown in Table 1. GTP, γ -GTP; TP, total protein; -, no change; n.d., not determined.

A summary of the results of the young rat study of 2TBP is given in Table 2. The NOAEL was concluded to be 100 mg/kg/day, at which no changes were observed.

2,4-di-*tert*-butylphenol (DTBP)

Newborn rat study

Two males and one female of the 300 mg/kg group were found dead on dosing days 3, 4, and 7. In this group, decrease in locomotor activity (12 males and 12 females), bradypnea (10 males and 10 females), and hypothermia (one male) were observed from the first dosing day, but then the incidence decreased, with disappearance after dosing day 7. Body weights of the 300 mg/kg group were lowered by 15-25% in males and by 9-20% in females during the dosing period, compared with the control values. There were no definitive changes in parameters for physical development and reflex ontogeny in any dose group. At the scheduled sacrifice, blood biochemical examination showed an increase in total bilirubin and a decrease in the A/G ratio in both sexes, an increase in γ -GTP in males, and an increase in total protein and BUN in females of the 300 mg/kg group. In the 300 mg/kg group, there was a 39-51% increase in relative liver weights, a 37-41% increase in relative kidney weights in both sexes, and a 24% decrease in relative spleen weights in males. In the 40 mg/kg group, 14% increases in relative weight of liver were found in females. On histopathological examination, various changes were observed in livers and kidneys in the 300 mg/kg group, as shown in Table 3. Furthermore, periportal fatty degeneration of hepatocytes was evident in one female given 40 mg/kg, and basophilic tubules in kidneys in one animal of each sex receiving 40 mg/kg and one control group male. Regarding

parameters of sexual development, a slight delay in preputial separation was noted in the 300 mg/kg group (the incidences were 0/5, compared with 2/6 in the control group at PND 42 [recovery-maintenance day 21]; 0/5, 3/6 at PND 43; 2/5, 5/6 at PND 44; 2/5, 6/6 at PND 46; 4/5, 6/6 at PND 47; and 5/5, 6/6 at PND 48). During this observation period, body weights were lowered by approximately 10% in males given 300 mg/kg than control levels, which was not statistically significant. In the last week of the recovery-maintenance period, there were no chemical-related changes on urinalysis in any dose group. At the end of the recovery period, changes noted in the scheduled-sacrifice group were not observed except for histopathological changes in the kidneys, significant in the 300 mg/kg group (Table 3).

A summary of the results of the newborn rat study of DTBP is shown in Table 4. Since fatty degeneration of hepatocytes and increase in liver weight were demonstrated at 40 mg/kg, the NOAEL was concluded to be 5 mg/kg/day.

Young rat study

No chemical-related changes were found in general condition, body weight, and food consumption at any dose. On urinalysis at the fourth week of dosing, an increase in urine volume, and a decrease in specific gravity and osmotic pressure were noted in both sexes of the 300 mg/kg group. At the scheduled sacrifice, hematological examination showed a decrease in hemoglobin and hematocrit, an increase in segmented neutrophils in females, and prolongation of PT and APTT in males at 300 mg/kg. On blood biochemical examination, there was an increase in total bilirubin in males given 300 mg/kg, and an increase in total cholesterol and phospholipid in females given 75 mg/kg and above. For organ weights, there were

Table 3 Histopathological findings for the newborn rat study of 2,4-di-*tert*-butylphenol

Dose (mg/kg/day)	Grade	Scheduled-sacrifice group				Recovery-maintenance group†	
		0	5	40	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	5/6	6/6	5/5
Liver							
– Fatty degeneration of periportal hepatocytes	+	0/0	0/0	0/1	0/0	0/0	0/0
	++	0/0	0/0	0/0	3/4	0/0	0/0
	+++	0/0	0/0	0/0	2/2	0/0	0/0
Kidneys							
– Basophilic tubules	+	1/0	n.d.	1/1	4/4	0/0	3/0
– Granular casts	+	0/0	n.d.	0/0	4/2	0/0	0/0
– Cystic dilatation of collecting tubules	+	0/0	n.d.	0/0	0/0	0/0	5/4
	++	0/0	n.d.	0/0	3/4	0/0	0/0
	+++	0/0	n.d.	0/0	2/2	0/0	0/0
– Cellular infiltration of neutrophils	+	0/0	n.d.	0/0	2/1	0/0	1/0
	++	0/0	n.d.	0/0	1/1	0/0	1/0
	+++	0/0	n.d.	0/0	1/1	0/0	0/0

†No histopathological examination was conducted at 5 and 40 mg/kg in the recovery-maintenance group. +, mild; ++, moderate; +++, marked; n.d., not determined.

increases in relative liver weights by 40–43% in both sexes given 300 mg/kg, and by 13% in females receiving 75 mg/kg. On histopathological examination, mild to marked changes in livers and kidneys were observed in both sexes from the 300 mg/kg group, as shown in Table 5. At the end of the recovery period, the increase in total cholesterol and phospholipid and renal histopathological changes observed in the scheduled-sacrifice group remained significant in the highest-dose group (Table 5).

The results of the young rat study are summarized in Table 4. Based on increase in the relative liver weights with some changes in blood biochemical parameters in females given 75 mg/kg, the NOAEL was concluded to be 20 mg/kg/day.

DISCUSSION

During development, many rapid and complex biological changes occur, which can have profound consequences on sensitivity to the effects of exogenous chemicals (Scheuplein *et al.* 2002). Although the neonatal body at birth is reasonably well prepared for the abrupt changes associated with parturition, and most functional systems possess a significant portion of their adult capacity (Dourson *et al.* 2002), it is known that the various functions remain immature in early postnatal period and that some organs and tissues, especially in the nervous, immune and reproductive systems, continue to develop after birth (NAS 1993). Therefore, it is important to evaluate toxic effects by exposure to chemicals during the early postnatal period as well as the fetal period for comprehensive risk assessment. However, economic issues and lack of human resources, arising from practical difficulties regarding protocols, have hindered routine implementation of toxicity studies using newborn animals. Our series of comparative analyzes on susceptibility of the newborn are therefore of particular importance for risk assessment.

In the present study on 2TBP and DTBP, there were no clear differences in toxicity profiles between the newborn and young rats in either case. For 2TBP, clinical signs such as a decrease in locomotor activity and ataxic gait, and effects on liver such as an increase in organ weight were observed. In the DTBP case, hepatic and renal toxicity (histopathological changes, increase in organ weight, etc.) were noted. As a characteristic effect of DTBP on male sexual development, slight delay in preputial separation was also observed in the newborn rat study. Preputial separation, an androgen-dependent process which is an early marker of puberty, represents a reliable non-invasive indicator of chemical-induced perturbation of male pubertal development in the rat (Gaytan *et al.* 1988). However, it is known that decreased body weights can result in non-specific delay in puberty (Ashby & Lefevre 2000). Since DTBP lowered body weights in the period of observation of preputial separation and there were no DTBP-related changes in weights or histopathology of the testes and epididymides, well known to be essentially androgen-dependent, no specific effect on male sexual development could be concluded in the present study. As for NOAELs of both chemicals, clear differences were observed between newborn and young rats, with values of 20 and 5 mg/kg/day in newborn rats, and 100 and 20 mg/kg/day in young rats for 2TBP and DTBP, respectively. Therefore, the susceptibility was four- to five-fold higher in newborn than in young rats.

Our previous analysis of 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005) showed dose-response curves to be very different between newborn and young rats. The same was recently reported for the widely used organophosphorus insecticide, chlorpyrifos (Zheng *et al.* 2000), as well as pyrethroid insecticides (Shafer *et al.* 2005). These data showed the importance of estimating unequivocally toxic levels (UETLs), defined for our comparative toxicity analysis as equivalent toxic doses inducing clear toxicity, including death, clinical toxic signs,

Table 4 Summary of the results of the newborn and young rat study of 2,4-di-*tert*-butylphenol

Newborn rat study				
Dose (mg/kg/day)	5	40	300	
Death	-	-	M: 2/12, F: 1/12	
Clinical signs	-	-	Decrease in locomotor activity bradypnea, hypothermia	
Body weight changes	-	-	9-25%↓	
Urinalysis	n.d.	n.d.	n.d.	
Hematological changes	-	-	-	
Blood biochemical changes	-	-	Various†	
Changes in relative organ weights	-	F: Liver 14%↑	Liver 39-51%↑, Kidney 37-41%↑ M: Spleen 24%↓	
Histopathological findings	-	F: Fatty degeneration in liver	Various changes in liver and kidney‡	
Developmental parameters	-	-	Slight delay in preputial separation	
Young rat study				
Dose (mg/kg/day)	5	20	75	300
Death	-	-	-	-
Clinical signs	-	-	-	-
Body weight changes	-	-	-	-
Urinalysis	-	-	-	UV↑ SG↓ OP↓
Hematological changes	-	-	-	Various§
Blood biochemical changes	-	-	F: Tcho↑ Pho↑	M: TB↑ F: Tcho↑ Pho↑
Changes in relative organ weights	-	-	F: Liver 13%↑	Liver 40-43%↑
Histopathological findings	n.d.	n.d.	-	Various changes in liver and kidney¶

Data on death are shown as no. of dead animals/no. of animals examined, according to sex. Statistically significant increases ($P < 0.05$) in body weights, urinalysis and blood biochemical parameters, and relative organ weights are shown as ↑, while decreases are shown as ↓. Changes observed only in males or females are shown as 'M' or 'F', respectively, while neither 'M' nor 'F' is mentioned in the case of changes noted in both sexes. †Increase in total bilirubin and decrease in the A/G ratio in both sexes, increase in γ -GTP in males, and increase in total protein and BUN in females were noted. ‡Various changes were observed as shown in Table 3. §Various hematological changes were noted such as decrease in hemoglobin and hematocrit and increase in segmented neutrophils in females and prolongation of PT and APTT in males. ¶Various changes were observed as shown in Table 5. OP: osmotic pressure; Pho: phospholipid; SG: specific gravity; TB: total bilirubin; Tcho: total cholesterol; UV: urine volume; -: no change; n.d.: not determined.

or critical histopathological damage (Koizumi *et al.* 2001). We here tried to apply this UETL approach to the present study. For 2TBP, clinical signs such as decrease in locomotor activity and ataxic gait were noted in most of the animals given 200 mg/kg (newborn rats) and 500 mg/kg (young rats) (Table 2). Furthermore, a 8-17% lowering of body weight was observed at 200 mg/kg in newborn rats, but not in the young rat study. Therefore, equivalent toxic effects to these observed at 500 mg/kg in young rats might be expected to appear at 100-150 mg/kg in newborn animals. The UETLs were concluded to be 100-150 and 500 mg/kg/day in newborn and young rats, respectively. In the case of DTBP, clear toxicity was observed at the top dose of 300 mg/kg in both newborn and young rat studies (Table 4), but the level of severity was very different, for example, deaths were only noted in the newborn cases. It was considered difficult to estimate the UETLs from the results of main studies only. However, the most critical endpoint for toxicity, mortality, was also noted at 100 mg/kg and more, and 500 mg/kg, in the dose-finding studies of newborn and young rats, respectively. Therefore, it would be possible to estimate the appropriate UETLs as the minimum lethal dose by taking the results of the dose-finding

studies into consideration. The UETLs were concluded to be 100 mg/kg/day for the newborn, and 500 mg/kg/day for young rats, at which one out of eight rats was found dead in both cases. These analyzes of UETLs, considering equivalence in toxic degree, showed 3.3-5.0 times higher susceptibility of newborn rats to 2TBP and DTBP than young rats, consistent with our analytical results for NOAELs.

Higher susceptibility of newborn rats was also demonstrated in our previous analyzes of five phenols (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol and 2,4,6-trinitrophenol) (Koizumi *et al.* 2001, 2002, 2003; Takahashi *et al.* 2004), considered mainly due to their poor metabolic and excretory capacity (Horster 1977; Cresteil *et al.* 1986). It has actually been reported that UDP-glucuronyltransferase and sulfotransferase activities, when 4-nitrophenol is used as the substrate, are lower in microsomes prepared from livers of newborn rats, and that the elimination rate of 2,4-dinitrophenol from serum of newborn rabbits is markedly slower than in young adults (Gehring & Buerge 1969; Matsui & Watanabe 1982). Unfortunately, there is no information on the toxicity mechanism and toxicokinetics of both 2TBP

Table 5 Histopathological findings for the young rat study of 2,4-di-*tert*-butylphenol

Dose (mg/kg/day)	Grade	Scheduled-sacrifice group†			Recovery group	
		0	75	300	0	300
No. of animals examined (Male/Female)		6/6	6/6	6/6	6/6	6/6
Liver						
- Centrilobular hypertrophy of hepatocytes	+	0/0	0/0	4/4	0/0	0/0
Kidneys						
- Basophilic tubules	+	0/0	0/0	1/4	0/0	3/1
	++	0/0	0/0	4/0	0/0	2/0
	+++	0/0	0/0	1/1	0/0	1/0
- Granular casts	+	0/0	0/0	5/2	0/0	4/0
	++	0/0	0/0	1/1	0/0	0/0
- Proteinaceous casts	+	0/0	0/0	5/1	0/0	2/0
	++	0/0	0/0	1/0	0/0	0/0

†No histopathological examination was conducted for the 5 and 20 mg/kg scheduled-sacrifice groups. +, mild; ++, moderate; +++, marked.

and DTBP; however, the immature functions involved in the toxicokinetics in newborn rats would be implicated in the higher susceptibility, as in the case of five phenols previously analyzed. While there are very little data on toxicokinetics of environmental chemicals in the newborn, relatively plentiful information has been reported in humans for pharmaceuticals which are clinically applied during the early postnatal period. Recently, Ginsberg *et al.* (2002) conducted comparative analysis of pharmacokinetic parameters for 45 drugs in both children and adults, and showed half-lives in children aged two months or under to generally be two-fold longer than in adults.

As for the susceptibility of the newborn to toxicity of chemicals, although it is generally important to take the sensitivity of target organs and tissues themselves (toxicodynamics) into consideration besides toxicokinetics, there are insufficient data on differences between newborn and young/adult animals. For appearance of toxicity, which is the outcome of toxicokinetics and toxicodynamics, some comparative studies have relied on LD₅₀ values (Goldenthal 1971; Sheehan & Gaylor 1990). However, it is not considered that information on acute toxicity at lethal dosage is appropriate when considering the susceptibility of newborn in risk assessment, because dose-response curves could differ, as mentioned above. With prolonged, subtoxic doses, which are basis for TDI or ADI, our series of comparative studies constitute the first systematic assessment, providing an important base for development of new methods of risk assessment of susceptibility of the newborn.

In conclusion, clinical signs and effects on the liver were observed for 2TBP, and hepatic and renal toxicity for DTBP. Although there were no clear differences in toxicity profiles between the newborn and young rats for both chemicals, the toxicity levels differed markedly. The susceptibility of the newborn to these chemicals appears to be 4-5 times higher than that of young animals.

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