ing the newborn period is not included in these studies, despite the significant possibility that the newborn are exposed to chemicals directly via mouthing toys and household materials, or having chemical-contaminated milk and baby food, and so on. In the routine repeated dose toxicity study, rats at approximately 5-6 weeks of age have generally been used, and this start period is largely a matter of practical convenience and feasibility. Rats much younger than this age, especially newborn rats, are so difficult to handle such as grouping, direct dosing and other testing or observation. Economic issues and lack of the human resource with this technical difficulty make it impossible to subject the newborn rat study to the routine one. Our series of comparative studies are the first systematic study to look into the direct effects of chemicals in newborn animals, and the comparative analysis on the susceptibility of the newborn rats to the toxicity of chemicals with that of young rats would give important information for considering the effects by chemical exposure during the newborn period in risk assessment.

In conclusion, the target organ of DBP and TBE was here found to be the liver in both newborn and young rats, but the doses at which the toxic signs began to appear were higher in newborn rats. In contrast, the doses at which clear toxicity was observed appeared to be lower in the newborn case. However, no special concern with regard to newborn risk is necessary in cases of chemicals which induce toxicity after biotransformation via hepatic cytochrome P450, because the tolerable daily intake (TDI) used for regulation is generally derived from NOAEL in toxicity studies in young/adult animals.

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REVISION AND ESTABLISHMENT OF JAPANESE DRINKING WATER QUALITY GUIDELINES FOR DI(2-ETHYLHEXYL) PHTHALATE, TOLUENE AND VINYL CHLORIDE – DIFFERENCES FROM THE LATEST WHO GUIDELINE DRAFTS –

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ABSTRACT — The revision of the Japanese drinking water quality guidelines was established in May 2003. The WHO drinking water quality guidelines for the 3rd edition were also revised and the draft has been open to the public since last year. Most guideline values of each chemical in both Japan and WHO were quite similar; however, there are different overt values for three chemicals. In this short communication, we describe them and discuss the reason for taking the different toxicity endpoints and derivation method for these three chemicals, di(2-ethylhexyl) phthalate, toluene and vinyl chloride.

KEY WORDS: Drinking water quality guidelines, Di(2-ethylhexyl) phthalate, Toluene, Vinyl chloride

INTRODUCTION

The revision of the Japanese drinking water quality guideline was established in May 2003 and implemented on May 2004. In this revising, regulated chemical lists were modified because of the past detection trend or exposure prospect. The chemicals already listed in the previous version were reevaluated and chemicals newly listed in this revision were assessed with the latest toxicity information. The Japanese guidelines derivation has referred to the concurrent WHO revision, and both of the general principles for the guidelines (GD) derivation are almost the same. Although most guideline values of chemicals in Japan were similar to those of WHO, some minor differences between WHO and Japan exist because of different default body weight application for the guideline calculation (50 kg/Japan vs. 60 kg/WHO). Furthermore, in some cases, different drinking water contribution ratios (allocation) to total exposure media were used for the guideline values calculation from tolerable daily intake (TDI) on account of the regional chemical exposure assessment. These differences were not owing to the difference of health risk assessment per se. However, the different guideline values for di(2-ethylhexyl) phthalate (DEHP), toluene and vinyl chloride between the Japanese guidelines revision (2003) and the latest rolling revision of WHO drinking water quality guideline were mainly caused by the health risk assessment variation. In this short communication, we describe the reason for taking the different toxicity endpoints or derivation method of the guidelines. Table 1 shows the guideline values for three chemicals of the WHO 2nd edition (WHO, 1996) established in 1994 and rolling revision in 2003, and previous and present Japanese versions.

DERIVATION OF GUIDELINE VALUES

Di(2-ethylhexyl) phthalate (DEHP)

As the guideline value of DEHP by the WHO 2nd edition, 0.008 mg/L was derived from a no observed

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adverse effect level (NOAEL) of 2.5 mg/kg/day in a rat feeding study (Morton, 1979) for 7 days according to no induction of hepatic peroxisome proliferation. The hepatic tumors were considered to be the most critical endpoint and hepatic peroxisome proliferation to be closely related to the carcinogenic mechanism. An uncertainty factor of 100 was applied only because of the animal most sensitive to peroxisome proliferation, and the allocation of 1% that was used as DEHP is generally not contained in food (WHO, 1996). For the latest WHO assessment, the guideline value of DEHP was not changed from the 2nd edition, because it was not listed for the detailed reevaluation.

In 1994, the Japanese government decided to use the same data and derivation method for domestic drinking water guidelines except for 10% allocation and 50 kg instead of 60 kg for human body weight. The guideline value was 0.06 mg/L.

However, the Japanese government established a TDI for DEHP in 2001 when high contamination was found in some specific foods and the health risk was deeply concerned (Koizumi *et al.*, 2001). In this assessment, TDI ranging from 40 to 140 μg/kg/day was established from a NOAEL of 3.7 mg/kg/day for testicular toxicity in a rat study (Poon *et al.*, 1997) and 14 mg/kg/day for reproductive toxicity in a mouse study (Lamb *et al.*, 1987), respectively, applying an uncertainty factor of 100 for intra- and interspecies differences. As for hepatic peroxisome proliferation, it was taken out for extrapolation to humans because IARC (2000) concluded that the hepatic tumor due to DEHP in rodents (in association with peroxisome proliferation) is not relevant to other animal species including humans (Group 3). Although it is clearly shown that there are strong species differences in testicular toxicity such as severely toxic in rats and guinea pigs, weakly in mice but not in hamsters, marmosets and cynomolgus monkeys, the potential of testicular toxicity in humans cannot be excluded at this moment. Therefore, the guideline of 0.1 mg/L was derived from 40 μg/kg/day of TDI using 10% of allocation, and 2 L of daily water intake for 50 kg body weight of the Japanese population.

Toluene

In 1994, WHO tried to re-assess the toxicity data of toluene and made the same conclusion as the previous value, 0.7 mg/L. A TDI of 0.223 mg/kg/day was derived using the lowest observed adverse effect level (LOAEL) for marginal hepatotoxicity in mice of 312 mg/kg/day (equivalent to 223 mg/kg/day, as there were 5 days per week) (NTP, 1990) and applying an uncertainty factor of 1,000 (100 for inter- and intra-species variation and 10 for the short duration of the study and use of a LOAEL instead of a NOAEL). This TDI yields a guideline value of 0.7 mg/L (rounded figure), allocating 10% of the TDI to drinking-water (WHO, 1996).

The Japanese government used the same data and derivation method for the domestic drinking water guideline except for 50 kg instead of 60 kg for human body weight. The guideline value was established as 0.6 mg/L in 1994.

For the new revision, the Japanese Government used a different toxicity endpoint, neurotoxicity, which is the most typical toxicity for toluene. In the case of neurotoxicity with histopathological changes as well as carcinogenicity and developmental toxicity without maternal toxicity, some additional uncertainty factors should be considered to derive a TDI. Toluene showed neuropathological effects in the brain consisting of neuronal cell necrosis in the dentate gyrus and Ammon's horn of the hippocampus at 1250 and 2500 mg/kg/day. NOAEL for neurotoxicity was 625 mg/kg/day (equivalent to 446 mg/kg/day, as there were 5 days per week) and a TDI of 0.0892 mg/kg/day was derived by application of an uncertainty factor of 5,000 including additional uncertainty factors of 5 for short exposure duration and 10 for neuropathological changes. This TDI yields a guideline value of 0.2 mg/L (rounded figure), allocating 10% of the TDI to drinking-water.

Table 1. Comparison of three guideline values (mg/L) between WHO and Japanese drinking water.

	WHO Guideline		Japanese Guideline	
	1994 (2nd ed.)	Revising 2003 (3rd ed.)	1994	2003
DEHP	0.008	0.008*	0.06	0.1
Toluene	0.7	0.7	0.6	0.2
Vinyl chloride	0.005	0.0003	No setting	0.002

*: No detailed reevaluation draft.

Vinyl chloride

It has been generally accepted that a mathematical model such as a linearized multistage is appropriate to estimate a low-dose cancer risk of a genotoxic carcinogen. There is sufficient evidence showing that vinyl chloride is a multiple site carcinogen and its metabolites are genotoxicants. Table 2 shows the incidences of hepatic tumor-related lesions in studies reported by Feron *et al.* (1981) and Til *et al.* (1991).

In the WHO 2nd edition, a linearized multistage model was applied to the incidence of angiosarcomas in female rats which was reported by Feron *et al.* (1981) only because of a good relationship with the human incidence at that time. An excess cancer risk at 10^{-5} was 0.010 mg/L. The guideline value was 0.005 mg/L, applying an uncertainty factor of 2 for double risk by exposure from birth (WHO, 1996).

On the other hand, in the WHO rolling revision, total liver tumors (angiosarcomas, hepatocellular carcinomas and neoplastic nodules) from the same study are incorporated to derive the guideline value including conversion to human equivalent doses (using the physiologically based pharmacokinetic (PBPK) model of U.S. EPA, 2000, Clewell *et al.*, 2001). A linear low-dose extrapolation was conducted by drawing a straight line between 10% of the low estimate dose (Benchmark dose approach) and the origin (zero dose). The results were nearly identical with those derived using the linearized multistage model. The concentrations in drinking-water of 0.0005 mg/L were calculated as being associated with excess risks of liver tumors of 10^{-5} for lifetime exposure beginning at adulthood. Exposure from birth would double this risk (U.S. EPA, 2000). This would result in a rounded guideline value of 0.0003 mg/L for a theoretical risk of 10^{-5}.

The guideline for vinyl chloride was not set in the previous Japanese guideline.

As described in Table 2, Feron *et al.* (1981) obtained clear evidence of carcinogenicity in rat liver in a three-dose setting study but the low dose of 1.7 mg/kg/day was still carcinogenic in female rats. The same group (Til *et al.*, 1991) conducted a further study up to 0.014 mg/kg/day and showed that the middle dose of 0.13 mg/kg/day was a non-carcinogenic dose. As both studies had been conducted under mostly the same experimental conditions, these data would be considered from a single study with doses ranging 1,000 times. For derivation of the newly established

Table 2. Summary incidence of hepatic tumor-related leisions for two rat carcinogenicity studies conducted by the same group.

	Til *et al.*, 1991				Feron *et al.*, 1981			
mg/kg/day	0	0.014	0.13	1.3	0	1.7	5.0	14.1
Male								
Neoplastic	0/99[a]	0/99	0/99	1/49	0/55	1/58	7*/56	23*/59
nodules	(0)[b]	(0)	(0)	(2.0)	(0)	(1.7)	(12.5)	(39.0)
Hepatocellular	0/99	0/99	0/99	3*/49	0/55	1/58	7*/56	23*/59
carcinoma	(0)	(0)	(0)	(6.1)	(0)	(1.7)	(12.5)	(39.0)
Angiosarcomas	0/99	0/99	0/99	1/49	0/55	1/58	2/56	8*/59
	(0)	(0)	(0)	(2.0)	(0)	(1.7)	(3.6)	(13.6)
Female								
Neoplastic	0/98	0/100	1/96	9*/49	2/57	26**/58	39*/59	44*/57
nodules	(0)	(0)	(1.0)	(18.4)	(8.8)	(44.8)	(66.1)	(77.2)
Hepatocellular	1/98	0/100	1/96	3/49	0/57	4*/58	19*/59	29*/57
carcinoma	(1.0)	(0)	(1.0)	(6.1)	(0)	(6.9)	(33.2)	(50.9)
Angiosarcomas	0/98	0/100	0/96	2/49	0/57	0/58	2/59	9*/57
	(0)	(0)	(0)	(4.1)	(0)	(0)	(3.4)	(15.8)
Total liver					2/57	28/58	49/59	56/57
tumors[c]					(8.8)	(48.2)	(83.1)	(98.2)

[a]: Number of lesion-bearing animals / number of analyzed animals.

[b]: Percentages of incidences.

[c]: The total number of animals with tumors derived from US IRIS(2000) / number of analyzed animals.

Statistically significant compared to the controls with *p<0.05 or **p<0.01 was reported in the original articles.

Japanese guideline value, the neoplastic nodules were not taken into account for the following reasons. As there was no diagnosis of nodular hyperplasia in those reports, there is a possibility that the neoplastic nodules may include not only hepatocellular adenoma but also nodular hyperplasia, which is not considered to be a neoplastic leision. The high incidence of neoplastic nodules at 1.7 mg/kg/day in females quickly dropped to less than half at 1.3 mg/kg/day and virtually no incidence at 0.13 mg/kg/day. This dose-response may not be appropriate for extrapolation to low doses. The incidence slope of total liver tumors mostly reflected the high incidence of neoplastic nodules rather than the real cancer incidence. In addition, because hepatocellular carcinomas and angiosarcomas originate from different cells, liver and vascular cells respectively, the evaluation of combined incidences may draw a conflicting conclusion. Therefore, the dose-response incidences of hepatocellular carcinoma in female rats were considered to be most appropriate for application to dose-response analysis, in view of data from the two reports. After dose conversion based on the PBPK model, an excess risk of 10^{-5} by the multistage model was calculated to be 0.0875 mg/kg/day as a virtual safety dose (VSD). The guideline of 0.002 mg/L was derived using 2 L of daily water intake for 50 kg body weight of the Japanese population. The allocation factor was not applied for the mathematical model approach because of large uncertainty caused by highly lower dose extrapolation.

DISCUSSION

Table 3 summarizes the derivation processes of all three chemicals. Although the detailed reevaluation draft for DEHP has not been published in the 3rd WHO water quality guideline, it was presumed that the derivation process would be same as the 2nd edition because were no changed guideline values. The general principle for the derivation of TDI and VSD is the same between Japan and WHO; however, the difference in the choice of critical endpoints leads to varied guideline values. In the Japanese assessment, testicular toxicity of DEHP and neurotoxicity of toluene were used to derive a TDI instead of their hepatotoxicity adopted by WHO. In the case of vinyl chloride, the same critical study was used for the guideline derivation, but the adopted neoplastic endpoints were differ-

Table 3. Summary of guideline value derivation in WHO (3rd ed.) and Japan (2003).

endpoint	NOAEL (mg/kg/day)	uncertainty factor					TDI or VSD* (mg/kg/day)	allocation (%)	body weight (kg)	water consump. (L)	guideline value (mg/L)
		inter-species	intra-species	use of LOAEL	study period	nature of toxicity					
DEHP(WHO)[a]											
hepatic peroxisome proliferation	2.5	10	10				0.025	1	60	2	0.008
DEHP(Japan)											
testicular toxicity	3.7	10	10				0.04	10	50	2	0.1
Toluene(WHO)											
hepatotoxicity	223	10	10	10			0.223	10	60	2	0.7
Toluene(Japan)											
neurotoxicity	446	10	10		5	10	0.0892	10	50	2	0.2
Vinyl chloride(WHO)											
total liver tumors (angiosarcoma, hepatocellular carcinoma and neoplastic nodules)											0.0003¶
Vinyl chloride(Japan)											
hepatocellular carcinoma							0.0875*		50	2	0.002

[a]: Derived from the 2nd edition.

¶: At the initial calculation from experimental animal data, the guideline concentration of 0.0005 mg/L was derived as 10^{-5} excess risk concentration during adulthood. Then the concentration was decreased to half because of doubled risk for exposure from birth.

*: Virtual safety dose corresponding to an excess cancer risk of 10^{-5}.

ent from each other because of the different interpretation on the cancer risk assessment. The adverse effects in experimental animals for the human health assessment are chosen by consideration of appropriate extrapolation to humans, which is expected from the nature of the toxicity, toxicity mechanism, etc. With regard to taking appropriate toxicity endpoints for derivation, the latest Japanese decision is considered to be more suitable on the basis of recent scientific consideration as described before. Because the revisions for the 3rd edition of water quality guidelines in the WHO are still ongoing, the assessment and the guideline value may be changed until the fixed version is published.

As for the derivation of the guideline value from the TDI, the estimation of the exposure contribution ratio (the allocation) is another important issue. In the case of DEHP, both levels of TDIs or NOAELs estimated in Japan and WHO are similar, although the critical endpoints are different. The guideline values were different at one order of degree from each other, because the allocation factor for drinking water of the TDI estimated in WHO was one-tenth of that in Japan. The allocation depends on environmental circumstances as well as chemical physical properties, and local exposure assessment is necessary for the estimation of the allocation factor of the respective chemical. Although the DEHP exposure contribution for drinking water in the WHO 2nd edition was estimated to be considerably lower, the allocation of 10% was applied in Japan as the default value when the exposure assessment was not elucidated.

Given the risk management of drinking water supplied by the Waterworks, the derivation of the guideline values of chemicals may be a regional issue. However, a large amount of drinking water bottled as mineral water has been circulating worldwide and the regulated values of chemicals will also be based on the drinking water guideline. Therefore the need for the international harmonization of chemical risk assessment will be required even more in the future.

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ORIGINAL ARTICLE

Susceptibility of newborn rats to 3-ethylphenol and 4-ethylphenol compared with that of young rats

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ABSTRACT **Newborn rat studies were conducted with oral administration of 3-ethylphenol (3EP) and 4-ethylphenol (4EP) on postnatal days (PND) 4–21 to allow comparison of no observed adverse effect level (NOAEL) and unequivocally toxic level (UETL) with those from 28-day studies of young rats starting at 5–6 weeks of age. In the newborn rat studies, slightly lowered body weight was observed after 3EP treatment, and deaths, hypoactivity, Straub tail, deep respiration and delayed righting reflex were clearly observed after 4EP treatment. In the young rat studies, salivation, staggering gait, changes in the liver including high values of liver weight and alanine aminotransferase or total cholesterol and the lesions in the forestomach were clearly observed after 3EP and 4EP treatments. NOAELs of 3EP and 4EP in the newborn rat studies appeared to be almost 3 times lower than those in the young rat studies. As a clear toxicity of 3EP was not observed in newborn rats, UETLs were not established for 3EP. Regarding 4EP, UETL of young rats was 4–5 times higher than that of newborn rats. In conclusion, newborn rats were 3–5 times more susceptible to 3EP and 4EP than young rats.**

Key Words: 3-ethylphenol, 4-ethylphenol, newborn rats, repeated-dose toxicity, young rats

INTRODUCTION

The possible toxic effect of chemical substances on the development of 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. In the EPA children's environmental health yearbook, US EPA (1998) has already stated comprehensively that children have their special vulnerability to certain toxic substances such as drugs and environmental chemicals. The special vulnerability in children to toxic substances may results from a combination of toxicokinetic, toxicodynamic and exposure factors, and kinetic factors are of importance mainly in the early postnatal period, largely as the result of immature elimination systems, i.e. metabolizing enzymes and/or renal function (Schwenk *et al.* 2002). There is much less information about differences between children and adults with regard to toxicodynamics (Schwenk *et al.* 2002). Regarding exposure factors, children play close to the ground and are constantly licking their fingers or mouthing toys or objects. As a result, mouthing becomes a potentially significant exposure route (US EPA 2002).

The potential toxic effects of chemicals cannot be anticipated from data on adults, and a data set on exposed children is essential for assessment of children's health. In this context, we have determined the toxicity of chemicals in newborn rats after direct dosing and compared it with that in young rats. We have already reported the differences in the susceptibility to toxicities of chemicals between newborn and young rats for 4-nitrophenol and 2,4-dinitrophenol (Koizumi *et al.* 2001), for 3-aminophenol (Koizumi *et al.* 2002), for 3-methylphenol (Koizumi *et al.* 2003), for tetrabromobisphenol A (Fukuda *et al.* 2004), for 2,4,6-trinitrophenol (Takahashi *et al.* 2004), for 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005). With regard to the no observed adverse effect level (NOAEL), these reports showed that the toxic response in newborn rats was at most 3–4 times (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol and 3-methylphenol) higher than that in young rats. On the other hand, the toxic response in newborn rats was 5 times (1,3-dibromopropane) and 8 times (1,1,2,2-tetrabromoethane) lower than that in young rats. The toxicological profiles of 4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, 1,3-dibromopropane and 1,1,2,2-tetrabromoethane were similar between newborn rats and young rats. The nephrotoxicity of tetrabromobisphenol A was specific for newborn rats. We also reported that the toxicity profiles induced by 2,4,6-trinitrophenol were markedly different between newborn and young rats.

3-Ethylphenol (3EP) is a photographic chemical intermediate and an intermediate for the cyan coupler of photographic paper (Horikawa *et al.* 1998). 4-Ethylphenol (4EP) is a chemical compound widely used as a source material of reactive polymers, antioxidants, drugs, agricultural chemicals and dyes (Chemical Products' Handbook 2004). These chemicals are listed in the 2004 OECD list of high production volume (HPV) chemicals (OECD 2004a). The HPV chemicals list contains those chemicals that are produced at levels greater than 1000 tons per year in at least one member country/region of OECD. Regarding the toxicity information on these two chemicals, only a few studies are available. Thompson *et al.* (1995) showed that 4EP was metabolized to a reactive quinone methide intermediate by rat liver enzymes and that this oxidation mechanism played a significant role in the cytotoxic effect of 4EP. This intermediate was subsequently trapped with glutathione to produce two diastereomeric conjugates. Recently, 28-day repeated dose oral toxicity studies of 3EP and 4EP in young rats were conducted as part of the Japanese Existing Chemical Safety Program and published in the annual toxicity

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testing report (MHLW 2001a,b), in which no observed effect level was evaluated.

In the present paper, we re-evaluated the toxicity of 3EP (MHLW 2001a) and 4EP (MHLW 2001b) in young rats in terms of NOAEL and unequivocally toxic level (UETL). We considered that the findings in the main test of repeated dose study and the dose-finding study were useful for characterizing the toxicity of chemicals. NOAEL is the highest tested dose in a study that did not produce any observable adverse effects and is expressed in terms of the weight of a test substance given daily per unit weight of a test animal. UETL has been used only for our comparative toxicity analysis as a clear toxic dose. It is generally not readily definable because it depends on the type of toxicity (Hirata-Koizumi *et al.* 2005). We determined the toxicity of 3EP and 4EP in newborn rats, compared and discussed NOAELs and UETLs of 3EP and 4EP for young and newborn rats.

MATERIALS AND METHODS

Chemicals

3EP (3-ethylphenol, CAS no. 620-17-7, purity 96.2%) was obtained from Taoka Chemical Co., Ltd. (Osaka, Japan) and 4EP (4-ethylphenol, CAS no. 123-07-9, purity 98.4% for the newborn rat study and 98.3% for the young rat study) was obtained from Maruzen Petrochemical Co., Ltd. (Tokyo, Japan) and they were dissolved in olive oil.

Animals

In the newborn rat study, pregnant SPF Crj:CD(SD)IGS rats (gestation day 14–15) were purchased from Atsugi Breeding Center, Charles River Japan (Yokohama, Japan) and allowed to deliver spontaneously. The day on which parturition was completed was designated as postnatal day (PND) 0. Pups (newborn rats) were separated from dams on PND 3 and were suckled by foster mothers. In the young rat study, four-week old males and females of the same strain were purchased from the same farm as in the newborn rat study.

The animals were maintained in an environmentally controlled room set at 20–26°C with a relative humidity of 45–65% 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 (CRF-1, Oriental Yeast, Tokyo, Japan or Laboratory MR Stock, Nosan Corporation, Yokohama, Japan) and water. The animals were euthanized by exsanguination under anesthesia using ether.

Study design

Time schedule for 3EP and 4EP studies is shown in Figure 1.

18-day repeated dose study in newborn rats

Dose-finding study. Twenty-four male and 24 female newborns for 3EP or 20 male and 20 female newborns for 4EP were randomly selected and assigned to four dose groups, including a control group. Six foster mothers for 3EP and five for 4EP were used. One foster mother suckled four male and four female pups. Newborn rats (6/sex/dose for 3EP, 5/sex/dose for 4EP) were given 3EP at 0, 30, 100 or 300 mg/kg/day or 4EP at 0, 100, 300 or 1000 mg/kg/day by gavage once a day on PND 4–21 (for 18 days) and killed on PND 22 after overnight starvation. General condition, body weights, hematology, blood biochemistry, necropsy and organ weights were examined. The similar study design was applied to the main study.

Main study. Forty-eight males and 48 females for each chemical for two autopsy groups (the end of the dosing period and the recovery-maintenance period) were randomly selected and assigned to four dose groups, including a control group. Twelve foster mothers were used for each chemical. One foster mother suckled four male and four female newborn rats up to weaning on PND 21. After weaning, newborn rats of the recovery-maintenance group were individually maintained for 9 weeks. Newborn rats (6/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 30, 100 or 300 mg/kg/day on PND 4–21 (for 18 days) and killed on PND 22 after overnight starvation. The dosage levels were determined based on the results of the dose-finding study. Recovery-maintenance groups (6/sex/dose for each chemical) given the same dosage were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks of age, almost the same age as young rats at the end of the recovery period.

General condition was observed at least once a day for newborn rats during the dosing period (separated from each foster mother) and during the recovery-maintenance period. Body weight was measured before dosing, more than two times per week during the dosing period and at seven-day intervals thereafter. Food consumption was measured about 2 times per week only during the recovery-maintenance period. Some developmental landmarks were assessed (OECD 2004b), such as piliation, incisor eruption, eye opening, testes descent and vaginal opening. All newborn rats were examined for abnormalities of reflex ontogeny; e.g. pupillary

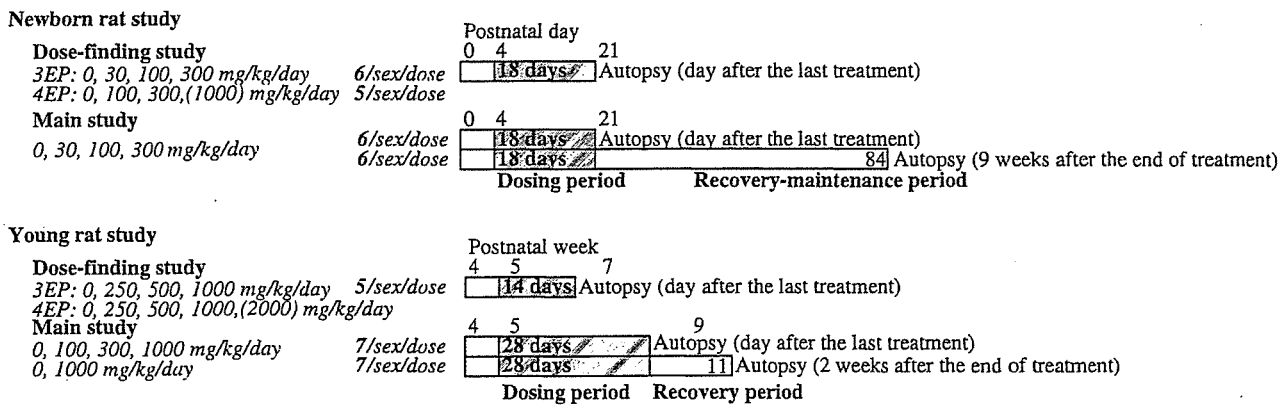

Fig. 1 Time schedule of newborn and young rat studies of 3-ethylphenol (3EP) and 4-ethylphenol (4EP).

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reflex, Preyer's reflex, corneal reflex, righting reflex and air righting reflex on PND 20 or 21.

In urinalysis, color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, urine sediment, specific gravity, osmotic pressure and volume of urine were examined in the late recovery-maintenance period. Newborn rats were killed on PND 22 or 85. On the day of the sacrifice, blood was collected from the abdominal aorta. Hematological parameters, such as the red blood cell count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte ratio, 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, alanine aminotransferase (ALT), γ-glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, cholinesterase, phospholipids, calcium, inorganic phosphorus, sodium, potassium and chloride levels in the serum, were also determined. After a gross examination, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenals, thyroids, lungs, testes/ovaries and epididymides/uterus were weighed. The organs were fixed with 10% buffered formation-phosphate and paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination. The studies using newborn rats were conducted at Gotemba Laboratory, Bozo Research Center Inc. (Gotemba, Japan) for 3EP and at Research Institute for Animal Science in Biochemistry and Toxicology (Sagamihara, Japan) for 4EP under Good Laboratory Practice (GLP) conditions (MHW 1988), and accordance with 'Guidelines for Animal Care and Use' of these laboratories.

28-day repeated dose study in young rats

Dose-finding study. Five-week-old rats (5/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 250, 500, 1000 or 2000 (only for 4EP) mg/kg/day for 14 days and killed the day following the last administration after overnight starvation. General condition, body weights, food consumption, hematology, blood biochemistry, necropsy and organ weights were examined.

Main study. Five-week-old rats (7/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 100, 300 or 1000 mg/kg/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 1000 mg/kg/day) (7/sex/dose for each chemical) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. The rats were examined for general condition, body weights, food consumption, urinalysis, hematology, blood biochemistry, necropsy findings, organ weights and histopathological findings. The study using young rats was conducted at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan) for 3EP and 4EP under GLP conditions (MHW 1988), and accordance with 'Guidelines for Animal Care and Use' of these laboratories.

Statistical analysis

Continuous data were analyzed with Bartlett's test for homogeneity of variance. If the data were homogeneous, one-way analysis of variance and Dunnett's test were conducted for group comparisons between the control and individual chemical-treated groups. If not homogenous or in case of quantitative urinalysis data, analysis was performed using the Kruskal–Wallis test. In consequence, if a significant difference was detected, the Dunnett type test or Mann–Whitney's *U*-test was conducted. In the newborn rat study, categorical data for general appearance and reflex ontogeny were analyzed by Fisher's exact probability test or Mann–Whitney's *U*-test. A probability less than 5% was considered statistically significant.

RESULTS

18-day study of 3EP in newborn rats

In the dose-finding study, body weights were considerably lowered in males (max. 9% decrease) and females (max. 6% decrease) at 300 mg/kg/day during the dosing period when compared to controls. However, the decreases were not statistically significant due to variations of the data.

Only slight changes were found in the main study as shown in the Table 1 and Figure 2. At 300 mg/kg/day, body weights were significantly lower than controls in males on PND 11–17 (max. 6% decrease) and females on PND 11–21 (max. 7% decrease). Significantly high value of relative liver weight was observed in males at 300 mg/kg/day and in females at 100 and 300 mg/kg/day at the end of the dosing period; however, it was not considered toxicologically significant because of the absence of changes in parameters of blood biochemistry and histopathological findings related to liver damage. There were no effects on the developmental landmarks at any dose. There were no effects of 3EP treatment at the end of the recovery-maintenance period.

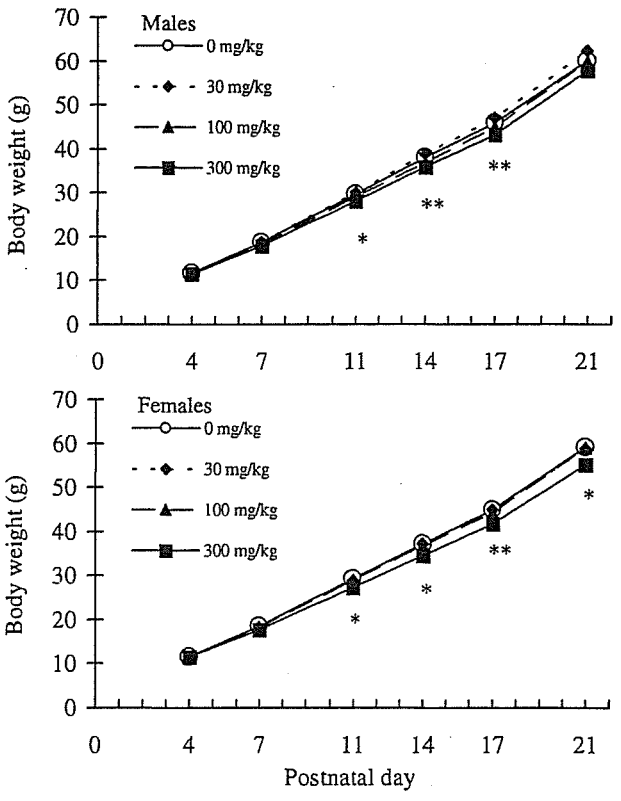

Fig. 2 Body weight curves in 18-day study of 3-ethylphenol (3EP) in newborn rats.

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Table 1 Main findings of 3-ethylphenol (3EP) at the end of the dosing in the newborn and the young rat main studies

	Newborn rat study (mg/kg/day)				Young rat study (mg/kg/day)			
	0	30	100	300	0	100	300	1000
Male								
No. animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	2
No. animals examined	6	6	6	6	6‡	7	7	7
ALT (IU/L)	36 ± 7	36 ± 4	41 ± 9	35 ± 5	24 ± 2	25 ± 3	27 ± 4	40 ± 2**
Total cholesterol (mg/dL)	85 ± 8	86 ± 17	83 ± 11	99 ± 18	55 ± 8	53 ± 9	59 ± 15	61 ± 7
Relative liver weight (g/100 g BW)	3.00 ± 0.16	3.14 ± 0.10	3.18 ± 0.11	3.42 ± 0.21**	3.11 ± 0.19	2.98 ± 0.14	3.36 ± 0.24	3.62 ± 0.25**
Relative kidney weight (g/100 g BW)	1.10 ± 0.09	1.08 ± 0.03	1.10 ± 0.06	1.05 ± 0.06	0.81 ± 0.02	0.80 ± 0.05	0.80 ± 0.11	0.91 ± 0.06**
Forestomach, hyperplasia	0	0	0	0	0	0	0	7
Female								
No. animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	5
No. animals examined	6	6	6	6	7	7	7	7
ALT (IU/L)	34 ± 3	30 ± 4	32 ± 4	30 ± 6	22 ± 4	22 ± 3	22 ± 2	28 ± 6*
Total cholesterol (mg/dL)	89 ± 10	90 ± 21	96 ± 18	94 ± 10	56 ± 15	57 ± 7	61 ± 7	76 ± 15**
Relative liver weight (g/100 g BW)	2.93 ± 0.10	3.03 ± 0.12	3.14 ± 0.10*	3.39 ± 0.17**	3.10 ± 0.14	3.09 ± 0.16	3.28 ± 0.18	3.68 ± 0.25**
Relative kidney weight (g/100 g BW)	1.07 ± 0.07	1.15 ± 0.08	1.13 ± 0.06	1.15 ± 0.05	0.82 ± 0.05	0.83 ± 0.03	0.85 ± 0.07	0.86 ± 0.04
Forestomach, hyperplasia	0	0	0	0	0	0	0	7

Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. BW, body weight.
†Staggering gait, prone/lateral position, tremor or soiled perigenital fur; ‡data from one animal were excluded because its hard palate was accidentally broken on day 23 of dosing.

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28-day study of 3EP in young rats

In the dose-finding study, one female showed staggering gait and a lateral position for three hours after the first dosing at 1000 mg/kg/day. At this dose, significantly high values of relative liver weight and ALT in males and relative liver weight and total cholesterol in females were observed. At 500 mg/kg/day, significantly high values of ALT in males and relative liver weight in females were observed.

In the main study (Table 1 and Fig. 3), adverse effects as below were found at 1000 mg/kg/day. Clinical signs, such as staggering gait, a prone/lateral position and soiled perigenital fur, were observed in 2/14 males and 5/14 females. Staggering gait and a prone and/or lateral position occasionally occurred 10 min after dosing and lasted one hour. Soiled perigenital fur was also observed in 1/14 males and 3/14 females at this dose. Body weight of males was significantly lowered on days 2 and 7 of dosing. In urinalysis, significantly high volumes of urine and water consumption and significantly low protein were observed in males and females at the end of the dosing period. In blood biochemistry, significantly high values of ALT in males and females and total cholesterol in females were observed. In the necropsy findings, thinning of the limiting ledge in the forestomach in 5/7 males and 2/7 females were observed at the end of the dosing period. Significantly high values of relative liver weight in males and females and relative kidney weight in males were observed at the end of the dosing period. Hyperplasia of the squamous cell in the forestomach was observed in all 7 males and all 7 females at the end of the dosing period. There were no effects of 3EP treatment at the end of the recovery period.

18-day study of 4EP in newborn rats

In the dose-finding study, deaths occurred at 300 mg/kg/day in one female each on days 6 and 8 of dosing, and at 1000 mg/kg/day in all rats by day 3 of dosing. In these dead rats, hypoactivity was observed and additionally, deep respiration, pale skin and/or dehydration were observed. In the surviving rats, hypoactivity during the dosing period was found in 3/5 males and 1/3 females at 300 mg/kg/day.

The main findings in the main study are shown in Table 2 and Figure 4. Clinical signs, such as hypoactivity, hypothermia, tremor, Straub tail, deep respiration and emaciation, were observed in 10/12 males and all 12 females at 300 mg/kg/day. Hypoactivity in males and females and hypothermia, tremor, Straub tail, deep respiration and emaciation in females were significantly more frequent at this dose and these clinical signs disappeared by day 9 of dosing for males and day 13 of dosing for females. At 300 mg/kg/day, 2/12 females were found dead on days 10 and 12 of dosing. One of them showed dark red lung and congestive edema of the lung and the other showed distention of the gastrointestinal tract and atrophy of the thymic cortex at necropsy. The delay in the righting reflex was observed in 4/12 males at 300 mg/kg/day, in 1/12 females at 100 mg/kg/day and in 1/10 females at 300 mg/kg/day. At 300 mg/kg/day, body weights of males and females were significantly lower on PND 7–21. Significantly high relative weight of the liver was observed in males and females at 300 mg/kg/day at the end of the dosing period. There were no changes in the parameters of blood biochemistry or histopathological findings related to liver damage. There were no effects of 4EP treatment at the end of the recovery-maintenance period.

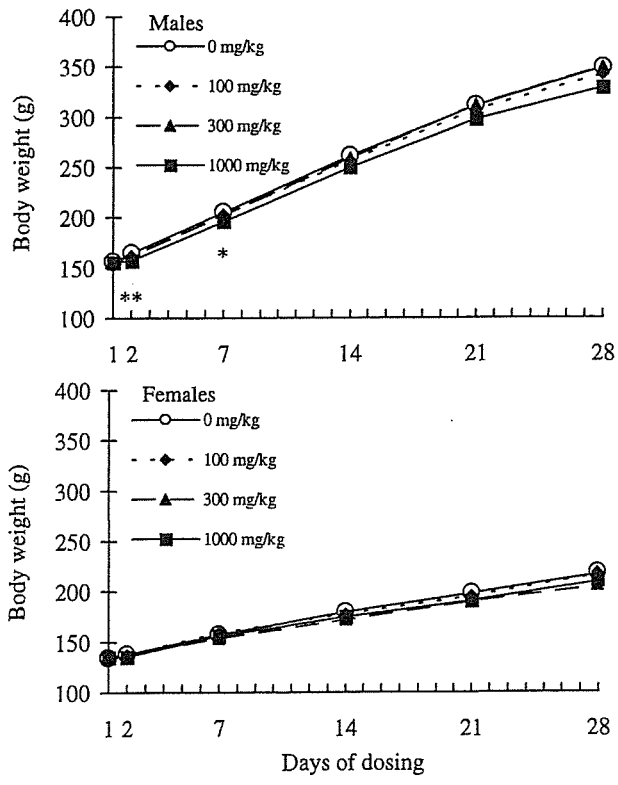

Fig. 3 Body weight curves in 28-day study of 3-ethylphenol (3EP) in young rats.

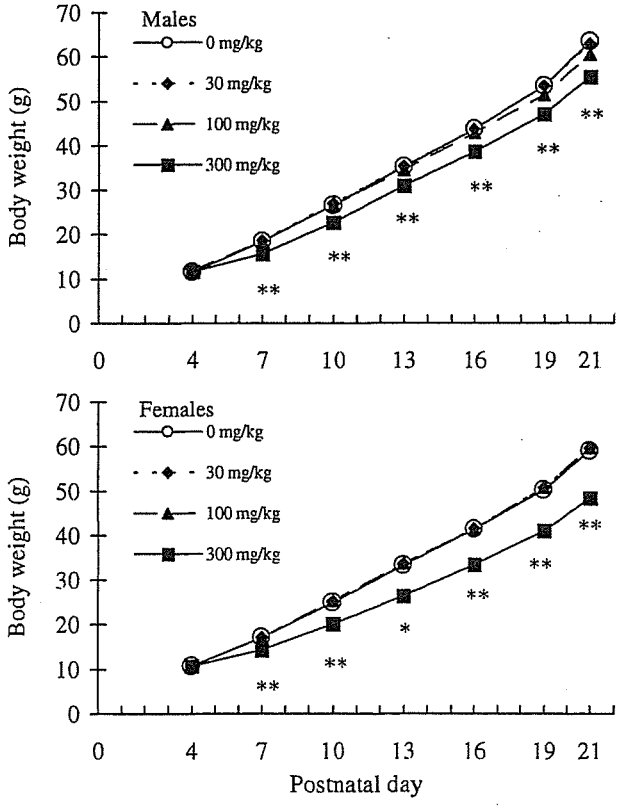

Fig. 4 Body weight curves in 18-day study of 4-ethylphenol (4EP) in newborn rats.

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Table 2 Main findings of 4-ethylphenol (4EP) at the end of the dosing in the newborn and the young rat main studies

	Newborn rat study (mg/kg/day)				Young rat study (mg/kg/day)			
	0	30	100	300	0	100	300	1000
Male								
No. animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	1‡	0	0	10	0	0	0	11
Death	0	0	0	0	0	0	0	0
Delayed righting reflex	0	0	0	4*				
No. animals examined	6	6	6	6	7	7	7	7
ALT (IU/L)	27 ± 7	21 ± 5	23 ± 2	25 ± 4	24 ± 3	24 ± 1	28 ± 3	41 ± 9**
Total cholesterol (mg/dL)	82 ± 13	83 ± 14	84 ± 8	91 ± 5	66 ± 6	58 ± 8	63 ± 9	68 ± 9
Relative liver weight (g/100 g BW)	3.37 ± 0.14	3.39 ± 0.22	3.40 ± 0.13	3.68 ± 0.16**	3.13 ± 0.18	3.28 ± 0.18	3.46 ± 0.16**	3.58 ± 0.17**
Relative kidney weight (g/100 g BW)	1.18 ± 0.05	1.17 ± 0.08	1.17 ± 0.06	1.22 ± 0.07	0.80 ± 0.05	0.79 ± 0.05	0.79 ± 0.05	0.89 ± 0.03**
Forestomach, hyperplasia	0	0	0	0	0	0	1	7
Female								
No. animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	12	0	0	0	9
Death	0	0	0	2§	0	0	0	0
Delayed righting reflex	0	0	1	1				
No. animals examined	6	6	6	5	7	7	7	7
ALT (IU/L)	19 ± 3	20 ± 3	20 ± 2	19 ± 1	22 ± 8	21 ± 2	20 ± 2	27 ± 4
Total cholesterol (mg/dL)	80 ± 11	84 ± 11	85 ± 12	85 ± 23	61 ± 13	69 ± 10	65 ± 5	82 ± 14**
Relative liver weight (g/100 g BW)	3.25 ± 0.12	3.26 ± 0.05	3.37 ± 0.11	3.63 ± 0.23**	3.07 ± 0.17	2.99 ± 0.15	3.12 ± 0.12	3.47 ± 0.21**
Relative kidney weight (g/100 g BW)	1.21 ± 0.11	1.17 ± 0.05	1.20 ± 0.05	1.26 ± 0.07	0.82 ± 0.04	0.84 ± 0.06	0.83 ± 0.05	0.88 ± 0.05
Forestomach, hyperplasia	0	0	0	0	0	0	0	6

Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. BW, body weight.

†Hypoactivity, hypothermia, tremor, straub tail, deep respiration or emaciation for newborn rats and salivation, staggering gait, prone/lateral position or soiled perigenital fur for young rats; ‡straub tail casually occurred on PND 9; §each female died on day 10 and 12 of dosing.

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28-Day study of 4EP in young rats

In the dose-finding study, 4/5 males and all 5 females at 2000 mg/kg/day died after the first dosing and the remaining 1/5 males was killed because of moribundity on day 3 of dosing. At 1000 mg/kg/day, 1/5 females showed soiled perineal fur on days 5–7 of dosing and then died on day 8 of dosing. The body weight of females was significantly lower on day 2 of dosing at 1000 mg/kg/day. Significantly high values of ALT and total cholesterol at 1000 mg/kg/day and significantly high value of ALT at 500 mg/kg/day were detected in males. Significantly low value of alkaline phosphatase and significantly high value of potassium at 1000 mg/kg/day were detected in females. In the necropsy findings for rats died during the dosing period, acute changes, such as red coloration of the lung, forestomach and kidney, thinning of the mucosa in the glandular stomach, discoloration of the liver and spleen, blood pooling in the urinary bladder and abdominal dropsy were observed at 2000 mg/kg/day and reddish spots of the glandular stomach and atrophy of the thymus and spleen were detected at 1000 mg/kg/day. For the surviving rats, thickening of the mucosa in the forestomach was observed in 2/5 males and 3/4 females at 1000 mg/kg/day at the end of the dosing period. At 1000 mg/kg/day, significantly high values of the relative liver weight in males and females and a significantly low value of relative spleen weight in females were observed. At 500 mg/kg/day, a significantly low value of relative spleen weight in females was observed.

In the main study (Table 2 and Fig. 5), clinical signs, such as salivation, staggering gait, a lateral position and soiled perigenital fur, were observed in 11/14 males and 9/14 females at 1000 mg/kg/day. At this dose, salivation for males and females was observed within 30 min after dosing daily from day 6 to the end of the dosing period. Staggering gait and a lateral position were occasionally observed in males and females for 1 h from a few minutes after dosing, and soiled perigenital fur was occasionally observed for males and females. Significantly low body weights from days 7–28 of dosing in males and from days 14–28 in females were also observed. In urinalysis, a significantly high volume of urine was observed in females at 1000 mg/kg/day at the end of the dosing period. In the blood biochemistry, significantly high values of ALT in males and total cholesterol in females at 1000 mg/kg/day were observed. In the necropsy findings, thinning of the mucosa in the glandular stomach in 5/7 males and 6/7 females and reddish spots in the glandular stomach in 1/7 females were observed at 1000 mg/kg/day at the end of the dosing period. Significantly high values of relative liver weight at 300 and 1000 mg/kg/day in males and at 1000 mg/kg/day in females were observed at the end of the dosing period. Significantly high value of relative kidney weight at 1000 mg/kg/day in males was observed at the end of the dosing period. Erosion, hyperplasia of squamous cells, degeneration of squamous cells and/or edema of the submucosa in the forestomach was observed in all 7 males at 1000 mg/kg/day. Hyperplasia of squamous cells in the forestomach was observed in 1/7 males at 300 mg/kg/day. Hyperplasia of squamous cells in the esophagus, degeneration of squamous cells, edema of the submucosa, granulation of the submucosa, hyperplasia of squamous cells and/or ulcer in the forestomach were observed in 6/7 females at 1000 mg/kg/day. There were no effects of 4EP treatment at the end of the recovery period except for the lowered body weight of males at 1000 mg/kg/day.

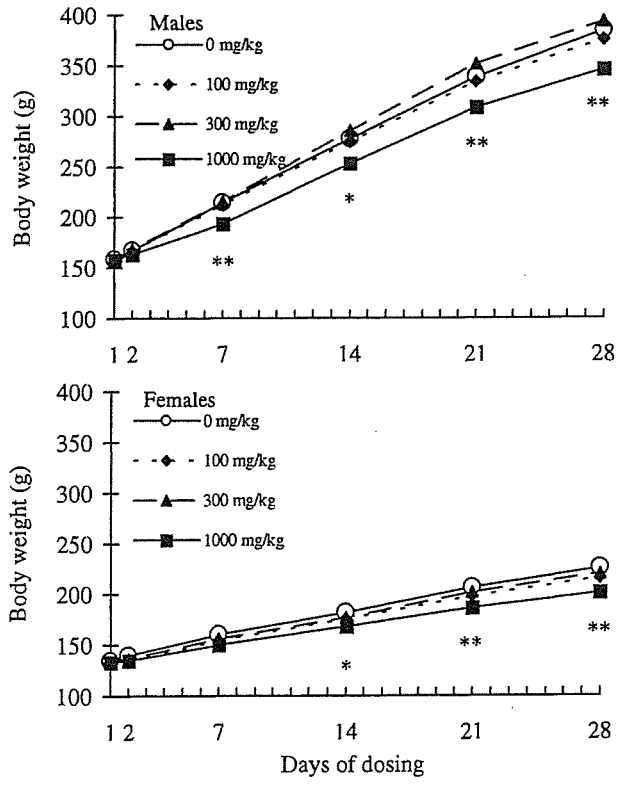

Fig. 5 Body weight curves in 28-day study of 4-ethylphenol (4EP) in young rats.

DISCUSSION

In the present paper, we determined the toxicity of 3EP and 4EP in newborn rats and reevaluated the toxicity of these chemicals in young rats, then compared the susceptibility of newborn rats in terms of NOAEL and UETL with that of young rats.

As for the administration of 3EP, NOAEL in the newborn rat study was concluded to be 100 mg/kg/day based on the lowered body weight at 300 mg/kg/day, although an increase in relative liver weight in females with no histopathological change and no changes in parameters of blood biochemistry related to liver damage was observed at 100 mg/kg/day in the main study. NOAEL in the young rat study was concluded to be 300 mg/kg/day based on the clinical toxic signs (staggering gait, prone/lateral position, tremor and soiled perigenital fur), changes in the liver (high values of weight and ALT or total cholesterol) and lesions in the forestomach at 1000 mg/kg/day. As clear toxicity did not appear in the newborn rat study even at the highest dose, we were not able to estimate UETL for 3EP.

As for the administration of 4EP, NOAEL in the newborn rat study was concluded to be 30 mg/kg/day based on the delay in the development of the righting reflex at 100 mg/kg/day. At 300 mg/kg/day, most animals showed clinical toxic signs and some females died in both the main and dose-finding studies. NOAEL in the young rat study was concluded to be 100 mg/kg/day, based on the lesions in the forestomach at 300 mg/kg/day. At 1000 mg/kg/day, clinical toxic signs were observed in all animals with the lesions in the forestomach. At this dose, no animal died in the main study but 1/5 females died in the dose-finding study (data not shown). When the dose of 1000 mg/kg/day for young rats was presumed as a UETL, which was the minimum lethal dose expecting the possibility of one female death, equivalent UETL for newborn rats was considered to be in the range of 200–250 mg/kg/day because 2/12

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and 2/5 females died at 300 mg/kg/day in the main and dose-finding newborn studies, respectively.

In the newborn rat studies, slightly lowered body weight was observed after 3EP treatment, and deaths, hypoactivity, Straub tail, deep respiration and a delayed righting reflex were clearly observed after 4EP treatment. In the young rat studies, salivation, staggering gait, changes in the liver, including high values of liver weight and ALT or total cholesterol and lesions in the forestomach were clearly observed after 3EP and 4EP treatments. As for NOAEL, the susceptibility of newborn rats to 3EP and 4EP was approximately 3 times higher than that of young rats. The reason that newborn rats had higher susceptibility than young rats could be that newborn rats have immature metabolic activity, thus oxidation and conjugation of 3EP or 4EP in their livers would occur less, and toxic effects of the parent chemicals would continue longer.

The change of the mucosa and lesions of the submucosa and squamous cells in the forestomach caused by the corrosiveness of 3EP and 4EP were observed in young rats, but not in newborn rats. Generally, the phenols have similar toxicological effects and phenol is a protoplasmic poison and extremely corrosive (Bloom & Brandt 2001; Manahan 2003). 3EP and 4EP are irritating to the eyes, skin, mucous membranes and upper respiratory tract (Lenga 1985). Histopathological findings were not observed in the newborn rat study at any dose. The fact could be expected from the assumption that the membrane of the gastrointestinal tract of newborn rats would be more quickly renewed than that of young rats because of a higher turnover rate of the gastric membrane in developing newborn rats (Majumdar & Johnson 1982).

Methylphenol is an analog chemical of ethylphenol. Methylphenols or cresols, including three isomers, were reviewed as to their toxicity, and they have strong skin irritation and induce symptoms of poisoning (ASTDR 1992; WHO 1995; Stouten 1998). These reviews show that 4-methylphenol is more toxic than 3-methylphenol on the repeated-dose toxicity. In the present study, severer lesions in the forestomach were found after administration of 4EP than with 3EP in young rats. 4EP was also more toxic than 3EP in the newborn rat study. Deaths occurred after administration of 4EP.

Based on NOAEL, the susceptibility of newborn rats to 3EP and 4EP appeared to be almost 3 times higher than that of the young rats, being consistent with our previous results for four chemicals, 4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol and 3-methylphenol, which showed 2–4 times differences in the toxic response between newborn and young rats. As for 3EP, unequivocal toxicity was not observed in the newborn rat study. As for 4EP, UETL in the young rat study was 4–5 times higher than that in the newborn rat study. In conclusion, newborn rats were 3–5 times more susceptible to 3EP and 4EP than young rats.

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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

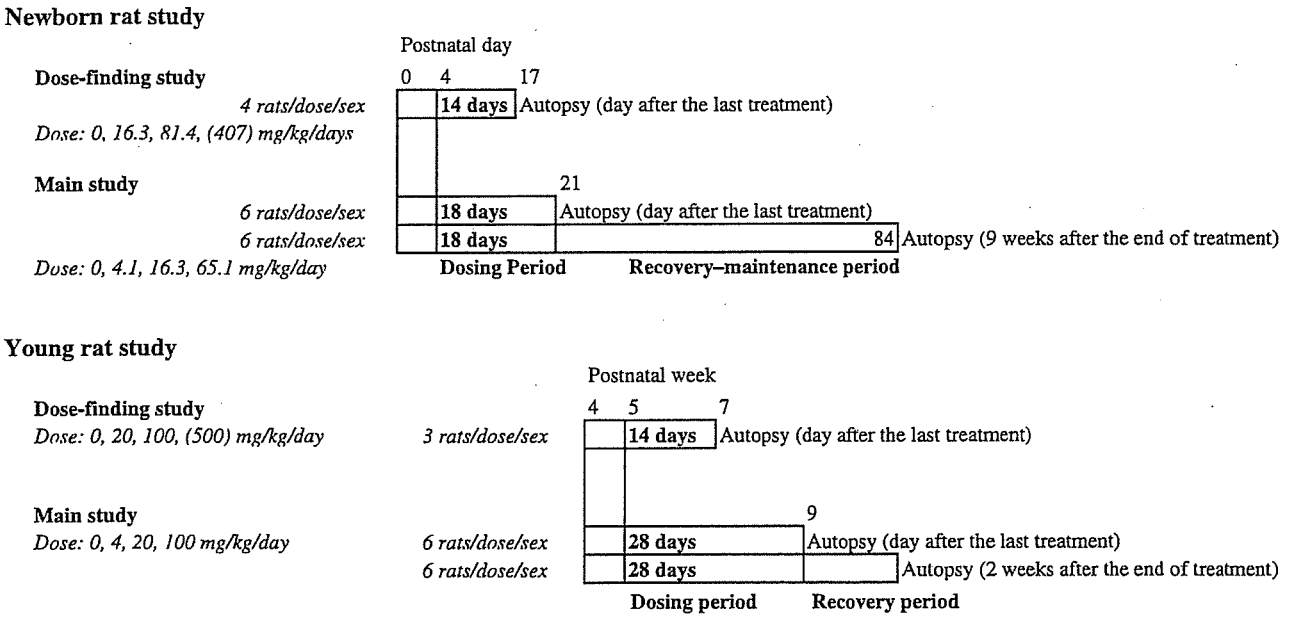

Fig. 1 Time schedule of the newborn and young rat studies.

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.