1日摂取量として10 μg/kg/dayを設定した。(以前は、1986年に米国EPAとは設定根拠となる試験は異なるものの、耐容1日摂取量は米国EPAと同じ50μg/kg/dayと設定していた。)その再評価に際しては、生殖発生毒性や内分泌かく乱作用は重要な因子であるとしているが、最も低用量で認められている精子数の減少や前立腺重量増加などの雄性生殖器系への影響については、他の機関で再現されていないことや多世代試験でも影響が認められていないことを根拠に耐容1日摂取量の算定根拠としなかった。また、子宮重量増加などの雌性生殖器への影響も卵巣摘出などの内分泌系のホメオスタシスを崩した条件での反応であり、正常状態での毒性学的意義も定かでないうえに、測定の定量性に乏しいと考えられている。そこで、最近の3世代試験(Tylら2002)の結果より得られる無毒性量として5 mg/kg/dayを耐容1日摂取量算定の出発点とし、不確実係数としては種差と個体差に基づく不確実係数:100と、上記低用量影響に関するデータの不確かさに基づく不確実係数:5を適用し、総合不確実係数:500(＝100×5)を適用することにより耐容1日摂取量として10 μg/kg/dayを算定している。

結論的には、一連のBPAによる低用量影響について、その生物学的な意味合いにおいて今後も注目されるべき問題であるという認識には至っているが、毒性学的、特にヒトへの健康影響を評価するうえに於いては、現時点で得られている知見は十分ではないと言える。つまり、動物実験で影響の認められた低用量暴露レベルを、耐容1日摂取量設定等の定量的なリスクアセスメントに使用するほどの科学的妥当性が示されていない状況であることを示している。しかし、低用量域における健康影響を否定するほどの材料もなく、ECの食品科学会議のように耐容1日摂取量を暫定値とせざるを得ない状況でもある。この時の低用量影響に関するデータの不確かさに基づく不確実係数:5が妥当であるかどうかについては議論の残るところであるが、内分泌かく乱問題が注目を浴びてから最初の指針値の設定であり、今後各種基準値の設定に少なからず影響を与えることになるであろう。この暫定耐容1日摂取量に従えば、現状のヒト暴露レベルはとりあえず安全であるといえるが、上述したように低用量影響が現れる動物実験暴露レベルとヒト暴露レベルとの差が小さいことを考慮すると、できるだけ早期に低用量発現機序の解明やヒトでの発現性に関する疑問が解決されることが望まれる。

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

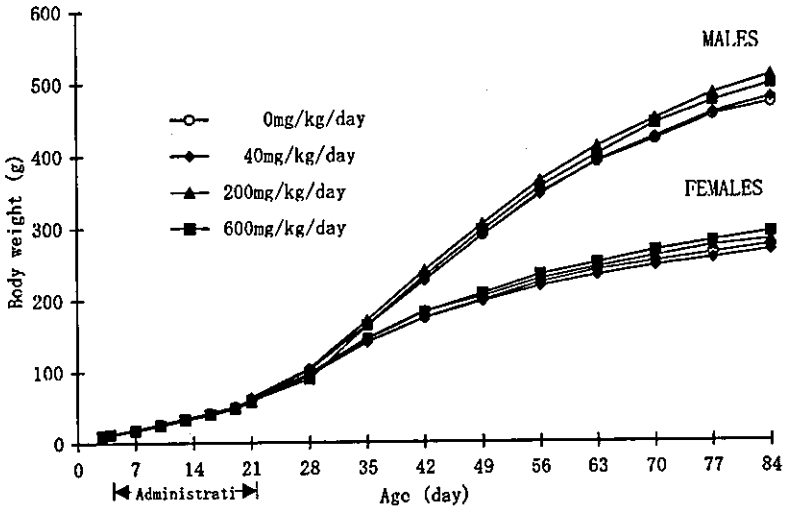


Fig. 1. Body weight changes of rats treated orally with TBBPA for 18 days from 4 days of age until weaning.

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,

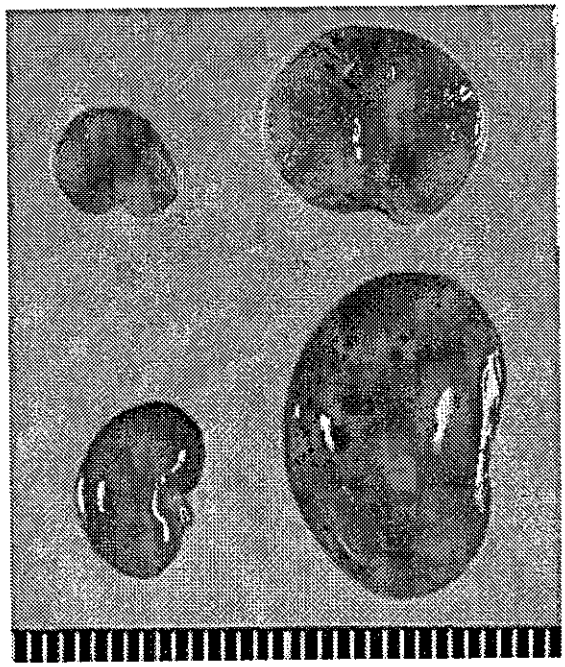

Fig. 2. Gross appearance of kidney (lower right) and its cross-section (upper right) in a 22-day-old rat treated with TBBPA (600 mg/kg body weight daily, orally) for 18 days. The kidney is markedly larger than that of a non-treated rat (left).

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.

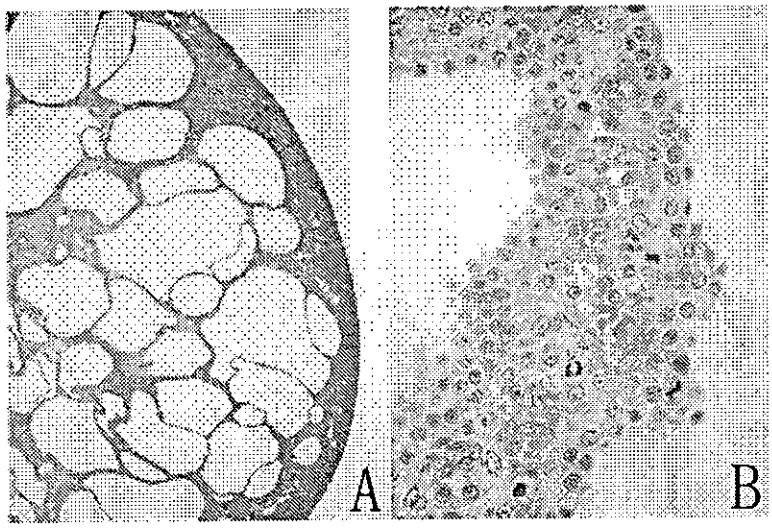


Fig. 3. Polyeystic renal lesion observed in a 22-day-old rat treated with TBBPA (600 mg/kg body weight daily, orally) for 18 days. H-E stain. (A) Dilatation of the tubules from the cortico-mudullary junction to the inner cortex, 40×; (B) hyperplasia of the tubular epithelium, 125×.

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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ORIGINAL ARTICLE

# Comparative toxicity study of 2,4,6-trinitrophenol (picric acid) in newborn and young rats

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**ABSTRACT** **The toxicity of oral 2,4,6-trinitrophenol (TNP) was determined in newborn rats, and compared with that in young rats. In newborn rats, males and females were given TNP at 0, 16.3, 81.4 or 407 mg/kg per day on postnatal days (PND) 4–17 for the dose-finding study, and at 0, 4.1, 16.3 or 65.1 mg/kg per day on PND 4–21 for the main study. Deaths, lower body weight (BW) and behavioral changes were found at 81.4 and 407 mg/kg per day in the dose-finding study, and lower BW was observed in males at 65.1 mg/kg per day during the dosing period of the main study. In young rats, 5-week-old males and females were given TNP at 0, 20, 100 or 500 mg/kg per day for 14 days as the dose-finding study and at 0, 4, 20 or 100 mg/kg per day for 28 days as the main study. Deaths were observed at 500 mg/kg per day in the dose-finding study. Deaths or changes in BW were not found at 100 mg/kg per day or less. At 100 mg/kg per day, hemolytic anemia and testicular toxicity were found. In conclusion, toxicity profiles induced by TNP were markedly different between newborn and young rats.**

**Key Words:** 2, 4, 6-trinitrophenol, newborn rats, picric acid, repeated-dose toxicity, young rats

## INTRODUCTION

The adverse effects of environmental chemicals including endocrine disruptors on not only contemporary but also future generations are causing increasing concern. The possible toxic effect of chemicals on fetuses and newborns has aroused great concern among the public, and the protection of fetuses and newborns has become a major scientific and political issue.

Comprehensive statements for children's health, considering their special vulnerability to certain toxic substances, are shown in the US Environmental Protection Agency Children's Environmental Health Yearbook (US EPA 1998). Infants and young children have greater respiratory and circulatory flow rates, as well as energy and fluid requirements than adults, giving rise to a greater potential for respiratory and intestinal exposure to chemicals per unit body weight (BW) (WHO 1986). Children live close to the ground because of their behavioral patterns of play and their height and perform hand-to-mouth activities, which would expose them to much larger amounts of pollutants in dust and soil (US EPA 1998). However, children could be less sensitive than adults to some chemicals (NRC 1993) because infants have more extracellular water that is the only avenue connecting cells with the outside world (Fomon *et al.* 1982), enough amounts of toxic metabolites are not produced in infants due to their immature metabolic capacities (Kearns & Reed 1989), or the developing brain has increased plasticity.

Because of these unique characteristics, children react differently from adults. Differences in susceptibility to toxicants between children and adults may result from a combination of toxicokinetic, toxicodynamic and exposure factors (Schwenk *et al.* 2002). The potential toxic effects of chemicals on children cannot be anticipated using data on adults, and a data set on exposed children is essential for the assessment of children's health. Although gathering information on the toxicity of chemicals in newborns is very important to evaluate children's health, toxicity data on chemical compounds in newborns are limited.

We have already reported the differences in the susceptibility to toxicities of chemicals between newborn and young rats (Koizumi *et al.* 2001, Koizumi 2002, Koizumi 2003;

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Fukuda *et al.* 2004). We demonstrated that the toxic response in newborn rats was at most four times (4-nitrophenol and 2,4-dinitrophenol), approximately three times (3-aminophenol), and three to four times (3-methylphenol) higher than that in young rats. The toxicological profiles of 4-nitrophenol (Koizumi *et al.* 2001), 2,4-dinitrophenol (Koizumi *et al.* 2001), 3-aminophenol(Koizumi *et al.* 2002), and 3-methylphenol (Koizumi *et al.* 2003) were similar in newborn rats and young rats. The nephrotoxicity of tetrabromobisphenol A was specific for newborn rats (Fukuda *et al.* 2004).

2,4,6-Trinitrophenol (TNP) was listed in the Organisation for Economic Co-operation and Development (OECD) High Production Volume Chemical Table in 1999, meaning that it is produced at levels greater than 1000 tonnes per year in at least one OECD member country. TNP is known as picric acid, has a yellow color and is explosive. This compound is used in the production of gunpowder, fireworks, agricultural chemicals and dyes, and is widely used in industry, by the military, and as a research/clinical chemistry reagent. Much of the human toxicity data showed that exposure to picric acid was primarily through inhalation of dust or through skin contact (Wyman *et al.* 1992). This chemical caused irritation of eyes, a transient yellowish appearance, and skin sensitization in humans (Health Council of the Netherlands 2002). Wyman *et al.* (1992) investigated the acute toxicity, distribution, and metabolism of TNP using Fischer 344 rats. The values of oral LD50 in male and female rats were 290 and 200 mg/kg, respectively. TNP was found to bring about severe acidosis during acute intoxication. Recently, a 28-day repeat dose oral toxicity study of this compound in young rats was conducted as part of the Japanese Existing Chemical Safety Program (MHLW 2001), in which the no observed effect level (NOEL) and toxicity profile of chemicals were evaluated.

In the present paper, we re-evaluated the toxicity of TNP in young rats (MHLW 2001), determined the toxicity of TNP in newborn rats, and compared the findings.

## MATERIALS AND METHODS

### Chemicals

TNP (2,4,6-trinitrophenol, CAS. no. 88-89-1, purity: 81.4%) was obtained from Mitsui Chemicals (Tokyo, Japan) and suspended in a 0.5% CMC-Na (carboxymethyl cellulose sodium salt; Nacalai Tesque, Kyoto, Japan or Iwai Chemicals, Tokyo, Japan) aqueous solution mixed with 0.1% Tween-80 (polyoxyethylene sorbitan monooleate; Nacalai Tesque, Kyoto, Japan or Difco Laboratories, Detroit, USA).

### Animals

In the newborn rat study, pregnant SPF Crj:CD(SD)IGS rats (gestation day 13) were purchased from Atsugi Breeding Center, Charles River Japan (Yokohama, Japan) and allowed to deliver spontaneously. The animals were maintained in an environmentally controlled room at 24 ± 2°C with a relative humidity of 55 ± 10% and a 12:12 h light/dark cycle. Newborn rats were separated from dams on postnatal day (PND) 3.

In the young rat study, 4-week-old males and females of the same strain were purchased from the same farm. The animals were maintained in an environmentally controlled room at 22 ± 2°C with a relative humidity of 55 ± 15% and a 12:12 h light/dark cycle.

All animals in the newborn and young rat studies were allowed free access to a sterilized basal diet (MF, Oriental Yeast, Tokyo, Japan) and water. Rats were euthanized by exsanguination under anesthesia using sodium pentobarbital in the newborn rat study and sodium thiopental in the young rat study.

### Repeated dose study in newborn rats

Time schedule of the newborn rat studies is shown in Figure 1.

#### *Dose-finding study*

Sixteen males and 16 females were randomly selected and assigned to four dose groups, including a control group. Four foster mothers were used. One foster mother suckled the four males and four females. Pups (4/sex per dose) were given TNP by gavage at 0, 16.3, 81.4 or 407 mg (as TNP)/kg per day on PND 4–17 (14 days) and killed on PND 18 after overnight starvation. General condition, BW, hematology, blood biochemistry, necropsy, and organ weights were examined.

#### *Main study*

Forty-eight males and 48 females for two autopsy groups (the ends of the dosing period and recovery-maintenance period) were randomly selected and assigned to four dose groups, including a control group. Twelve foster mothers were used. One foster mother suckled the four males and four females up to weaning on PND 22. After weaning, rats of the recovery-maintenance group were individually maintained for 9 weeks. Pups (6/sex per dose) were given TNP by gavage at 0, 4.1, 16.3 or 65.1 mg (as TNP)/kg per day on PND 4–21 (18 days), and killed after overnight starvation following the last treatment. The dosage levels were determined based on the results of the dose-finding study in newborn rats. Recovery-maintenance groups (6/sex per dose) given the same dosages were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks, almost the same age as at the end of the recovery period of the main study of young rats.

General condition was observed two times per day (before and after administration) for pups (separated from each foster mother) and foster mothers during the dosing period, and

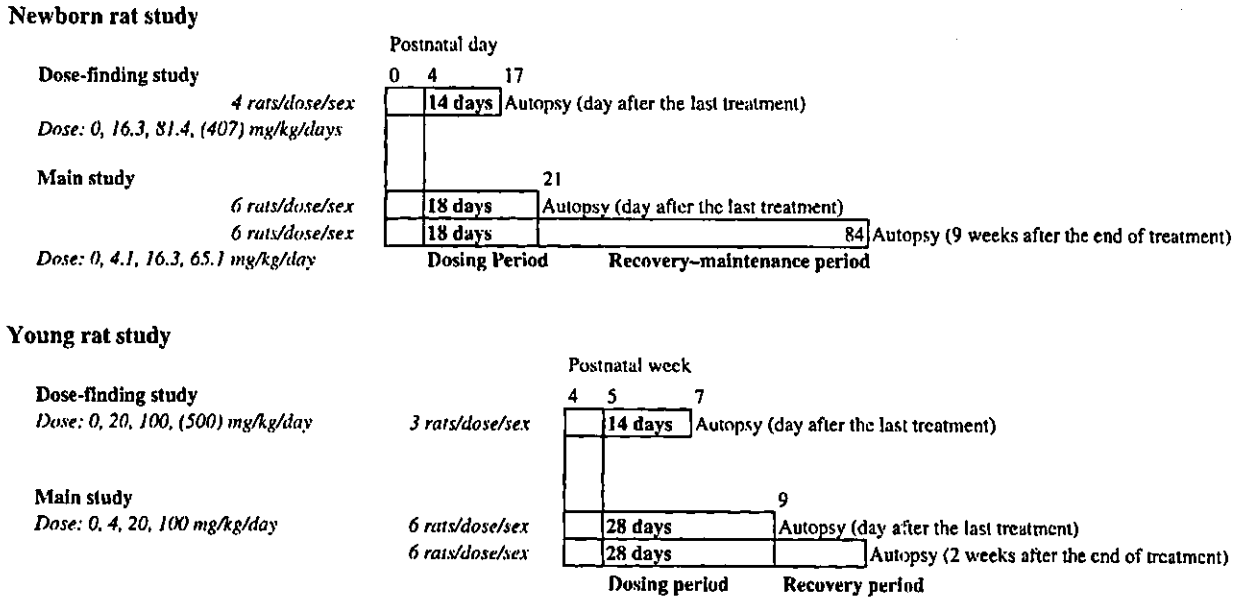


**Fig. 1** Time schedule of the newborn and young rat studies.

daily for pups during the recovery-maintenance period. BW and food consumption were measured more than two times per week. All pups were examined for developmental landmarks; pinna detachment on PND 4, piliation on PND 8, incisor eruption on PND 10, gait and eye opening on PND 15, testes descent on PND 21, preputial separation on PND 42, and/or vaginal opening on PND 42. BW was measured on the day of testes descent, preputial separation and/or vaginal opening. All pups were examined for the assessment of reflex ontogeny; surface righting reflex and ipsilateral flexor reflex on PND 5, visual placing response on PND 16, and Preyer's reflex on PND 28.

In urinalysis, color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, urine sediment, specific gravity, osmotic pressure and volume of urine were examined only at the end of the recovery-maintenance period. Rats were killed on PND 22 or PND 85. On the day that the rats were killed, blood was collected from the abdominal vein. Hematological parameters, such as the red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), platelet counts, reticulocyte ratio (Ret), differential leukocyte count, and blood clotting parameters, such as prothrombin time and activated thromboplastin time, were determined. The blood biochemical parameters, such as the total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase, phospholipids, calcium, inorganic phosphorus, sodium, potassium and chloride levels in serum, were also determined. After a gross examination, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenals, thyroids, lungs, testes, epididymides and/or ovaries were weighed. The organs were fixed with 10% buffered formation-phosphate (2.5% glutaraldehyde's prefixation for the eyes, Bouin's prefixation for the testes and epididymis) and paraffin sections were routinely prepared and stained with hematoxylin–eosin for microscopic examination. The study using newborn rats was conducted at Panapharm Laboratories Co., Ltd. (Uto, Japan) under Good Laboratory Practice (GLP) conditions (OECD 1981; MHW 1988).

### Repeated dose study in young rats

Time schedule of the young rat studies is shown in Figure 1.

#### *Dose-finding study*

Five-week-old rats (3/sex per dose) were given TNP by gavage at 0, 20, 100 or 500 mg (as TNP)/kg per day for 14 days and killed the day following the last administration after overnight starvation. General condition, BW and food consumption, hematology, necropsy, and organ weights were examined.

#### *Main study*

Five-week-old rats (6/sex per dose) were given TNP by gavage at 0, 4, 20 or 100 mg (as TNP)/kg per day for 28 days and killed after overnight starvation following the last

treatment. The dosage levels were determined based on the results of the dose-finding study in young rats. Recovery groups (0 or 100 mg/kg per day) (6/sex per dose) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. Rats were examined for general condition, BW, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings. The study using young rats was conducted at Kashima Laboratory, Mitsubishi Chemical Safety Institute Ltd. (Kashima, Japan) under GLP conditions (MHW 1988; OECD 1997).

***Statistical analysis***

Continuous data were analyzed with Bartlett's test for homogeneity of variance. If the data were homogeneous, Dunnett's test was conducted for group comparisons between control and individual TNP-treated groups. If not homogenous, the data were analyzed using Steel's test. Quantitative data for histopathology were analyzed with Mann–Whitney's *U*-test or Fisher's exact test. In the newborn rat study, the chi-square test was conducted for physical and sexual development and reflex ontogeny. The 0.05 or 0.01 level of probability was used as the criterion for significance.

## RESULTS

**Repeated dose study in newborn rats (dose-finding study)**

Death occurred at 81.4 mg/kg per day in one male on day 3 of the dosing period, two females on days 6 and 7 of the dosing period, and at 407 mg/kg per day in all rats by day 4 of the dosing period. In these dead rats, hypoactivity, bradypnea and hypothermia were observed. Only hypoactivity was found in surviving rats at 81.4 mg/kg per day on days 3, 5, or 8 of the dosing period. Yellowish fur was observed in all TNP-treated rats.

A significantly lower BW (max. 16% decreased) in males, and suppression of weight gain (max. 35% decreased) in females were noted at 81.4 mg/kg per day. The organ weights are summarized in Table 1. At 81.4 mg/kg per day, a significantly higher relative weight of the liver (13% increased) and lower relative weight of the kidney (14% decreased) were observed in males.

No consistent changes related to the administration of TNP in hematological or blood biochemical parameters or necropsy findings were found at any doses.

**Repeated dose study in newborn rats (main study)**

There were no deaths throughout the experimental period in males and females, even at 65.1 mg/kg per day. Yellowish fur was observed in all TNP-treated rats. A significantly lower BW (max. 7% decreased) was found in males on days 4 and 8 of the dosing period at 65.1 mg/kg per day. During the recovery-maintenance period, no dose-dependent effects on BW and food consumption were observed.

No toxicological effects of TNP on physical development, reflex ontogeny, and sexual maturation were detected at any doses in the newborn rat study.

The organ weights are summarized in Table 1. Significantly higher relative weights of the liver in males and females (13 and 12% increased, respectively) were observed at 65.1 mg/kg per day.

No consistent changes related to the administration of TNP were found in hematological or biochemical parameters, urinalysis or histopathological findings.

**Repeated dose study in young rats (dose-finding study)**

All male rats and one female rat at 500 mg/kg per day died by day 2 of the dosing period. No death was found at 20 and 100 mg/kg per day. Yellowish fur was observed in all TNP-treated rats. BW of males and females at 20 and 100 mg/kg per day were not significantly different from controls during the dosing period.

The results of hematological examinations are summarized in Table 2. Significantly lower values of Hb and Ht, and a higher value of Ret were detected in females at 100 mg/kg per day.

The organ weights are summarized in Table 3. At 100 mg/kg per day, a significantly higher value of relative spleen weight (14% increased) in males, and a significantly higher value of relative liver weight (18% increased) in females were observed.

**Repeated dose study in young rats (main study)**

There were no deaths throughout the experimental period even at 100 mg/kg per day. Yellowish fur was observed in all TNP-treated rats. A yellowish color change of urine was also found in all TNP-treated groups during the dosing period and this coloration disappeared during the recovery period. BW of males and females in the TNP-treated groups were not significantly different from controls during the dosing and recovery periods. No consistent changes in food consumption were found in the TNP-treated groups.

The results of hematological examinations are summarized in Table 2. Significantly higher values of WBC and Ret and lower values of RBC and Hb were observed in males at 100 mg/kg per day. At this dose, significantly higher values of WBC, MCV and Ret, and lower values of RBC, Hb and MCHC were also found in females.

The organ weights are summarized in Table 3. Significantly higher values of relative liver weight (12% increased) and relative spleen weight (45% increased) and significantly lower value of relative epididymides weight (21% decreased) were observed in males at 100 mg/kg per day at the end of the dosing period. A Significantly lower value of relative epididymides weight at 100 mg/kg per day was also

**Table 1** Organ weights in the newborn rat study of 2,4,6-trinitrophenol

| | Dose-finding study† | | | Main study‡ | | | |
|---|---|---|---|---|---|---|---|
| Dose (mg/kg per day) | 0 | 16.3 | 81.4 | 0 | 4.1 | 16.3 | 65.1 |
| Males | | | | | | | |
| No. animals | 4 | 4 | 3 | 6 | 6 | 6 | 6 |
| Body weight§ (g) | 48.9 ± 3.7 | 47.7 ± 2.6 | 42.3 ± 2.0* | 63.4 ± 4.9 | 63.0 ± 2.8 | 63.7 ± 5.7 | 61.8 ± 4.8 |
| Liver (g) | 1.73 ± 0.14 | 1.67 ± 0.13 | 1.70 ± 0.13 | 2.69 ± 0.22 | 2.74 ± 0.14 | 2.79 ± 0.24 | 2.97 ± 0.38 |
| (g/100 g BW) | (3.55 ± 0.10) | (3.49 ± 0.12) | (4.01 ± 0.13)** | (4.25 ± 0.16) | (4.35 ± 0.12) | (4.38 ± 0.08) | (4.79 ± 0.28)** |
| Spleen (g) | 0.21 ± 0.04 | 0.21 ± 0.02 | 0.17 ± 0.01 | 0.34 ± 0.07 | 0.35 ± 0.06 | 0.38 ± 0.04 | 0.37 ± 0.06 |
| (g/100 g BW) | (0.44 ± 0.07) | (0.45 ± 0.05) | (0.40 ± 0.03) | (0.54 ± 0.07) | (0.56 ± 0.08) | (0.60 ± 0.05) | (0.60 ± 0.05) |
| Kidneys (g) | 0.58 ± 0.03 | 0.56 ± 0.04 | 0.43 ± 0.05** | 0.74 ± 0.12 | 0.73 ± 0.08 | 0.77 ± 0.03 | 0.73 ± 0.12 |
| (g/100 g BW) | (1.18 ± 0.04) | (1.17 ± 0.05) | (1.02 ± 0.08)** | (1.16 ± 0.12) | (1.16 ± 0.09) | (1.21 ± 0.10) | (1.18 ± 0.12) |
| Epididymides (mg) | – | – | – | 57.6 ± 4.6 | 55.4 ± 6.0 | 57.6 ± 7.3 | 50.3 ± 3.7 |
| (mg/100 g BW) | – | – | – | (91.1 ± 6.9) | (87.9 ± 7.2) | (91.3 ± 16.4) | (81.9 ± 7.9) |
| Testes (mg) | – | – | – | 326 ± 47 | 302 ± 27 | 319 ± 22 | 295 ± 20 |
| (mg/100 g BW) | – | – | – | (513 ± 54) | (479 ± 26) | (504 ± 44) | (478 ± 27) |
| Females | | | | | | | |
| No. animals | 4 | 4 | 2 | 6 | 6 | 6 | 6 |
| Body weight§ (g) | 45.2 ± 2.2 | 47.5 ± 3.1 | 38.6 | 59.0 ± 3.3 | 59.6 ± 2.3 | 57.0 ± 4.6 | 58.8 ± 5.3 |
| Liver (g) | 1.57 ± 0.08 | 1.72 ± 0.09 | 1.64 | 2.46 ± 0.22 | 2.44 ± 0.24 | 2.33 ± 0.25 | 2.75 ± 0.28 |
| (g/100 g BW) | (3.48 ± 0.25) | (3.62 ± 0.10) | (4.23) | (4.18 ± 0.35) | (4.09 ± 0.29) | (4.09 ± 0.19) | (4.67 ± 0.19)* |
| Spleen (g) | 0.20 ± 0.03 | 0.20 ± 0.04 | 0.17 | 0.32 ± 0.04 | 0.33 ± 0.04 | 0.29 ± 0.05 | 0.37 ± 0.05 |
| (g/100 g BW) | (0.43 ± 0.04) | (0.43 ± 0.06) | (0.44) | (0.54 ± 0.05) | (0.55 ± 0.07) | (0.51 ± 0.08) | (0.62 ± 0.03) |
| Kidneys (g) | 0.55 ± 0.02 | 0.57 ± 0.05 | 0.43 | 0.69 ± 0.05 | 0.69 ± 0.06 | 0.66 ± 0.06 | 0.70 ± 0.05 |
| (g/100 g BW) | (1.22 ± 0.06) | (1.20 ± 0.06) | (1.12) | (1.17 ± 0.09) | (1.16 ± 0.08) | (1.16 ± 0.10) | (1.20 ± 0.06) |

†Rats were killed on postnatal day (PND) 18; ‡rats were killed on PND 22; §body weight (BW) after overnight starvation follow the last dosing. Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. –, no data.