to the groups with single chemical administration using a least squares method (assuming zero interaction).
Step 2. Calculate the mean response $\bar{y}$ for simultaneous administration groups and the estimate $\hat{y}$ of expected response corresponding to $\bar{y}$ under zero interaction.
Step 3. Calculate the test statistic T and the critical value $t(\nu, \alpha)$ with the adjusted degrees of freedom ν using the Welch correction, where α is the significance level.
Step 4. If $T > t(\nu, \alpha)$, then the relationship is judged as synergistic with significance level α.

6. SIMULATION STUDY

A simulation study was performed to evaluate the performance of the proposed tests.

6.1. Common setup

Let y_{ij} be the response variable obtained from the jth animal of the ith group, where the number of groups with individual chemical administration is seven, being the same as the triangular design shown in Table 2, whereas that of the simultaneous administration groups is one, two, and three for Cases 1, 2, and 3, respectively. The total number k of groups is therefore eight, nine, or ten, depending on the case. The number of animals was fixed at six to coincide with the number used in the endocrine disruptor experiment for the case study in the next section.

It is assumed that y_{ij}, $i = 1, 2, \ldots, k, j = 1, 2, \ldots, 6$, were distributed independently as normal with mean μ_i and variance σ_i^2 and that the dose–response relationship was linear when each chemical was singly administered. As an alternative to the proposed test, we considered an analysis of variance test for interaction in a regression model with interaction, i.e. the null hypothesis was $H_0 : \beta_{AB} = 0$ for the following model:

$$E\{y_{ij}\} = \beta_0 + \beta_A d_A + \beta_B d_B + \beta_{AB} d_A d_B \tag{4}$$

where d_A, d_B are the doses of chemicals A and B, respectively, administered to the ith group. Robustness was examined by comparing the proposed t-tests with Welch correction (Proposed-W test) and without Welch correction (Proposed-T test) with the analysis of variance test (Regression test). Other common simulation conditions were as follows:

- repetition of simulation, 10 000 times
- dose setting for singly administered groups,

$$(d_A, d_B) = (0,0)\ (0,1)\ (0,2)\ (0,3)\ (1,0)\ (2,0) \text{ or } (3,0).$$

- parameter values: $\beta_0 = \beta_A = \beta_B = 1$
- nominal significance level: 5 per cent.

6.2. Alternative hypothesis

Three cases of simultaneous administration groups were considered, i.e.:

Case 1: One group with $(d_A, d_B) = (1.0, 1.0)$.
Case 2: Two groups with $(d_A, d_B) = (1.0, 1.0), (1.5, 1.5)$.
Case 3: Three groups with $(d_A, d_B) = (1.0, 1.0), (1.0, 2.0), (2.0, 1.0)$.

Table 3. Setup of the strength of synergy in alternative hypothesis. Δ_i represents the strength of synergy on the ith group with simultaneous administration of two chemicals. Model (1) corresponds to the null hypothesis, whereas models (2) through (9) represent alternative hypothesis

		Model								
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Case 1	Δ_1	0.0	0.3	0.5	1.0	1.5	2.0			
Case 2	Δ_1	0.0	0.3	0.5	1.0	0.3	0.5	1.0	0.3	0.5
	Δ_2	0.0	0.45	0.75	1.5	0.675	1.125	2.25	0.9	1.5
Case 3	Δ_1	0.0	0.3	0.5	1.0	0.3	0.5	1.0	0.3	0.5
	Δ_2	0.0	0.45	0.75	1.5	0.6	1.0	2.0	0.9	1.5
	Δ_3	0.0	0.45	0.75	1.5	0.6	1.0	2.0	0.9	1.5

The number of simultaneous administration groups is therefore different, depending on the case, and $\bar{y}$ is the mean of the observed responses of 1, 2, or 3 groups, depending on Cases 1, 2, or 3, respectively. The strength of the synergism is represented by the parameters Δ_1, Δ_2, and Δ_3, which are defined as the difference between the expected value of y_{ij} of the simultaneous administration groups and the one under the null hypothesis, i.e. Equation (3). If we adopt Equation (4) as an alternative model such as models (5), (6) and (7) in Table 3, then $\Delta_i = \beta_{AB} d_A d_B$, where d_A, d_B are the doses of A and B of the ith group, respectively.

Δs in Table 3 were selected as the simulation setting. As all Δs are obviously zero for model (1), this implies the null hypothesis. For models (2)–(4) the Δs are proportional to $d_A + d_B$, while for models (5)–(7) the Δs are proportional to $d_A d_B$, being advantageous for the analysis of variance test. Models (8) and (9) use steeper Δs.

6.3. Power under variance homogeneity

Table 4 summarizes the results of the simulation, where all the σ_i^2s are the same. It is theoretically natural that powers in Case 1 are the same between the two tests. In other settings, it is noted that the proposed tests are slightly inferior to the analysis of variance test in power under variance homogeneity.

Table 4. Probability (%) to realize significance. Type I error in model (1) and powers in other models. 'Proposed-W' and 'Proposed-T' are proposed tests with or without Welch correction, respectively

		Model								
Case	Test	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Case 1	Proposed-W	5.4	9.7	17.3	49.9	82.5	96.7			
	Proposed-T	4.9	10.6	20.2	61.0	91.7	99.5			
	Regression	4.9	10.6	20.2	61.0	91.7	99.5			
Case 2	Proposed-W	5.2	18.0	41.6	92.2	26.3	60.6	99.3	37.9	77.4
	Proposed-T	4.9	18.8	44.0	93.7	27.2	63.4	99.4	39.6	80.3
	Regression	5.0	17.9	42.5	93.4	30.1	68.8	99.8	46.9	88.2
Case 3	Proposed-W	4.9	24.1	54.1	98.5	33.5	73.0	100.0	57.3	94.9
	Proposed-T	5.1	24.2	54.4	98.6	34.4	73.8	100.0	58.1	95.2
	Regression	4.8	24.0	54.5	98.5	36.1	75.8	100.0	62.8	97.0

Table 5. Type I error (%) under heteroscedasticity. $\sigma_i^2 = \sigma^2 + \mu_i \times \gamma$. 'Proposed-W' and 'Proposed-T' are proposed test with or without Welch correction, respectively

Case	Test	$\gamma = 0$	$\gamma = 1$	$\gamma = 2$	$\gamma = 3$
Case 1	Proposed-W	5.4	5.9	5.6	5.7
	Proposed-T	4.9	6.3	6.2	6.1
	Regression	4.9	6.3	6.2	6.1
Case 2	Proposed-W	5.2	6.1	6.1	6.1
	Proposed-T	4.9	7.0	7.6	7.8
	Regression	5.0	8.0	8.2	8.5
Case 3	Proposed-W	4.9	6.4	6.2	6.4
	Proposed-T	5.1	7.4	7.4	7.3
	Regression	4.8	7.6	7.7	7.6

6.4. Robustness against variance heterogeneity

Variance heterogeneity will likely occur in real situations, and we therefore examine the robustness of the proposed test against variance heterogeneity by considering the case in which within-group variance σ_i^2 is as follows:

$$\sigma_i^2 = \sigma^2 + \mu_i \gamma \tag{5}$$

The parameter γ takes values 1, 2, and 3. Simulation results are summarized in Table 5, where, even though all tests appear to be liberal, the proposed test is the most robust one.

7. CASE STUDY

As a case study, we selected an 'ovariectomized rodent uterotrophic assay' containing seven groups with six animals per group, examining the quantitative endpoint of uterine weight gain in response to the estrogenicity of administered test chemicals. The dose setting was $(d_A, d_B) = (0.0, 0.0)$, $(10.0, 0.0)$, $(20.0, 0.0)$, $(0.0, 10.0)$, $(0.0, 20.0)$, $(5.0, 5.0)$, and $(10.0, 10.0)$, i.e. two simultaneous administration groups were included. The major interest was whether or not the combined effect was synergistic. The observed uterine weights were averaged over two simultaneous administration groups and compared with the response for $(d_A, d_B) = (7.5, 7.5)$ estimated under the null hypothesis based on the data from groups with individual chemical administration.

The observed mean response was $\bar{y} = 1606.4$ with standard error $V(\bar{y}) = 59.4$, and the estimated response was $\hat{y} = 1450.0$ with standard error $V(\hat{y}) = 39.7$. The degrees of freedom using Welch correction were 19, and therefore the one-sided p-value corresponding to the observed value $T = 2.190$ was $p = 0.021$. From these results we judged that the combined action was synergistic—a judgment accepted as reasonable by researchers conducting this experiment.

8. CONCLUSION AND DISCUSSIONS

One issue elicited from endocrine disruptor studies is how to judge the occurrence of synergism among chemicals. Here, we considered exchangeable cases in which two chemicals induce the same type of response on animals.

A linear model was assumed regarding the experiment data using a proper transformation of response and/or doses of chemicals, with synergism being defined as the case in which there occurs a higher response for simultaneous administration of chemicals than the corresponding response expected from the linear model without interaction. Based on this consideration, we proposed a triangular design for an animal experiment and a statistical test for judging synergism.

Results of a simulation study indicate that the proposed test is not superior in power to the analysis of variance test in the regression model with interaction, yet it is nevertheless robust against heteroscedasticity for type I error. By applying the proposed test to actual experimental data, a judgment was made that the combined effect of test chemicals was synergistic. Researchers who conducted the experiment in question agreed this was a reasonable finding.

When considering the molecular mechanism of the receptor-ligand system, it is obvious that the simplest *in vitro* system has only a slight possibility to produce a synergistic response because at the ligand binding site the best ligand is always interfered with by a less potent ligand. The occurrence of synergism is always speculated when the system is complex and has multiple signal pathways, most of which are in a 'black box'.

It is for this reason that a study aimed at detecting possible synergism will likely be an *in vivo* experiment. And yet, such *in vivo* studies always have limitations in their size, sometimes due to (i) the limited capacity of the animal facility, or (ii) manpower limitations. This affects the ability to complete each process within a certain timeframe in order to minimize the circadian variables. The proposed experimental design using a limited number of animals together with a robust statistical analysis method is therefore expected to be useful to many researchers for detecting possible synergistic effects in *in vivo* assays.

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Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Abstract

Twelve male and female rats per group were exposed to the rubber accelerator 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 8, 20 or 50 mg/kg bw/day. Males were dosed for a total of 49 days beginning 14 days before mating. Females were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. At 50 mg/kg bw/day, deaths were observed in two males and three females. Lowered body weight gain and food consumption were noted in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day. Mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation were observed in males and females at 20 and 50 mg/kg bw/day. No effects of DTG were found on the estrous cyclicity, precoital interval, copulation, fertility and gestational indices, numbers of corpora lutea and implantations, or gestation length. A significant decrease in the number, body weight and viability of offspring and increase in the incidence of fetuses with external malformations were found at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were observed. These data suggest that DTG may be teratogenic. The NOAELs of DTG for general and developmental toxicity in rats are 8 and 20 mg/kg bw/day, respectively.

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

1. Introduction

The basic rubber accelerator 1,3-di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the United States [1,2]. DTG is known as a selective sigma ligand [3]. In this context, many pharmacological studies of DTG were performed [3–12]. Ligands that interact with sigma sites have been shown to produce hypothermia [4–6]. Hypothermia induced by DTG was detected following subcutaneous or intracerebroventricle injection in rats [5,6] and intraperitoneal injection in mice [4]. The intraperitoneal injection of DTG potently reduced the pain behavior in the acute but increased pain behavior in the tonic phase in the formalin test in mice [7]. Intraperitoneal injection of DTG produced significant but short-lived increases in the withdrawal latencies in mice [4]. Bastianetto et al. [8] showed that unilateral intranigral injection caused circulating behavior in rats and suggested that sigma sites play a role in movement and posture through their association with brainstem and forebrain motor control circuits. Decreased locomotor activity induced by intraperitoneal injection [9,10], increased bladder capacity induced by intravenous injection in the anaesthetized condition [11] and no change in immobility time in open field after intraperitoneal injection [12] were also reported in rats given DTG. Toxicological studies on DTG have given little information on acute animal toxicity [13]: intraperitoneal LD50 was 25 mg/kg bw in mice; oral LD50 was 500 mg/kg bw in rats; lowest published lethal dose of oral administration was 80 mg/kg bw in rabbits; and the lowest published lethal dose was 120 mg/kg bw after oral administration in mammals, species unspecified. At the present time, no information is available for the reproductive and developmental toxicity of DTG. It is generally assumed that the results of animal test on chemical toxicity are relevant to human health [14]. As such, the testing for reproductive and developmental toxicity

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in animal models is an important part of the overall toxicology. The present study was conducted to obtain information on the effects of DTG on reproductive and developmental parameters in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15] and in accordance with the principles for Good Laboratory Practice [16,17] and "Guidance for Animal Care and Use" of Panapharm Laboratories Co., Ltd.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxic studies, including reproductive and developmental toxicity studies, and historical control data are available. Males and females at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimated to the laboratory for 13 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Vaginal smears of each female were recorded and only females showing a 4-day estrous cycle were used in the experiment. Male and female rats were distributed on a random basis into four groups of 12 males and 12 females each. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared using wooden chips as bedding (White Flake; Charles River Japan, Inc.).

Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water ad libitum and maintained in an air-conditioned room at 24 ± 2 °C, with a relative humidity of 55 ± 10%, a 12-h light/12-h dark cycle and ventilation with 13–15 air charges per hour.

2.2. Chemicals and dosing

DTG was obtained from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). DTG, a white powder, is slightly soluble in hot water and alcohol, soluble in chloroform and very soluble in ether, and its melting point is 179 °C, specific gravity is 1.10 and molecular weight is 239.3 [2]. The DTG (Lot No. 30J08) used in this study was 99.6% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 8, 20 or 50 mg/kg bw. The dosage levels were determined based on the results of our previous dose-finding study, the 14-day repeated dose toxicity study in rats given DTG by gavage at 0, 10, 20, 40 or 80 mg/kg bw/day, in which deaths were found at 80 mg/kg bw/day, decreased locomotor activity, mydriasis, tremor and salivation were observed at 40 and 80 mg/kg bw/day, and no adverse effects were detected at 10 and 20 mg/kg bw/day (data not shown). DTG was suspended in 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. Males (12 rats/group) were dosed for a total of 49 days beginning 14 days before mating. Females (12 rats/group) were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 10 ml/kg body weight based on the latest body weight during the re-mating and mating period in males and females or the body weight on day 0 of pregnancy in females after copulation. Control rats were given 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. The stability of formulations has been confirmed for up to 8 days. During use, the formulations were maintained under such conditions for less than 7 days, and the target concentration was 96.5 to 101.4%.

2.3. Observations

All rats were observed daily for clinical signs of toxicity. The body weight was recorded twice a week in males, and twice a week during the pre-mating and mating periods, on days 0, 7, 14 and 21 of pregnancy and on days 0 and 4 of lactation in females. Food consumption was recorded twice weekly during the pre-mating period in males, and twice weekly during the pre-mating period, on days 1, 7, 14 and 21 of pregnancy and on days 1 and 4 of lactation in females. The rats were euthanized by exsanguination under anesthesia on the next day of the last administration in males and on day 4 of lactation in females. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. In males, the testes and epididymides were weighed. In females, the numbers of corpora lutea and implantation sites and weight of the ovaries were recorded. The testes and epididymides were fixed with Bouin's solution and preserved in 10% neutral buffered formalin, and the ovaries were stored in 10% neutral buffered formalin. Histopathological evaluations were performed on hematoxylin–eosin-stained tissue sections of these organs.

Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the pre-mating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the mating period, 2 weeks, had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence for successful mating. Once insemination was confirmed, the females were checked for signs of parturition before noon from day 20 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed by 12:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded. Gender was determined on live pups examined grossly and individually weighed on PNDs 0 and 4. On PND 4, the pups were euthanized by exsanguination under anesthesia and gross internal examinations were performed.

2.4. Data analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. The body weight, body weight gain, food consumption, length of estrous cycles, precoital interval, gestation length, weight of the organs, relative organ weight, numbers of corpora lutea, implantations and live and dead pups, total number of pups and weight of live pups were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. If homogeneous the data were analyzed using Dunnett's multiple comparison test to compare the mean of the control group with that of each dosage group. If not, the DTG-treated groups were compared with that of the control group with Steel's multiple comparison test. The implantation, delivery and viability indexes, and incidence of pups with anomalies and individual anomalies were analyzed with Wilcoxon's rank sum test. The mortality, copulation, fertility and gestation indexes, and sex ratio of pups were analyzed with Fisher's exact test. The 5% level of probability was used as the criterion for significant.

3. Results

Table 1 shows the findings in male rats given DTG. At 50 mg/kg bw/day, one male died after six administrations and one male died after seven administrations. These dead rats showed mydriasis, decreased locomotor activity, bradypnea, a prone position and tremor 10–20 min after the administration of DTG. In surviving males, mydriasis, decreased locomotor activity, bradypnea and prone position on days 1–9 of the administration period, tremor during the whole period of administration and salivation on days 22–49 of the administration period were also observed at 50 mg/kg bw/day. Salivation was noted on days 28–49 of the administration period at 20 mg/kg bw/day. A significant decrease in the body weight gain was found on days 1–8 (81% decrease) and days 15–22 (48% decrease) of the administration period at 50 mg/kg bw/day. At this dose, significantly lower food consumption on days 7–8 (20% decrease) and days 14–15 (7% decrease) of the administration period was also observed.

Table 1
Findings in male rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of male rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	30 ± 7	33 ± 7	25 ± 7	6 ± 9**
Days 8–15	29 ± 5	32 ± 5	32 ± 7	24 ± 7
Days 15–22	23 ± 6	25 ± 8	23 ± 7	12 ± 11**
Days 22–29	19 ± 9	22 ± 7	25 ± 8	19 ± 5
Days 29–36	22 ± 6	22 ± 6	23 ± 7	18 ± 8
Days 36–43	15 ± 8	12 ± 9	13 ± 5	14 ± 7
Days 43–50	19 ± 8	19 ± 7	13 ± 4	13 ± 11
Food consumption (g/day/rat)[a]				
Days 7–8	25 ± 3	26 ± 3	26 ± 2	20 ± 3**
Days 14–15	29 ± 2	30 ± 2	29 ± 3	27 ± 3*
Days 29–30	27 ± 2	27 ± 3	28 ± 3	25 ± 2
Days 35–36	28 ± 2	29 ± 2	29 ± 2	27 ± 2
Days 42–43	26 ± 3	25 ± 3	27 ± 4	27 ± 3
Days 49–50	28 ± 4	29 ± 3	28 ± 2	28 ± 3

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

Table 2 presents the findings in female rats given DTG. At 50 mg/kg bw/day, two females died after the first administration and one female died after normal delivery of her pups on day 22 of pregnancy. Mydriasis, decreased locomotor activity, bradypnea, prone position, and tremor and salivation 10–20 min after the administration of DTG were observed in females died after the first administration. These clinical signs and salivation were found during pregnancy and on day of parturition in a female which died after parturition. In surviving females, mydriasis, decreased locomotor activity, bradypnea and prone position on day 1 of the administration period to day 0 of lactation, tremor on day 1 of the administration period to day 5 of pregnancy and salivation on day 4 of pregnancy to day 3 of lactation were observed at 50 mg/kg bw/day. Mydriasis, decreased locomotor

Table 2
Findings in female rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of female rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
No. of deaths during pregnancy	0	0	0	1
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	19 ± 8	17 ± 7	11 ± 6*	−1 ± 9**
Days 8–15	10 ± 7	15 ± 8	20 ± 5**	15 ± 10
Days 0–7 of pregnancy	34 ± 6	31 ± 6	33 ± 4	28 ± 8
Days 7–14 of pregnancy	34 ± 5	34 ± 4	36 ± 3	30 ± 10
Days 14–21 of pregnancy	85 ± 17	100 ± 14	105 ± 9*	42 ± 21**
Days 0–4 of lactation	20 ± 19	14 ± 16	22 ± 9	16 ± 13
Food consumption (g/day/rat)[a]				
Days 7–8	22 ± 3	21 ± 2	19 ± 2**	13 ± 3**
Days 14–15	20 ± 4	22 ± 3	22 ± 2	20 ± 2
Days 6–7 of pregnancy	22 ± 3	23 ± 2	23 ± 3	17 ± 3**
Days 13–14 of pregnancy	23 ± 2	24 ± 3	25 ± 2	22 ± 5
Days 20–21 of pregnancy	24 ± 4	26 ± 3	29 ± 3*	21 ± 5
Days 3–4 of lactation	41 ± 5	41 ± 3	46 ± 4*	32 ± 6**

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

Table 3
Reproductive findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of pairs	12	12	12	10
Length of estrous cycles (day)[a]	4.0 ± 0.2	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2
Precoital interval (day)[a]	3.0 ± 1.0	2.7 ± 1.0	2.4 ± 1.1	2.2 ± 1.0
Copulation index (%)[b]				
Male	100	91.7	100	100
Female	100	91.7	100	100
Fertility index (%)[c]	100	100	91.7	100
Gestation index (%)[d]	100	100	100	90.0
Gestation length (day)[a]	22.6 ± 0.5	22.3 ± 0.5	22.5 ± 0.5	22.6 ± 0.5
Weight of testes (g)[a]	3.24 ± 0.34	3.34 ± 0.19	3.31 ± 0.28	3.30 ± 0.24
Relative weight of testes[a,e]	0.60 ± 0.05	0.62 ± 0.07	0.63 ± 0.07	0.68 ± 0.07*
Weight of epididymides (g)[a]	1.16 ± 0.10	1.21 ± 0.06	1.21 ± 0.12	1.23 ± 0.07
Relative weight of epididymides[a,e]	0.22 ± 0.02	0.22 ± 0.02	0.23 ± 0.03	0.25 ± 0.02**
Weight of ovaries (mg)[a]	101 ± 8	106 ± 6	101 ± 11	102 ± 10
Relative weight of ovaries[a,e]	30 ± 2	31 ± 2	28 ± 3	32 ± 2

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = (no. of rats copulated/no. of pairs) × 100.
[c] Fertility index (%) = (no. of females pregnant/no. of females copulated) × 100.
[d] Gestation index (%) = (no. of females with parturition/no. of females copulated) × 100.
[e] Relative weight = organ weight/100 g of body weight.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

activity, bradypnea and prone position on days 2–3 of the administration period, and salivation on day 14 of pregnancy to day 3 of lactation were observed at 20 mg/kg bw/day. Body weight gain was significantly lowered on days 1–8 of the pre-mating period at 20 mg/kg bw/day (42% decrease) and on days 1–8 of the pre-mating period (105% decrease) and days 14–21 of pregnancy (49% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significantly higher body weight gain was observed on days 8–15 of the pre-mating period and days 14–21 of pregnancy. Food consumption was significantly reduced on days 7–8 of the pre-mating period at 20 mg/kg bw/day (14% decrease) and on days 7–8 of the pre-mating period (41% decrease) and days 3–4 of lactation (24% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significant increase in the food consumption was observed on days 20–21 of pregnancy and days 3–4 of lactation.

The reproductive findings in rats given DTG are presented in Table 3. No effects of DTG were observed on the length of estrous cycles, precoital interval and gestation length. One pair did not copulate at 8 mg/kg bw/day, one female did not become impregnated at 20 mg/kg bw/day and one female did not deliver any pups at 50 mg/kg bw/day; however, no significant differences were noted in the copulation, fertility or gestation index between the control and DTG-treated groups. The weights of the testes and epididymides, and absolute weight and relative weight of the ovaries in the DTG-treated groups did not differ from the control group. The relative weights of the testes (13% increase) and epididymides (14% increase) were significantly higher at 50 mg/kg bw/day.

The developmental findings in rats given DTG are shown in Table 4. There was no significant difference in the numbers of corpora lutea, implantations and stillborns, implantation index, sex ratio of live pups, viability index on day 0 of lactation and body weight of live pups on day 4 of lactation between the control and DTG-treated groups. The numbers of pups delivered (45% decrease) and live pups delivered (45% decrease) and delivery index (43% decrease) were significantly lowered at 50 mg/kg bw/day. At this dose, the viability index on day 4 of lactation (34% decrease) and body weight of live male (16% decrease) and female (19% decrease) pups on day 0 of lactation were also significantly decreased. Two dams with totally litter loss were observed. No poor maternal behavior or nursing was observed in dams at 50 mg/kg bw/day. No histopathological changes were found in the testes, epididymides and ovaries in the DTG-treated groups. External anomalies in pups of rats given DTG are also presented in Table 4. No fetuses with external malformations were observed in the control and groups given DTG at 8 and 20 mg/kg bw/day. At 50 mg/kg bw/day, fetuses with external malformations were found in 10 out of the 65 fetuses and in 3 out of the 9 litters. Oligodactyly was observed in four pups in two litters. A kinked tail was found in six pups in one litter and a short tail and anal atresia was observed in one pup in each letter. Although there was no significant difference in the incidence of fetuses with individual malformations between the control and 50 mg/kg bw/day groups, a significantly higher incidence of total number of fetuses with external malformations was noted at this dose.

Table 4
Developmental findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of litters	12	11	11	9
No. of implantations[a]	14.3 ± 2.6	16.2 ± 1.9	15.9 ± 1.4	14.2 ± 3.6
Implantation index (%)[b]	92.2	94.7	97.6	90.9
No. of pups delivered[a]	13.0 ± 2.4	15.2 ± 2.0	14.7 ± 1.4	7.2 ± 4.1**
No. of live pups delivered[a]	13.0 ± 2.4	15.1 ± 1.9	14.7 ± 1.4	7.2 ± 4.1**
No. of stillborns	0	0.1 ± 0.3	0	0
Delivery index (%)[c]	91.0	93.3	92.2	51.7**
Sex ratio of live pups (males/females)	71/85	84/82	80/82	31/34
Viability index (%)[d,e]				
Day 0 of lactation	100	99.5	100	100
Day 4 of lactation	99.4	99.4	100	65.4**
Body weight of male pups during lactation (g)[a]				
Day 0	7.4 ± 0.7	6.9 ± 0.6	7.3 ± 0.6	6.2 ± 1.0**
Day 4	11.9 ± 1.3	11.1 ± 1.0	11.7 ± 1.0	11.0 ± 2.3
Body weight of female pups during lactation (g)[a]				
Day 0	7.0 ± 0.7	6.6 ± 0.6	6.8 ± 0.7	5.7 ± 0.8**
Day 4	11.4 ± 1.3	10.5 ± 1.0	11.0 ± 0.9	10.5 ± 2.0
External examination of pups				
No. of pups (litters) with malformations	0	0	0	10 (3)*
Oligodactyly	0	0	0	4 (2)
Kinky tail	0	0	0	6 (1)
Short tail	0	0	0	1
Anal atresia	0	0	0	1

[a] Values are given as the mean ± S.D.
[b] Implantation index (%) = (no. of implantations/no. of corpora lutea) × 100.
[c] Delivery index (%) = (no. of live pups delivered/no. of implantations) × 100.
[d] Viability index on day 0 of lactation (%) = (no. of live pups delivered/total no. of pups delivered) × 100.
[e] Viability index on day 4 of lactation (%) = (no. of live pups on day 4 of lactation/no. of live pups delivered) × 100.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

4. Discussion

The present study was conducted to obtain initial information on the possible effects of DTG on reproduction and development in rats. The data show that DTG exerts developmental toxicity and suggest that DTG possesses teratogenic potential.

DTG was given to males during the pre-mating and mating periods and to females during the pre-mating, mating, pregnancy and shortly after parturition. The dosage used in the present study was sufficiently high such that it should be expected to induce general toxic and neurobehavioral effects. As expected, general toxicity, such as decreases in body weight gain and food consumption, was found at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females. Decreases in the body weight gain and food consumption during the early administration period, and thereafter, significant increases in body weight gain and food consumption were observed in females at 20 mg/kg bw/day. One possible explanation for increased body weight gain during late pregnancy at 20 mg/kg bw/day may be higher number of pups and higher net weight gain during pregnancy at this dose compared with the controls. Such recovery did not occur at the highest dose. Neurobehavioral effects, such as mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and salivation, were also observed at 20 and 50 mg/kg bw/day. DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,18,19]. It was reported that systemic injection of DTG caused neurobehavioral changes in rats [5,6,9,10]. The present study shows that the oral administration of DTG also induces neurobehavioral changes, and it is neurobehaviorally toxic at 20 and 50 mg/kg bw/day in rats.

Higher relative weights, but not the absolute weight, of the testes and epididymides were observed at 50 mg/kg bw/day. Body weights of male rats on the day of scheduled sacrifice were 537 and 485 g in the control and 50 mg/kg bw/day groups, respectively. It seems likely that the higher relative weights of the testes and epididymides at the highest dose were due to secondarily lowered body weight but not due to the direct effects of DTG on the male reproductive organs. Other male reproductive parameters were not significantly changed, even at the highest dose. These findings suggest that DTG is not reproductively toxic to male rats. It seems unlikely that DTG exerts reproductive toxicity to female rats when administered during the pre-mating, mating, pregnancy and early lactation period, because no adverse effects on the maternal reproductive parameters, including estrous cyclicity, precoital interval, copulation

index, fertility index, gestation index, gestation length and ovarian weight, were caused by the administration of DTG in females.

As for the developmental indexes, decreases in the numbers of total pups and live pups delivered, delivery index, viability on PND 4 and body weight of live pups on PND 0 were detected at 50 mg/kg bw/day. These findings indicate that DTG is toxic to the survival and growth of offspring and exerts developmental toxicity at 50 mg/kg bw/day in rats.

In the present study, the teratogenic effect of DTG is strongly suggested by the external examinations of pups. At 50 mg/kg bw/day, a significant increase in the total number of fetuses with external malformations was noted; however, incidences of fetuses with individual types of external malformations at this dose were not significantly different from those in the control group. The external malformations observed in the present study are of the types that occur spontaneously among control rat fetuses reported in the literature [20–23]. In the present study, only external examination in the newborn rats was performed, and no internal or skeletal examinations were performed. Even animals not ordinarily carnivorous, including nonhuman primates, are likely to eat dead and moribund offspring, as well as those with malformations that involve skin lesions allowing the loss of body fluids or the exposure of viscera [24]. To accurately evaluate the prenatal developmental toxicity including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals [24,25]. The present study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15], and this screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. In order to further evaluate the developmental toxicity, including teratogenicity, of DTG in rats, a prenatal developmental toxicity study is currently in progress.

In conclusion, DTG caused decreased body weight gain and food consumption at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females, neurobehavioral changes at 20 and 50 mg/kg bw/day in both sexes, and changes in developmental parameters at 50 mg/kg bw/day. DTG is suggested to be teratogenic. The NOAELs of DTG for general and developmental toxicity were 8 and 20 mg/kg bw/day, respectively, in rats.

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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) from postnatal days (PD) 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 PNDs 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 PNDs 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

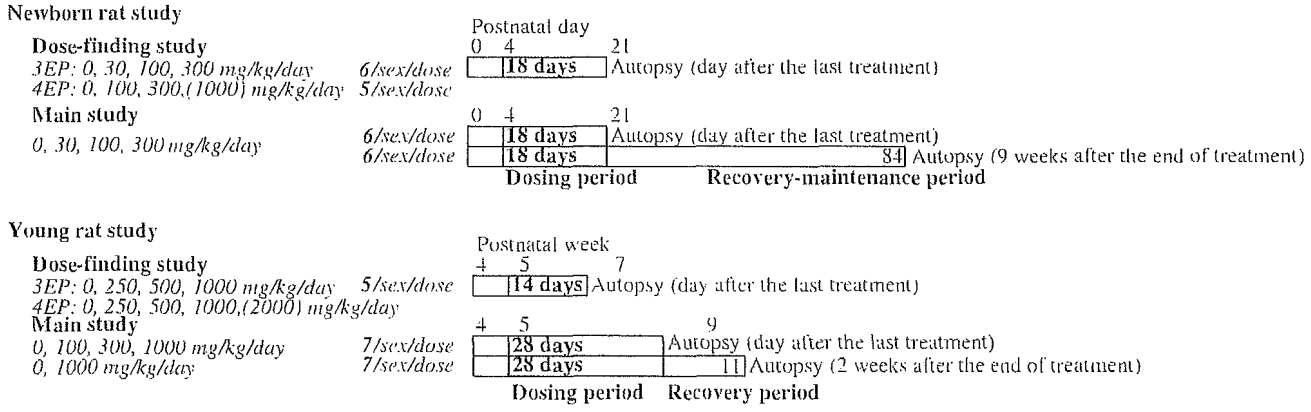

Fig. 1 Time schedule of newborn and young rat studies of 3-ethylphenol (3EP) and 4-ethylphenol (4EP).

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 recorded in males from PND 11–17 (max. 6% decrease) and females from PNDs 11–21 (max. 7% decrease) were significantly lower than controls. 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.

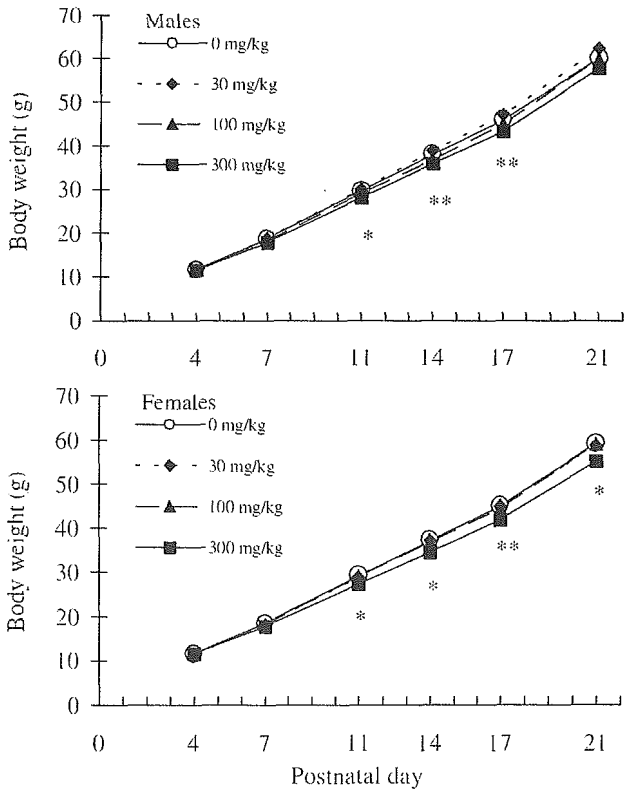

Fig. 2 Body weight curves in 18-day study of 3-ethylphenol (3EP) in newborn rats.

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. of animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	2
No. of 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. of animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	5
No. of 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.

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 PNDs 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.

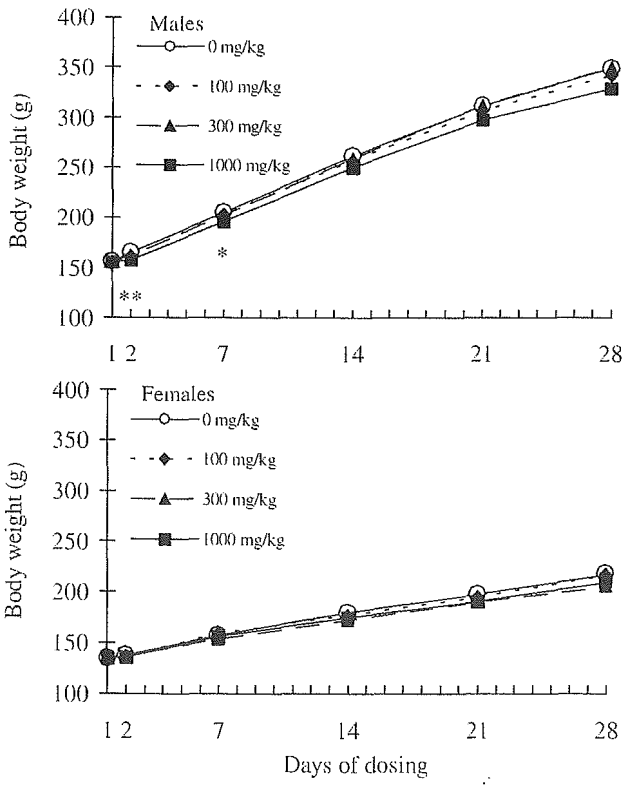

Fig. 3 Body weight curves in 28-day study of 3-ethylphenol (3EP) in young rats.

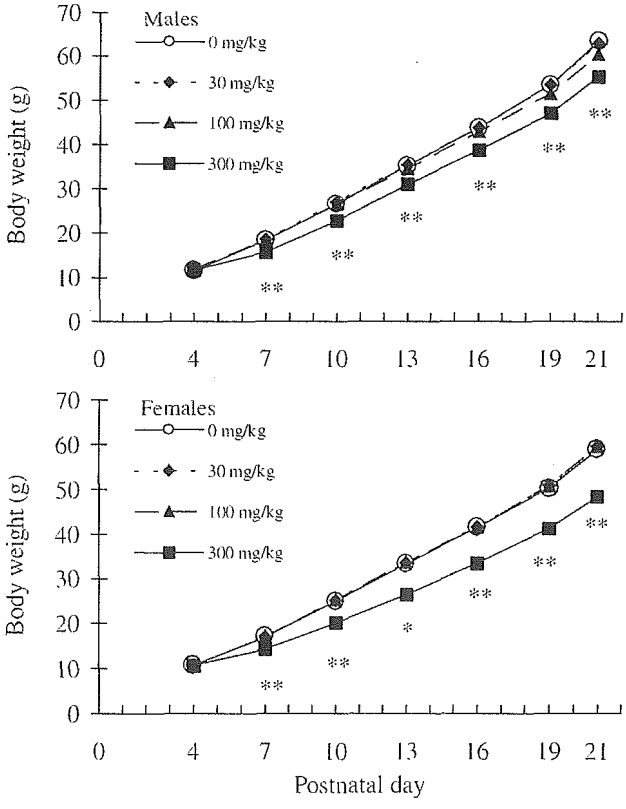

Fig. 4 Body weight curves in 18-day study of 4-ethylphenol (4EP) in newborn rats.

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. of 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. of 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. of 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. of 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.

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.

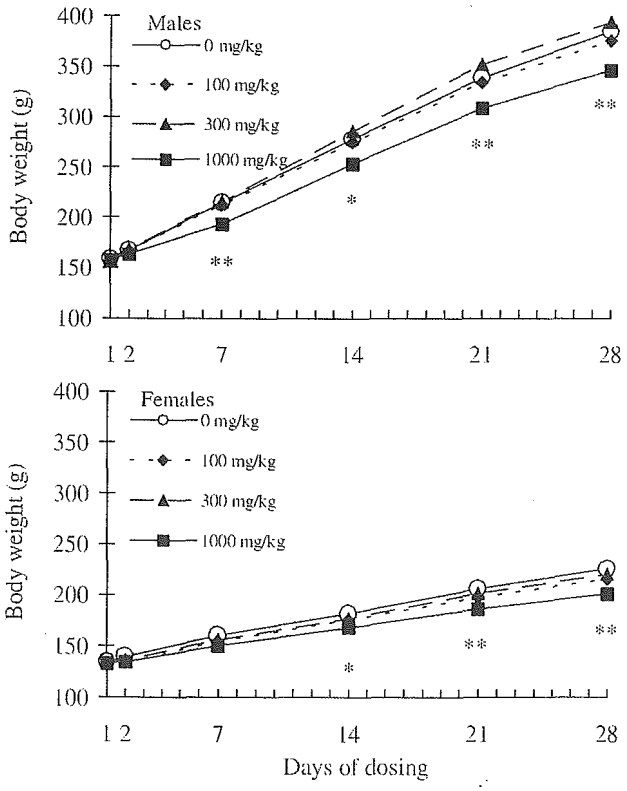

Fig. 5 Body weight curves in 28-day study of 4-ethylphenol (4EP) in young rats.

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

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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