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Original Article

Repeated dose and reproductive/developmental toxicity of perfluoroundecanoic acid in rats

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ABSTRACT — Perfluoroalkyl acids (PFAAs) are environmental contaminants that have received attention because of their possible effects on wildlife and human health. In order to obtain initial risk information on the toxicity of perfluoroundecanoic acid (PFUA), we conducted a combined repeated dose toxicity study with the reproduction/developmental toxicity screening test (OECD test guideline 422). PFUA was administered by gavage to rats at 0 (vehicle: corn oil), 0.1, 0.3 or 1.0 mg/kg/day. At 1.0 mg/kg/day, body weight gain was inhibited in both sexes, and there was a decrease in fibrinogen in both sexes and shortening of the activated partial thromboplastin time in males. An increase in blood urea nitrogen and a decrease in total protein in both sexes and increases in alkaline phosphatase and alanine transaminase and a decrease in albumin in males were observed at 1.0 mg/kg/day. Liver weight was increased in males at 0.3 mg/kg/day and above and in females at 1.0 mg/kg/day, and this change was observed after a recovery period. In both sexes, centrilobular hypertrophy of hepatocytes was observed at 0.3 mg/kg/day and above and focal necrosis was observed at 1.0 mg/kg/day. In reproductive/developmental toxicity, body weight of pups at birth was lowered and body weight gain at 4 days after birth was inhibited at 1.0 mg/kg/day, while no dose-related changes were found in the other parameters. Based on these findings, the no observed adverse effect levels (NOAELs) for the repeated dose and reproductive/developmental toxicity were considered to be 0.1 mg/kg/day and 0.3 mg/kg/day, respectively.

Key words: Perfluoroundecanoic acid, Repeated dose toxicity, Reproductive and developmental toxicity, Screening test, Rat

INTRODUCTION

Perfluoroalkyl acids (PFAAs) are environmental contaminants that have received attention because of their possible effects on wildlife and human health in recent years; PFAAs are very stable in the environment, have bioaccumulation potential, and have been detected in environmental media and biota in many parts of the world, including oceans and the Arctic; and many researchers have revealed their toxic effects, including hepatotoxicity and reproductive/developmental toxicity in laboratory animals, as reviewed by ATSDR (2009) and Hirata-Koizumi *et al.* (2012). In particular, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the most effective surfactants among PFAAs (Lau *et al.*, 2007), and many toxicological effects of PFOS and PFOA have been revealed (reviewed in ATSDR, 2009, and fully introduced in Hirata-Koizumi *et al.*, 2012). PFOS and PFOA have now been regulated worldwide, and the manufacture, import and use of PFOS were essentially prohibited in the EU in 2008 (DIRECTIVE 2006/122/EC) and in Japan in 2010 (Japanese law, 2009). As with PFOS, there is growing momentum to strengthen the regulation of PFOA.

Perfluoroundecanoic acid (PFUA, C11) is one of the higher homologue chemicals of PFOA, and PFUA is used as an alternative to PFOA, which is used as a processing aid in the manufacture of fluoropolymers (EPA, 2013a). Although the annual production and import volume of PFUA was not available, that of perfluoroalkyl carboxylic acids (PFCAs, C2-C10) in Japan was reported to be 1,000

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to 10,000 tons in 2007 and less than 1,000 tons in 2010 (CHRIP, 2013). The production and import volume of PFUA is considered to have fallen in recent years globally (EPA, 2013b). However, it is necessary to be concerned about the toxicological potential of PFUA even though its production and import volume has been reduced, due to its very persistent and highly bioaccumulative characteristics (ECHA, 2012). Moreover, long-chain (C9-C20) PFCAs can be detected in the environment as degradates from commercial fluorotelomers (Environment Canada, 2010). In humans, total exposure to PFUA is not available, but the mean concentration of PFUA in human serum collected in the U.S. was < 1 ng/ml (Calafat *et al.*, 2006, 2007a and 2007b; Kuklenyik *et al.*, 2004), and the maximum concentration in breast milk was 0.056 ng/ml (So *et al.*, 2006), as summarized by ATSDR (2009). In Sweden, estimated dietary exposure to PFUA increased (88, 158 and 212 pg/kg/day in 1999, 2005 and 2010, respectively) along with an increase in the quantified concentration of PFUA in fish products (Vestergren *et al.*, 2012). Domingo *et al.* (2012) summarized that the major dietary source of the estimated intake of PFUA was fish and shellfish.

In order to obtain initial risk information on the toxicity of PFCAs, which have a longer chain than PFOA (C8), we have carried out a series of screening tests on the toxicity of PFCAs (C11-C18), and the result for perfluorooctadecanoic acid (PFOdA, C18) has been already published (Hirata-Koizumi *et al.*, 2012). Here, we show initial risk information on the repeated dose and reproductive/developmental toxicity of PFUA (C11).

MATERIALS AND METHODS

This study was performed in compliance with OECD guideline 422 "Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test," and in accordance with the principles for Good Laboratory Practice (MOE *et al.*, 2003, 2008) at the BOZO Research Center (Shizuoka, Japan). The experiment was performed in accordance with the Japanese regulations on animal welfare (Japanese law, 2005).

Animals and housing conditions

Crl:CD(SD) rats (8 weeks old) were purchased from Atsugi Breeding Center (Charles River Laboratories Japan, Inc., Kanagawa, Japan). This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimatized to the laboratory for 15 days and subjected to treatment at 10 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. In addition, vaginal smears of each female were recorded, and only females showing a normal estrous cycle were used in the experiment. One day before the initial treatment, the rats were distributed into four main groups of 12 males and 12 females, and two additional satellite groups (control and highest dose groups) of five females, each by stratified random sampling based on body weight. For males, 5/12 animals each in the main groups of control and highest dose were used as the satellite groups.

Throughout the study, animals were maintained in an air-conditioned room set at 20-27°C, with relative humidity set at 31-69%, a 12-hr light/dark cycle, and ventilation with > 10 air changes/hr. A basal diet (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. The rats were housed individually, except for mating and nursing periods. From day 17 of pregnancy to the day of sacrifice, individual dams and/or litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc.).

Chemicals and dosing

PFUA (CAS RN: 2058-94-8) was obtained from Wako Chemical, Ltd. (Miyazaki, Japan), stored in a light-blocking bottle and kept at room temperature. The PFUA (Lot no. TSM0481) used in this study was 98.5% pure, and stability during the study was verified by gas chromatography. The test article was suspended in corn oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and administered to the animals by gastric intubation. Control rats received the vehicle alone. Dosing solutions were prepared at least once per eight days, stored under refrigeration until dosing, and dosed at room temperature, as stability under these conditions has been confirmed. The concentrations of PFUA in the formulations were within the acceptable range (97.0-101.8%).

The dose levels were chosen based on the results of a 14-day dose range-finding study conducted at levels of 2, 6, 20, 60, 200, and 600 mg/kg/day. In this range-finding study, deaths were observed in 5/5 males and 4/5 females at 20 mg/kg/day, and in all animals at 60 mg/kg/day or more, and an increase in liver weight in both sexes and increases in ALP and BUN in males were observed at 2 and 6 mg/kg/day. PFAAs including PFUA are persistent and bioaccumulative (ATSDR, 2009). Taking into account that the length of the dosing period in the present study was about three times than that in the dose range-finding study, the highest dose in the present study was set at 1.0 mg/kg/day. Finally, the dose levels of PFUA in

the present study were set as 0.1, 0.3 or 1.0 mg/kg/day.

Twelve males per group were dosed for 42 days, beginning 14 days before mating. After the administration period, 5 of 12 males per group were reared for the recovery period of 14 days without administration of PFUA, as satellite groups. The main group females were dosed for 41-46 days, beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. Females in the satellite group were given PFUA for 42 days, followed by the recovery period of 14 days. The first day of dosing was designated as day 0 of administration and the day after the final dose was designated as day 0 of the recovery period. The volume of each dose was adjusted to 5 ml/kg body weight based on the latest body weight.

Observations

All rats were observed daily for clinical signs of toxicity. Body weight was recorded twice a week in all males and in the satellite group females, and twice a week during the premating period, on days 0, 4, 7, 11, 14, 17, and 20 of pregnancy and on days 0 and 4 of lactation in main group females. Food consumption was recorded twice a week in all males and in satellite group females, and twice a week during the premating period, on days 1, 4, 7, 11, 14, 17, and 20 of pregnancy and on days 2 and 4 of lactation in main group females. Functional observation battery (FOB) in all animals was recorded once a week during the administration period, as follows: (i) home cage observation; posture, convulsion, and abnormal behavior, (ii) in-the-hand observation; ease of removal from cage and handling, fur and skin condition, eye ball, secretion from nose and/or eye, visible mucous membrane, lacrimation, salivation, piloerection, pupil diameter, and respiration, and (iii) open field observation; arousal, ambulation, posture, shivering, convulsion, rearing frequency, excreta, stereotypical behavior, and abnormal behavior.

Five animals in each group were subjected to the following observations and examinations unless noted otherwise. Sensory reactions for pupillary reflex, approximation reflex, tactile reflex, auditory reflex, pain reflex, righting reflex and width of the landing legs, grip strength of fore and hind limbs, and spontaneous motor activity were tested in main group males on day 37 of administration, in main group females on day 4 of lactation, and in satellite group males and females on day 37 of administration and on day 8 of the recovery period. Fresh urine was sampled from animals using a urine-collecting cage during the last weeks of the dosing and recovery periods. The 4-hr urine samples were collected soon after dosing under fasting (water was allowed *ad libitum*), and the 20-hr urine samples were collected, food and water being allowed *ad libitum*.

After 16-20 hr (overnight) of fasting, the main group of rats was euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 4 of lactation in females, and satellite group rats were euthanized on the day of the completion of the recovery period. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta. Major organs were removed from all animals, and the brain, thyroid, thymus, heart, liver, spleen, kidney, adrenal glands, testis, epididymis were weighed. The numbers of corpora lutea and implantation sites were counted in all main group females. The testes and epididymides were fixed with Bouin's solution and in 10% phosphate-buffered formalin. Other organs were stored in 10% phosphate-buffered formalin. The cerebrum and cerebellum, pituitary gland, spinal cord, sciatic nerve, thyroid, parathyroid, adrenal glands, thymus, spleen, mandibular lymph nodes, mesenteric lymph node, heart, lung, trachea, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, kidney, bladder, testis, epididymis, uterus, seminal vesicle, sternum, and femur were histopathologically evaluated for five males and females in the control and the highest groups, and organs with macroscopically abnormal findings were also examined histopathologically. The organs for histopathological evaluations were processed routinely for embedding in paraffin, and sections were prepared for staining with hematoxylin–eosin. Test substance-related histopathological changes were found in the liver in males and females, and in the stomach in males; therefore, the liver in all animals and the stomach in all males were also examined histopathologically.

The 4-hr urine samples were tested for color, pH, protein, glucose, ketone body, bilirubin, occult blood, urobilinogen, and urinary sediment. Urinary sediment was stained and examined microscopically. The 20-hr urine samples were tested for osmotic pressure. Urine volume for 4-hr and 20-hr was measured. In the collected blood samples the red blood cell (RBC) count, hemoglobin, platelet count, and white blood cell count were measured. In addition, mean corpuscular volume (MCV), hematocrit, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte rate, and differential leukocyte rates were calculated. Prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were determined. Blood chemistry was tested for alkaline phosphatase (ALP), total protein, albumin, albumin/globulin (A/G) ratio, total bilirubin, blood

urea nitrogen (BUN), creatinine, glucose, total cholesterol, triglycerides, phospholipid, Na, K, Cl, Ca, inorganic phosphate, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactase dehydrogenase (LDH), and gamma-glutamyltransferase (γ-GTP).

In the main group, daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the premating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the 2-week mating period had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence of successful mating. Once insemination was confirmed, the females were checked twice a day for signs of parturition from day 21 to day 24 of pregnancy. One female in the 0.1 mg/kg/day treatment group did not deliver and did not have implantation. Because of infertility, data for that female for the period corresponding to gestation were excluded from statistical analysis. Other females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed by 17:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and live pups were sexed and individually weighed on PNDs 0 and 4. Pups were inspected for external malformations on PND 0. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross internal examinations were performed.

Data analysis

Statistical analysis of pups was carried out using the litter as the experimental unit. Mean and standard deviation in each dose group were calculated for body weight, food consumption, water consumption, number of feces, rearing frequency, width of the landing legs, grip strength, spontaneous motor activity, urine volume, hematological test results, blood biochemical test results, absolute and relative organ weights, estrous cycle length, length of gestation, numbers of corpora lutea and implantations, implantation index, total number of pups born, number of male and female pups, number of live and dead pups, live birth index, live pups and viability index on day 4 of lactation, and body weight of pups. These were analyzed with Bartlett's test or F-test for homogeneity of variance. If they were homogeneous, the data were analyzed using Dunnett's test or Student's t-test to compare the mean of the control group with that of each dosage group, and if they were not homogeneous, a Dunnett-type rank test or Aspin-Welch t-test was applied. The copulation index, fertility index, gestation index, sex ratio of pups, and data for sensory reactions of reflexes were analyzed with Yates' chi-square test. The 5% levels of probability were used as the criterion for significance. Unless otherwise noted, there are statistically significant differences in the changes described in the following Results section.

RESULTS

Parental toxicity

No deaths were observed in any of the groups. A decrease in grip strength of the forefoot was observed in males and females at 1.0 mg/kg/day in the recovery period. No other treatment-related effects on clinical signs of toxicity, FOB, sensory reactivity, or spontaneous motor activity were observed in males and females in the main and satellite groups (data not shown).

Body weight changes in each group are shown in Figs. 1 and 2. In males at 1.0 mg/kg/day, body weight gains decreased during the dosing period and during the recovery period. In females at 1.0 mg/kg/day, body weight gains decreased during the lactation period in the main group and during the dosing period and the recovery period in the satellite group, and lowered body weight was observed on days 38 and 41 of the dosing period and on days 0-13 of the recovery period in the satellite group. No effects on body weight in male and female groups were observed at any other dosing. Food consumption (data not shown) was decreased on day 4 of the delivery period at 1.0 mg/kg/day in females. Urinalysis revealed no significant differences in any parameters between the control and treatment groups in males and females in the main and satellite groups (data not shown).

Table 1 shows hematological findings in male and female rats. At 1.0 mg/kg/day, low values of fibrinogen and APTT were observed in males of the main and satellite groups, and a low value of fibrinogen was observed in females of the main group. The other significant changes in hematological findings were incidental because they were slight without related changes or did not occur in a dose-dependent manner.

Blood biochemical findings are shown in Table 2. At 1.0 mg/kg/day in the main group, increases in BUN and ALP and decreases in total protein and albumin were observed in males, and an increase in BUN and a decrease in total protein were observed in females. At 1.0 mg/kg/day in the satellite group, increases in BUN and ALP in males and females, and a decrease in total protein in females were observed. The other changes with statistical significances in blood biochemical findings were incidental because they were slight without related changes or did not occur in a dose-dependent manner.

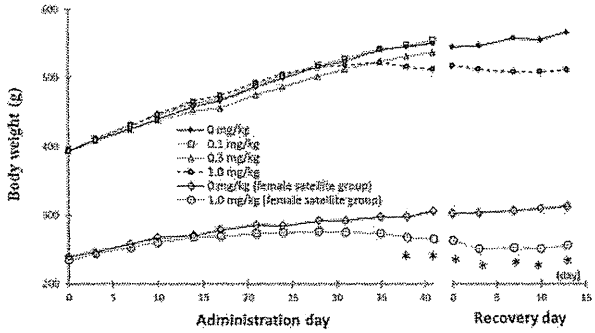

Fig. 1. Body weight of males in main groups and satellite groups for recovery period and females in satellite groups.
*: Significantly different from the control, $p \leqq 0.05$.

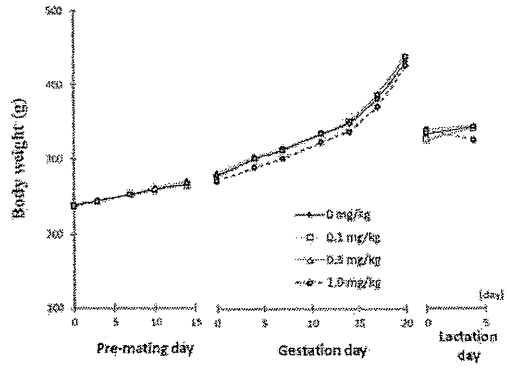

Fig. 2. Body weight of females in main groups.

Organ weights in males and females are shown in Table 3. Relative weight of the liver was increased at 0.3 mg/kg/day in main group males, and absolute and relative weights of the liver were increased in males and females at 1.0 mg/kg/day in main and satellite groups. Absolute and relative weights of the spleen were decreased at 1.0 mg/kg/day in main group males. Enlargement of the liver in two males and a dark red focus in the stomach in three males were observed at 1.0 mg/kg/day in the main group. No other treatment-related findings at necropsy were observed in males and females in main and satellite groups. Histopathological findings are shown in Table 4. Possibly treatment-related changes were observed in the liver and stomach: In the main groups, centrilobular hypertrophy of hepatocytes in males and females were observed at 0.3 mg/kg/day and above, diffuse vacuolation of hepatocytes in males, and minimal focal necrosis in males and females were observed at 1.0 mg/kg/day, and in the satellite groups, minimal diffuse vacuolation of hepatocytes in males, centrilobular hypertrophy/degeneration of hepatocytes in males and females, and Glisson's sheath cell infiltration in females were observed at 1.0 mg/kg/day. In the glandular stomach, minimal erosion was observed in 3/7 males at 1.0 mg/kg/day. Although a similar change was observed in 2/6 control females, the possibility that PFUA treatment affected the stomach in males could not be ruled out. The findings in other organs were considered to be incidental in main and satellite groups, because there was no dose-dependent increase in incidence or severity. On reproductive organs, no treatment-related histopathological changes were found in the epididymides, testis, and uterus in PFUA-treated groups.

Reproductive and developmental findings

There were no significant differences in the mean estrous cycle and in the incidence of females with a normal estrous cycle between the control and PFUA groups either in the main or recovery group (data not shown). The data for reproductive and developmental parameters are shown in Table 5. Reproduction performance of parental rats, delivery and nursing were not significantly different between the control and PFUA-treated groups. Regarding the general appearance of pups, there were no abnormal findings in any groups. The body weights of male and female pups on PNDs 0 and 4 were lowered at 1.0 mg/kg/day. There were no significant differences in the sex ratio of live pups or the viability index on PND 4. At gross pathology in pups on PND 4, thymic remnant in the neck was observed in one male and one female at 0.3 mg/kg/day, and in two females at 1.0 mg/kg/day, and these were considered to be incidental because of the low incidence. There were no other changes in gross internal findings of pups in any PFUA-treated groups.

DISCUSSION

The present study of rats was conducted to examine the possible effects of PFUA on reproduction and development as well as the possible general toxic effects. The dosage of PFUA used in this study was sufficiently high to be expected to induce general toxic effects in parental animals. The following results suggest that the liver is a sensitive target organ. The weight of the liver was increased in males at 0.3 mg/kg/day and above, and in females at 1.0 mg/kg/day, and centrilobular hypertrophy of hepatocytes was observed in both sexes at 0.3 mg/kg/day and above, focal necrosis and/or diffuse vacuolation of hepatocytes were also found in the 1.0

Table 1. Hematological findings

		Main group				Satellite group	
	Group	0 mg/kg/day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	0 mg/kg/day	1.0 mg/kg/day
Males							
Number of animals		5	5	5	5	5	5
WBC	(10^2/μl)	121.2 ± 31.4	94.8 ± 21.1	127.6 ± 35.4	129.8 ± 23.5	73.4 ± 26.8	111.6 ± 19.5*
RBC	(10^4/μl)	830 ± 40	846 ± 25	852 ± 20	869 ± 23	894 ± 34	886 ± 47
HGB	(g/dl)	15.6 ± 0.4	15.7 ± 0.6	15.4 ± 0.4	15.6 ± 0.7	16.0 ± 0.4	15.3 ± 0.9
MCV	fl	52.5 ± 1.8	51.4 ± 1.7	50.6 ± 0.7	50.1 ± 1.4*	50.9 ± 1.5	49.4 ± 1.9
MCH	pg	18.8 ± 0.5	18.6 ± 0.8	18.1 ± 0.4	17.9 ± 0.4*	17.9 ± 0.3	17.3 ± 0.7
Platelet	(10^4/μl)	98.7 ± 3.7	121.4 ± 5.2**	109.2 ± 8.8	111.2 ± 8.8*	107.8 ± 12.4	122.7 ± 18.6
APTT	(sec)	22 ± 4.1	19.2 ± 1.9	20.8 ± 4.2	16.6 ± 0.7*	20.4 ± 1.7	17.2 ± 2.6*
Fibrinogen	mg/dl	294 ± 20	273 ± 35	283 ± 31	200 ± 23**	304 ± 35	245 ± 22*
Females							
Number of animals		5	5	5	5	5	5
WBC	(10^2/μl)	143.4 ± 43.8	128.7 ± 25.4	151.8 ± 33.5	159.2 ± 45.1	58.6 ± 14.9	65.1 ± 13.6
RBC	(10^4/μl)	702 ± 46	680 ± 67	692 ± 50	645 ± 51	830 ± 30	846 ± 56
HGB	(g/dl)	13.1 ± 1.0	13.5 ± 1.0	13.5 ± 1.1	13.2 ± 0.8	15.4 ± 0.4	15.4 ± 1.0
MCV	fl	52.7 ± 1.3	56.7 ± 4.5	55.0 ± 1.2	58.0 ± 3.1*	51.4 ± 1.4	50.1 ± 1.2
MCH	pg	18.6 ± 0.5	20.0 ± 1.6	19.5 ± 0.6	20.5 ± 1.1*	18.6 ± 0.6	18.2 ± 0.6
Platelet	(10^4/μl)	159.4 ± 27.4	141.0 ± 22.7	164.8 ± 19.6	161.8 ± 30.9	130.6 ± 13.7	125.7 ± 18.1
APTT	(sec)	17.6 ± 1.8	17.5 ± 2.4	17.9 ± 2.3	15.2 ± 3.3	17.9 ± 2.3	17 ± 2.9
Fibrinogen	mg/dl	335 ± 53	319 ± 95	282 ± 49	228 ± 42*	207 ± 10	176 ± 31

Values are given as the mean ± S.D.
*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

mg/kg/day group. In rodents, it is clear that the hepatic response to exposure to many perfluoroalkyl compounds is initiated by the activation of the nuclear hormone receptor, PPARα (ATSDR, 2009), and PFUA activates mouse PPARα *in vitro* (Wolf *et al.*, 2012). The hepatic proliferative responses, including an increase in the liver weight and centrilobular hypertrophy of hepatocytes, observed in the present study might have been initiated by the activation of PPARα, although there is a scientific consensus that compounds which are peroxisome proliferators in rodents have little or no effect on human liver (IARC, 1995). Regarding the toxicity of PFAAs, the involvement of mechanisms other than PPARα has been suggested (Peters and Gonzalez, 2011), so further research on the toxicity mechanism of PFUA is desired.

Effects on the body weight of adult males/females and pups were observed only at 1.0 mg/kg/day. In adult animals, suppression of body weight gain was observed in males/females in the administration and/or recovery periods, although not in females in the premating and gestation periods. It is considered that these body weight changes were a direct effect of PFUA because they were not related to food consumption. There is a possibility of maternal-fetal/infant transfer of PFUA, because maternal-fetal transfer and maternal-infant transfer of PFOA through breast milk have been observed in rats (Hinderliter *et al.*, 2005). Because there was no difference in the length of the gestation period in dams dosed at 1.0 mg/kg/day compared to the controls, and because sup-

Table 2. Blood biochemical findings

		Main group				Satellite group	
	Group	0 mg/kg /day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	0 mg/kg/day	1.0 mg/kg/day
Males							
Number of animals		5	5	5	5	5	5
AST	(IU/l)	67 ± 9	70 ± 4	73 ± 17	77 ± 6	62 ± 9	73 ± 12
ALT	(IU/l)	31 ± 3	32 ± 3	34 ± 3	39 ± 7*	31 ± 5	37 ± 5
ALP	(IU/l)	427 ± 12.6	461 ± 85	514 ± 96	1021 ± 179**	379 ± 95	707 ± 152**
Total cholesterol	(mg/dl)	56 ± 14	47 ± 8	34 ± 6**	46 ± 11	55 ± 18	53 ± 13
Triglyceride	(mg/dl)	48 ± 10	70 ± 42	41 ± 9	46 ± 16	52 ± 17	45 ± 27
Phospholipid	(mg/dl)	90 ± 13	82 ± 14	65 ± 9*	87 ± 11	87 ± 19	92 ± 21
BUN	(mg/dl)	13 ± 2	14 ± 3	15 ± 1	21 ± 4**	17 ± 2	23 ± 5*
Na	(mmol/l)	147 ± 2	146 ± 2	147 ± 1	145 ± 1	145 ± 1	143 ± 1**
Cl	(mmol/l)	108 ± 2	108 ± 1	109 ± 1	109 ± 3	107 ± 1	108 ± 1
Ca	(mg/dl)	10.1 ± 0.2	10.0 ± 0.3	10.0 ± 0.3	9.7 ± 0.2*	9.9 ± 0.3	9.5 ± 0.3
Total protein	(g/dl)	6.2 ± 0.2	6.0 ± 0.3	6.1 ± 0.1	5.5 ± 0.3**	6.3 ± 0.1	5.8 ± 0.5
Albumin	(g/dl)	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.0	2.6 ± 0.1*	2.7 ± 0.1	2.8 ± 0.2
A/G		0.80 ± 0.07	0.86 ± 0.03	0.93 ± 0.05**	0.88 ± 0.06	0.77 ± 0.04	0.93 ± 0.09**
Females							
Number of animals		5	5	5	5	5	5
AST	(IU/l)	84 ± 21	92 ± 12	86 ± 15	81 ± 12	59 ± 4	68 ± 11
ALT	(IU/l)	53 ± 9	55 ± 12	50 ± 18	49 ± 1	26 ± 4	28 ± 4
ALP	(IU/l)	219 ± 72	242 ± 42	286 ± 176	263 ± 18	158 ± 28	289 ± 54**
Total cholesterol	(mg/dl)	60 ± 11	52 ± 13	41 ± 13*	49 ± 8	78 ± 16	64 ± 14
Triglyceride	(mg/dl)	54 ± 11	38 ± 12	41 ± 18	60 ± 25	28 ± 11	20 ± 3
Phospholipid	(mg/dl)	112 ± 13	94 ± 18	80 ± 20*	98 ± 11	141 ± 20	108 ± 15*
BUN	(mg/dl)	13 ± 2	13 ± 4	16 ± 3	19 ± 2**	20 ± 3	29 ± 7*
Na	(mmol/l)	141 ± 1	141 ± 2	143 ± 1	142 ± 1	143 ± 1	143 ± 1
Cl	(mmol/l)	106 ± 1	107 ± 2	108 ± 2	108 ± 2*	109 ± 1	112 ± 2
Ca	(mg/dl)	10.3 ± 0.2	10.2 ± 0.4	10.3 ± 0.1	10.0 ± 0.3	10.2 ± 0.3	9.9 ± 0.2
Total protein	(g/dl)	6.2 ± 0.2	5.8 ± 0.3*	6.0 ± 0.1	5.6 ± 0.2**	6.7 ± 0.2	5.8 ± 0.3**
Albumin	(g/dl)	2.8 ± 0.2	2.8 ± 0.2	2.8 ± 0.1	2.7 ± 0.2	3.1 ± 0.2	2.9 ± 0.3
A/G		0.85 ± 0.05	0.92 ± 0.05	0.89 ± 0.07	0.91 ± 0.11	0.87 ± 0.04	1.01 ± 0.09*

Values are given as the mean ± S.D.
*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

pression of body weight gain in females during pregnancy was not observed, the lowered body weight on PND 0 was considered a direct effect of PFUA due to intrauterine exposure. Also in other PFCAs, low values of body weight of pups at birth without effects on the body weight of dams in the gestation period were observed (Butenhoff *et al.*, 2004; Loveless *et al.*, 2009). The lowered body weight on PND 4 was considered to be a direct effect of PFUA by ingestion of breast milk, as well as a secondary effect of PFUA caused by the lowered body weight in

Table 3. Organ weights

Dose (mg/kg/day)		Main Group				Satellite Group	
		0 (control)	0.1	0.3	1.0	0 (control)	1.0
Males							
No. of animals examined		5	5	5	5	5	5
Brain	(g)	2.18 ± 0.08	2.18 ± 0.09	2.15 ± 0.08	2.17 ± 0.08	2.09 ± 0.04	2.14 ± 0.13
	(%)[a]	0.42 ± 0.03	0.4 ± 0.03	0.41 ± 0.01	0.44 ± 0.05	0.39 ± 0.03	0.44 ± 0.03**
Throid[b]	(mg)	22.4 ± 1.8	25.7 ± 2.5	21.1 ± 2.9	22.3 ± 3.5	23.5 ± 3.8	18.7 ± 2.9
	(%)[a]	4.3 ± 0.4	4.7 ± 0.4	4.1 ± 0.6	4.5 ± 0.7	4.4 ± 0.5	3.8 ± 0.4
Thymus	(mg)	297 ± 90	432 ± 173	342 ± 106	260 ± 61	250 ± 80	251 ± 67
	(%)[a]	57 ± 19	79 ± 27	66 ± 21	53 ± 16	47 ± 17	51 ± 11
Heart	(g)	1.52 ± 0.1	1.5 ± 0.2	1.51 ± 0.03	1.38 ± 0.17	1.46 ± 0.17	1.29 ± 0.19
	(%)[a]	0.29 ± 0.02	0.28 ± 0.04	0.29 ± 0	0.28 ± 0.02	0.28 ± 0.02	0.27 ± 0.02
Liver	(g)	15.12 ± 2.14	16.45 ± 2.06	17.54 ± 0.73	20.95 ± 2.56**	14.19 ± 1.56	19.85 ± 3.03**
	(%)[a]	2.88 ± 0.27	3.02 ± 0.19	3.39 ± 0.16**	4.18 ± 0.19**	2.67 ± 0.22	4.07 ± 0.36**
Spleen	(g)	0.84 ± 0.16	0.76 ± 0.09	0.79 ± 0.05	0.65 ± 0.09*	0.72 ± 0.11	0.72 ± 0.04
	(%)[a]	0.16 ± 0.03	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01*	0.14 ± 0.02	0.15 ± 0.01
Kidney[b]	(g)	3.43 ± 0.31	3.44 ± 0.38	3.51 ± 0.08	3.4 ± 0.17	3.51 ± 0.31	3.32 ± 0.43
	(%)[a]	0.65 ± 0.06	0.63 ± 0.04	0.68 ± 0.03	0.68 ± 0.06	0.66 ± 0.03	0.68 ± 0.04
Adrenal[b]	(mg)	64 ± 13	70 ± 8	68 ± 3	58 ± 9	61 ± 9	46 ± 8*
	(%)[a]	12 ± 2	13 ± 1	13 ± 1	12 ± 1	12 ± 2	9 ± 1
Testis[b,c]	(g)	3.34 ± 0.21	3.57 ± 0.26	3.48 ± 0.28	2.98 ± 0.86	3.49 ± 0.26	3.57 ± 0.35
	(%)[a]	0.63 ± 0.07	0.67 ± 0.05	0.68 ± 0.06	0.62 ± 0.17	0.66 ± 0.03	0.74 ± 0.07*
Epididymis[b,c]	(mg)	1339 ± 84	1420 ± 112	1368 ± 199	1578 ± 950	1337 ± 51	1388 ± 87
	(%)[a]	252 ± 21	265 ± 25	268 ± 36	335 ± 220	252 ± 11	288 ± 34
Females							
No. of animals examined		5	5	5	5	5	5
Brain	(g)	1.99 ± 0.05	1.97 ± 0.08	1.98 ± 0.09	2 ± 0.04	1.96 ± 0.09	1.86 ± 0.06
	(%)[a]	0.64 ± 0.04	0.66 ± 0.03	0.65 ± 0.05	0.67 ± 0.06	0.68 ± 0.08	0.78 ± 0.02**
Throid[b]	(mg)	17.2 ± 1.8	19.2 ± 3.2	17.5 ± 3	16.9 ± 0.7	17.2 ± 2.7	14.7 ± 1
	(%)[a]	5.5 ± 0.8	6.5 ± 1.1	5.8 ± 1.1	5.6 ± 0.6	6 ± 1	6.2 ± 0.3
Thymus	(mg)	192 ± 16	170 ± 102	243 ± 82	249 ± 58	245 ± 98	147 ± 59
	(%)[a]	61 ± 4	56 ± 32	79 ± 24	82 ± 14	85 ± 39	62 ± 23
Heart	(g)	1.02 ± 0.08	0.96 ± 0.06	0.92 ± 0.04	0.94 ± 0.11	0.86 ± 0.05	0.73 ± 0.03**
	(%)[a]	0.33 ± 0.03	0.32 ± 0.01	0.3 ± 0.01	0.31 ± 0.02	0.29 ± 0.02	0.31 ± 0.01
Liver	(g)	10.56 ± 0.68	10.61 ± 0.48	10.55 ± 1.48	12.76 ± 1.00**	7.22 ± 0.38	8.63 ± 1.04*
	(%)[a]	3.37 ± 0.12	3.57 ± 0.13	3.46 ± 0.36	4.21 ± 0.15**	2.48 ± 0.14	3.64 ± 0.47**
Spleen	(g)	0.62 ± 0.06	0.65 ± 0.16	0.65 ± 0.1	0.66 ± 0.15	0.49 ± 0.05	0.43 ± 0.05
	(%)[a]	0.2 ± 0.02	0.22 ± 0.05	0.22 ± 0.02	0.21 ± 0.03	0.17 ± 0.02	0.18 ± 0.01
Kidney[b]	(g)	2.24 ± 0.42	1.96 ± 0.18	2.06 ± 0.19	2.05 ± 0.09	1.89 ± 0.14	1.93 ± 0.17
	(%)[a]	0.72 ± 0.14	0.66 ± 0.06	0.68 ± 0.07	0.68 ± 0.04	0.64 ± 0.02	0.81 ± 0.07**
Adrenal[b]	(mg)	82 ± 4	84 ± 10	89 ± 14	80 ± 13	70 ± 8	49 ± 5**
	(%)[a]	26 ± 2	28 ± 5	30 ± 5	26 ± 3	25 ± 5	21 ± 1

Values are given as the mean ± S.D.

[a]: Ratio of organ weight to body weight (relative organ weight). [b]: Values are represented as the total weights of the organs on both sides. [c]: Organ weight was measured for all animals (number of examined animals: 7 at 0 and 1.0 mg/kg/day and 12 at 0.1 and 0.3 mg/kg/day in the main group, and 5 at 0 and 1.0 mg/kg/day in the recovery group.)

*: Significantly different from the control, $p \leqq 0.05$. **: Significantly different from the control, $p \leqq 0.01$.

Table 4. Histopathological findings

	Males						Females					
	Main				Satellite		Main				Satellite	
Dose (mg/kg/day)	0	0.1	0.3	1.0	0	1.0	0	0.1	0.3	1.0	0	1.0
Heart												
Number examined	5	0	0	5			5	0	0	5		
Cardiomyopathy (minimal)	1			1			0			0		
Kidney												
Number examined	5	0	1	5			5	0	0	5		1
Dilatation, pelvic	0		1	0			1			0		1
(minimal)			1									1
(moderate)							1					
Regeneration, tubular	4		1	1			1			1		0
(minimal)	3		1	1			1			1		
(mild)	1											
Liver												
Number examined	7	12	12	7	5	5	12	12	12	12	5	5
Vacuolation, hepatocytes, diffuse	0	0	0	3	0	1	0	0	0	0	0	0
(minimal)				2		1						
(mild)				1								
Necrosis, focal (minimal)	0	0	0	2	0	0	0	0	0	2	0	0
Cell infiltration, Glisson's sheath (mild)	0	0	0	0	0	0	0	0	0	0	0	2
Microgranuloma	4	3	1	2	3	3	1	1	0	2	4	4
(minimal)	4	3	1	2	3	3	1	1		2	4	2
(mild)												2
Degeneration, hepatocytes, centrilobular (minimal)	0	0	0	0	0	3	0	0	0	0	0	3
Hypertrophy, hepatocytes, centrilobular	0	0	3	7	0	5	0	0	1	11	0	5
(minimal)			2						1	8		
(mild)			1	2		3				3		2
(moderate)				5		2						3
Spleen												
Number examined	5	0	0	5			5	0	0	5		
Hematopoiesis, extramedullary (minimal)	4			1			5			4		
Stomach												
Number examined	7	12	12	7	5	5	6	0	0	5		
Erosion, glandular stomach (minimal)	0	0	0	3	0	0	2			0		
Thymus												
Number examined	5	0	0	5			5	1	0	5		
Atrophy, lymphoid (mild)	0			0			0	1		0		
Thyroid												
Number examined	5	0	0	5			5	0	0	5		
Ectopic thymus (minimal)	0			0			0			1		
Cyst, ultimobranchial (minimal)	1			2			2			0		
Testis												
Number examined	5	0	0	5								
Not remarkable	5			5								
Epididymis												
Number examined	5	1	0	5								
Granuloma, spermatic	1	1		1								
(minimal)		1		1								
(mild)	1											
Uterus												
Number examined							5	1	0	5		
Dilatation, lumina (minimal)							0	1		0		

Table 5. Reproductive and developmental parameters

	0 mg/kg/day	0.1 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day
Number of animals (males/females)	12/12	12/12	12/12	12/12
Copulation index (males/females) (%)	100/100	100/100	100/100	100/100
Fertility index (%)	100	91.7	100	100
Gestation index (%)	100	100	100	100
Number of pregnant animals	12	11	12	12
Gestation length (days)	22.0 ± 0.3	22.1 ± 0.5	22.1 ± 0.5	21.7 ± 0.2
Number of corpora lutea	15.8 ± 1.9	16.8 ± 1.8	16.2 ± 1.9	16.2 ± 1.5
Number of implantation sites	14.6 ± 2.0	15.5 ± 3.3	15.0 ± 1.9	15.3 ± 1.6
Implantation index (%)	92.0 ± 5.5	91.0 ± 15.4	92.8 ± 6.0	94.8 ± 4.4
Number of litters	12	11	12	12
Number of live pups on PND 0	13.9 ± 2.2	14.5 ± 3.4	13.1 ± 3.1	13.5 ± 2.2
Live birth index (%)	98.9 ± 2.6	97.3 ± 5.3	93.2 ± 18.2	97.9 ± 4.1
Sex ratio	0.51	0.47	0.55	0.52
Number of live pups on PND 4	13.7 ± 1.9	14.0 ± 3.2	12.8 ± 3.1	13.4 ± 2.2
Viability index (%)	98.5 ± 2.8	97.1 ± 3.3	97.7 ± 5.9	99.4 ± 2.2
Body weight of male pups (g)				
on PND 0	6.7 ± 0.3	6.7 ± 0.6	6.4 ± 0.5	5.8 ± 0.3**
on PND 4	10.5 ± 0.5	10.1 ± 1.8	10.2 ± 1.2	8.5 ± 0.7**
Body weight of female pups (g)				
on PND 0	6.4 ± 0.4	6.3 ± 0.6	6.1 ± 0.6	5.6 ± 0.2**
on PND 4	9.9 ± 0.6	9.7 ± 1.7	9.5 ± 0.8	8.3 ± 0.7**

Values are given as the mean ± S.D.
**: Significantly different from the control, $p \leqq 0.01$.

dams. In the PFOA oral dose study (Abbott *et al.*, 2007), the reduction of postnatal weight gain appeared to depend on PPARα expression.

The elimination rate of PFOA in female rats is approximately 40 times faster than in male rats (ATSDR, 2009). Organic anion transport proteins play a key role in PFCAs (C4 to C10) renal tubular reabsorption (Han *et al.*, 2012), and the slower elimination of PFOA in male rats compared to female rats has been attributed to sex hormone modulation of organic anion transporters in the kidney (ATSDR, 2009). In the present study, there were slight gender differences in the hepatotoxicity of PFUA: liver weight increased in males at 0.3 mg/kg/day and above and in females at 1.0 mg/kg/day, and histopathological findings observed in the 1.0 mg/kg/day groups were more numerous and severer in males than in females. The gender differences in hepatotoxicity observed in the present study are considered to be attributable to faster elimination in female rats, as with other PFCAs.

Increased liver weight and hepatocellular hypertrophy, induced by activation of PPARα, were generally observed in previous studies on PFAAs. Significant peroxisome proliferative activity seems to require a carbon length more than 7 (ATSDR, 2009). In gavage studies of PFAAs in male rats, which are more sensitive than females, the following results were observed; for PFOA (C8), increased liver weight and hepatocellular hypertrophy at 5 mg/kg/day for 28 days (Cui *et al.*, 2009); for perfluorononanoic acid (C9), increased liver weight at 1 mg/kg/day for 14 days (Fang *et al.*, 2012); for perfluorododecanoic acid (C12), increased liver weight at 0.02 mg/kg/day for 110 days (Ding *et al.*, 2009). In the current study of PFUA (C11), increased liver weight and centrilobular hypertrophy of hepatocytes were observed from 0.3 mg/kg/day for 42 days. In consideration of differences in the administration period or doses in these studies, the intensity of the liver toxicity of PFUA (C11) was estimated to be between C9 and C12, suggesting that the toxic potency of PFAAs (C8-C12) increases by lengthening their carbon chain. This is because hydrophobicity, which increases as carbon length increases, seems to favor biliary enterohepatic recirculation, resulting in more protracted toxicity (ATSDR, 2009). In contrast, 42-day administration of PFOdA (C18) increased liver

weight at 200 mg/kg/day but not at 40 mg/kg/day in male rats (Hirata-Koizumi *et al.*, 2012). In comparison with other PFAAs (C8-C12), including PFUA (C11), PFOdA induced liver toxicity at higher doses, and this may be due to the low absorption of PFOdA into the body.

At 1.0 mg/kg/day in the main group, the following effects on hematological and blood biochemical parameters were observed; a decrease in fibrinogen was observed in males and females, but increases in APTT and PT were not observed, suggesting that there would be no toxicologically significant effects on the blood coagulation system; decreases in fibrinogen, total protein and albumin observed in males and/or females may be due to reduced synthesis in the damaged liver; the increase in BUN observed in males and females could be due to increased hepatic protein catabolism, because urinalysis parameters and the gross and microscopic appearance of the kidneys were not changed; and the increase of ALP in males was related to the histopathological findings in the liver. These effects except for the decrease in fibrinogen in females were observed also at the end of the recovery period, and the increase of ALP was observed in females only after the recovery period. Moreover, in histopathological findings, centrilobular degeneration of hepatocytes in both sexes and Glisson's sheath cell infiltration in females were observed only at the end of the recovery period, and in females, centrilobular hypertrophy of hepatocytes was more serious at the end of the recovery period. These results suggest that the whole body elimination of PFUA in rats, as well as other PFCAs, is slow. There are some reports indicating that PFCAs are secreted in bile and undergo extensive reabsorption from the gastrointestinal tract (Kudo *et al.*, 2001; Vanden Heuvel *et al.*, 1991a, 1991b; reviewed in ATSDR, 2009). In general, PFCAs with longer carbon chains (C4-C10) have a longer half-life (Hirata-Koizumi *et al.*, 2012). Although the elimination half-life of PFUA is unknown, the half-life after intravenous injection of perfluorodecanoic acid (PFDeA, C10) in rats was about 40 to 60 days (Ohmori *et al.*, 2003), It is estimated that the half-life of PFUA is longer than the recovery period, 14 days, and it is reasonable that some effects of PFUA appear after the recovery period. The above findings may be effects of PFUA caused by enterohepatic recirculation, which lasted through the dosing and recovery periods. The decrease in grip strength of the forefoot observed in males and females at 1.0 mg/kg/day in the satellite group was considered a secondary effect related to suppression of body weight gain.

In conclusion, the NOAEL for repeated dose toxicity is considered to be 0.1 mg/kg/day based on the observed centrilobular hypertrophy of hepatocytes in both sexes at 0.3 mg/kg/day, and the NOAEL for reproductive/developmental toxicity is considered to be 0.3 mg/kg/day based on the lowered body weight of pups at birth and body weight gain at 4 days after birth inhibited at 1.0 mg/kg/day.

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An antioxidant, N,N′-diphenyl-p-phenylenediamine (DPPD), affects labor and delivery in rats: A 28-day repeated dose test and reproduction/developmental toxicity test

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ABSTRACT

A 28-day repeated dose toxicity test and reproduction/developmental toxicity test for N,N′-diphenyl-p-phenylenediamine (DPPD) were conducted in [Crl:CD(SD)] SPF rats. Male and female rats were dosed with DPPD by gavage for 28 days at 0, 100, 300, or 1000 mg/kg bw/day or for a total of 42–46 days at 0, 8, 50, or 300 mg/kg bw/day. No significant adverse effects were observed in the repeated dose toxicity study up to 1000 mg/kg bw/day in both sexes. In the reproduction/developmental toxicity study, two females showed piloerection, hypothermia, and pale skin; one died and the other showed dystocia on day 23 of pregnancy at 300 mg/kg bw/day. Another female delivered only three live pups at 300 mg/kg bw/day. A significantly prolonged gestation period was observed at 50 and 300 mg/kg bw/day. The NOAELs of repeated dose toxicity and reproduction/developmental toxicity were considered to be 1000 and 8 mg/kg bw/day, respectively.

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1. Introduction

N,N′-diphenyl-p-phenylenediamine (DPPD; CAS: 74-31-7), a gray or dark gray powder, is used as a polymerization inhibitor and antioxidant (HSDB, 2012). The antioxidative activity of DPPD is implemented by the donation of a hydrogen to a radical derivative and breaking the autocatalytic cycle (Chemicalland21, 2012). DPPD is widely used in rubber, oils, and feedstuffs, especially for tires in the rubber industry due to its color and stability (Chemicalland21, 2012; HSDB, 2012). Occupational exposure to DPPD may occur through inhalation and dermal contact with this compound at workplaces where DPPD is produced or used (HSDB, 2012). DPPD was detected at a high rate in leachate samples from landfills containing plastic and rubber waste at concentrations of 0.1–13 ng/L (Hasegawa and Suzuki, 2005) and was found in air samples taken from one location at 0.002–0.009 ng/m^3 (MOE, 2005) in Japan. Therefore, exposure to DPPD via the environment is also anticipated.

The oral acute toxicity of DPPD is low with LD_{50} values of 2370 mg/kg bw in rats (Marhold, 1986) and 18,000 mg/kg bw in mice (Labor Hygiene and Occupational Diseases, 1966). A long-term feeding study also showed the relatively low toxicity of DPPD in rats (Hasegawa et al., 1989). Rats were fed a diet containing 0.5% or 2% of DPPD (194 or 857 mg/kg bw/day in males; 259 or 1024 mg/kg bw/day in females) for 104 weeks, and a dose dependent reduction in body weight gain (not associated with decreased food consumption) and a significant decrease in relative weight of the liver were observed in both sexes. Calcium deposition in the kidney in males was the only significant histopathological change. Erythrocyte count, hemoglobin, and hematocrit were significantly increased in the female treatment groups while they were dose dependently decreased in males. In this study, an autopsy was carried out 8 weeks after the cessation of DPPD administration; therefore, some difficulty exists in interpreting study results.

As for reproductive and developmental effects, a study in the 1950s showed that feeding doses of commercial grade of DPPD at 0.025%, 0.10%, 0.40%, and 1.60% prolonged the gestation period in

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; COX, cyclooxygenase; DPPD, N,N′-diphenyl-p-phenylenediamine; HPV, high production volume; NSAID, non-steroidal anti-inflammatory drug; OECD, Organisation for Economic Co-operation and Development.

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all treatment groups in rats (Oser and Oser, 1956). In this study, female rats were fed DPPD from 2 weeks before mating (a total administration period was not specified). Although fertility was not affected by the DPPD treatment, mortality of pups at birth was increased. In a later study by Marois (1998), daily doses of 20–40 mg of DPPD/animal from the 14th day of pregnancy prolonged the gestation period and caused stillbirths in rats (Marois, 1998). In these comparable studies, the fertility effects of DPPD in males were not assessed, and detailed study methods were not fully described.

DPPD is a high production volume (HPV) chemical with production or importation exceeding 1000 tonnes per year in Organisation for Economic Co-operation and Development (OECD) member countries and is listed in the most recent OECD HPV list for investigation of its environment and human health effects under the OECD Cooperative Chemical Assessment Programme (OECD, 2012). Although some early studies briefly showed DPPD toxicity, further reliable information was necessary to assess the human health effects of DPPD. Therefore, DPPD was selected as a target substance for the Safety Examination of Existing Chemicals in Japan. The present paper reports the results of the repeated dose toxicity screening test and reproductive/developmental toxicity screening test of DPPD in rats.

2. Materials and methods

The 28-day repeated dose study was performed at the Research Institute for Animal Science (RIAS) in Biochemistry & Toxicology (Kanagawa, Japan) in compliance with "the notice on the test method concerning new chemical substances (November 21, 2003, No. 1121002, Pharmaceutical and Food Safety Bureau, MHLW; No.2, Manufacturing Industries Bureau, METI; No. 031121002, Environmental Policy Bureau, MOE)" and "the standard for the test facility conducting tests concerning new chemical substances, etc. (November 21, 2003, No. 1121003 Pharmaceutical and Food Safety Bureau, MHLW; No. 3 Manufacturing Industries Bureau, METI; No. 031121004 Environmental Policy Bureau, MOE)". Animals were treated in accordance with "the regulations for animal experimentation in RIAS" and the test was conducted with the approval of "the Animal Care and Use Committee of RIAS".

The reproduction/developmental toxicity study was performed at the Food and Drug Safety Center, Hatano Research Institute (Kanagawa, Japan) in compliance with OECD Guideline 421 Reproduction/Developmental Toxicity Screening Test, along with the above described notice and standard. Animals were treated in accordance with "the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973)", "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88 of the Ministry of Environment, dated April 28, 2006)", "Guidelines for Proper Conduct of Animal Experiments (June 1, 2006)", and "the Guideline for Animal Experiment in Hatano Research Institute, Food and Drug Safety Center".

2.1. Animals

2.1.1. The 28-day repeated dose study

Male and female [Crl:CD(SD)] SPF rats were purchased from Atsugi Breeding Center, Charles River Japan, Inc., (Kanagawa, Japan). Five-week-old male and female rats (male: 152–172 g; female: 130–147 g) found to be in good health were selected for use. Male and female rats were distributed into four groups on a random basis. Animals were reared on a basal diet, Labo MR Stock; NOSAN corporation (Tokyo, Japan) and water *ad libitum* and were housed individually. Animals were maintained in an air-conditioned room at a room temperature of 21.9–23.0 °C, relative humidity of 55–61%, 12-h light/dark cycle, and 10 and more air changes per hour.

2.1.2. The reproduction/developmental study

Male and female [Crl:CD(SD)] SPF rats were purchased from Atsugi Breeding Center, Charles River Japan, Inc., (Kanagawa, Japan). Ten-week-old male and female rats (male: 370.2–446.9 g; female: 220.4–265.2 g) found to be in good health were selected for use. Vaginal smears of each female were examined, and only females showing a 4-day or 5-day estrous cycle were used. Male and female rats were distributed into four groups on a random basis. Animals were reared on a basal diet, CE-2; CLEA Japan, Inc. (Tokyo, Japan) and water *ad libitum* and were housed individually, except for mating and lactation periods. Animals were maintained in an air-conditioned room at a room temperature of 21.5–23.5 °C, relative humidity of 47–67%, 12-h light/dark cycle, and 15 air changes per hour.

2.2. Chemicals and dosing

2.2.1. The 28-day repeated dose study

DPPD (Lot No. 307605R, purity: 99.87%) was obtained from Seiko Chemical (Tokyo, Japan). Male and female rats (5 or 10 rats/sex/group) were dosed once daily by gastric intubation with DPPD at a dose of 0 (control: methylcellulose), 100, 300, or 1000 mg/kg bw for 28 days. After the dosing period, five rats per each sex at 0 and 1000 mg/kg bw/day were reared for 14 days without administration of DPPD as the recovery groups. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight.

2.2.2. The reproduction/developmental study

DPPD (Lot No. KWR0015, purity 100%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Male and female rats (13 rats/sex/group) were dosed once daily by gastric intubation with DPPD at a dose of 0 (control: sodium carboxymethyl cellulose), 8, 50, or 300 mg/kg bw. Males were dosed for a total of 42 days beginning 14 days before mating, and females were dosed for a total of 42–46 days beginning 14 days before mating to day 4 of lactation throughout mating and gestation periods. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight.

2.3. Observations

2.3.1. The 28-day repeated dose study

The first day of dosing was designated as day 1 of administration. All rats were observed daily for clinical signs of toxicity. Clinical signs in detailed observation in all animals were recorded one day before the administration period and once a week during the administration period. Sensory reactions for a sight reaction, hearing reaction, sense of touch reaction, pain reaction, pupil reflex, and righting reflex were recorded on day 27 of the administration period and on day 13 of the recovery period. Grip strength of fore and hind limbs was tested by a grip strength meter (MK-380R/FR, Muromachi Kikai Co., Ltd., Tokyo Japan) and spontaneous motor activity was recorded by an infrared-ray passive sensor system (SUPERMEX, Muromachi Kikai Co., Ltd., Tokyo Japan) on day 27 of the administration period and on day 13 of the recovery period. Body weight was recorded on days 1, 7, 14, 21, and 28 of the administration period, on days 7 and 14 of the recovery period, and on the day of necropsy. Food consumption was recorded once a week during both administration and recovery periods. Fresh urine was sampled from animals on day 22 of the administration period and on day 8 of the recovery period. Urine samples were tested for color, pH, protein, glucose, ketone bodies, bilirubin, occult blood, and urobilinogen.

Rats were euthanized by exsanguination under anesthesia 1 day after the final administration or 1 day after completion of the recovery period. External surfaces of the rats were examined. Abdomen and thoracic cavities were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta of fasted rats. Collected blood samples were examined for hematology by an automated hematology analyzer (XT-2000i, Sysmex Co., Kobe, Japan) and automatic coagulometer (KC-10A, Amelung, US). Serum biochemistry was tested by an automatic analyzer (JCA-BM8, JEOL, Tokyo, Japan) and automated electrolyte analyzer (NAKL-132, TOA electronics Ltd., Tokyo, Japan). The brain, thymus, heart, liver, spleen, kidney, adrenal gland, thyroid gland, pituitary gland, testis, epididymis, and ovary were isolated and weighed. Histopathological evaluations were performed on these organs in addition to the eye ball, spinal cord, lung, trachea, stomach, intestines, prostate, seminal vesicle, vagina, uterus, urinary bladder, sciatic nerve, lymph nodes, and bone marrow (femur) in control and highest dose groups.

2.3.2. The reproduction/developmental study

The first day of dosing was designated as day 1 of administration or day 1 of the premating period. The day of successful mating was designated as day 0 of the pregnancy period. The day on which parturition was completed by 11:00 was designated as day 0 of the lactation period. All rats were observed daily for clinical signs of toxicity. Body weight was recorded once a week during the administration period, and on the day of autopsy in males, and once a week during the pre-mating and mating periods, on days 0, 7, 14, and 21 of pregnancy, on days 0 and 4 of the lactation period and on a day of autopsy in females. Food consumption was recorded on days 1–2, 7–8, 13–14, 29–30, 35–36, and 41–42 of the administration period in males, and on days 1–2, 7–8, and 13–14 of the pre-mating period, on days 0–1, 7–8, 14–15, and 20–21 of the pregnancy period, and on days 3–4 of the lactation period in females. 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 2-week mating period had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered as evidence of successful mating. Once insemination was confirmed, females were checked for signs of parturition before 11:00 from day 21 of pregnancy. Females were allowed to deliver spontaneously and nurse their pups until day 5 of the lactation period. Litter size and

numbers of live and dead pups were recorded, and live pups were sexed and individually weighed on days 0 and 4 of the lactation period. Pups were inspected for external malformations on day 0 of the lactation period.

Rats were euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 5 of the lactation period in females. External surfaces of rats were examined. Abdomen and thoracic cavities were opened, and gross internal examination was performed. The testis, epididymis, prostate, and seminal vesicle were isolated from all males, and the testis and epididymis were weighed and histopathologically examined. The ovary, uterus, vagina, and mammary gland were isolated, and the ovary was weighed and histopathologically examined. Organs were stored in 10% formalin with 0.1 M phosphate buffer. Organs that showed gross pathological changes were histopathologically examined. The numbers of corpora lutea and implantation sites were counted. On day 5 of the lactation period, pups were euthanized by exsanguination under anesthesia, and gross external and internal examinations were performed.

2.4. Data analysis

To assess the homogeneity of data, parametric data were analyzed with Bartlett's test or the *F*-test. When homogeneity was recognized, data were analyzed using a one-way analysis of variance or the Student's *t*-test. Non-homogeneous data were analyzed with Kruskal–Wallis's rank test or the Aspin–Welch *t*-test. Nonparametric data were analyzed with Kruskal–Wallis's rank test or Mann–Whitney's U test. The Dunnett test or Dunnett type test was used to assess multiple comparisons. Fisher's exact test was used to assess categorical data. Five per cent levels of probability were used as the criterion for significance. Statistical analysis of pups was carried out using the litter as the experimental unit in the reproductive/developmental study.

2.5. Evaluation of bilirubin measurements by the diazo method

In the repeated dose study, bilirubin levels significantly increased without being related to toxicological effects in males. Because both bilirubin and DPPD contain –NH substitutes, the interference of DPPD with bilirubin measurements was anticipated. The interference of DPPD with bilirubin measurements was tested as follows. Serum samples were taken from untreated male rats, and 0.2 mL of DPPD at 0.001, 0.01, 0.1, and 1 mg/mL (1:1 aceton and dimethyl sulfoxide) was added to 0.5 mL serum of rats. In addition, rat liver S9 was added to DPPD at 0.1 mg/mL to test the interference of DPPD metabolites. Bilirubin levels were measured by the diazo method, the same method as that of the repeated dose study.

3. Results

3.1. The 28-day repeated dose study

No deaths were observed in any groups. There were no effects on the clinical observation, detailed clinical observation, sensory function, motor activity, body weight, or hematological findings. Food consumption significantly decreased in the fourth week at 300 mg/kg bw/day and in the third and fourth weeks at 1000 mg/kg bw/day in males (Table 1). Table 2 presents the urinary examination in rats given DPPD at the end of the administration period. Protein levels significantly decreased in all treatment groups, but this was not dose dependent and was considered to be due to spontaneously occurring higher levels in control groups.

As shown in Table 3, no effects were found in the hematological examination in rats dosed with DPPD for 28 days. Table 4 presents serum biochemistry in rats given DPPD at the end of the administration period. Total bilirubin significantly increased in all treatment groups at the end of the treatment period in males, but it was not observed at the end of the recovery period. When DPPD was added to rat serum, bilirubin levels measured by the diazo method increased in a concentration-related manner with or without the rat S9 mix (Table 5). Therefore, increased bilirubin levels in this study were considered to be due to interference by DPPD. In females, γ-GTP significantly decreased (0.63 IU/L) at 1000 mg/kg bw/day at the end of the administration period, but it was within the background data of the facility (0.31–2.06 IU/L) and was not considered to be toxicologically significant. This change was not observed at the end of the recovery period.

Table 6 shows the incidence of histopathological findings in rats. At necropsy, slight hydrometra in the uterus was found in one female at 300 mg/kg bw/day, and dilatation of the lumen was histopathologically observed in the uterus of this female at the end of administration period; however, no gross or histopathological effects in the uterus were observed at 1000 mg/kg bw/day. Relative and absolute weights of the thyroid gland in males and absolute weight of the kidney in females significantly increased at 100 mg/kg bw/day, but histopathological changes were not significantly different in these organs at the end of the administration period. No other effects were observed in organ weights in both sexes. In the histopathological examination, no significant changes were observed in both sexes.

3.2. The reproduction/developmental study

There were no effects on body weight, body weight gain, and food consumption. Neither death nor clinical toxicity was observed in males. One female in the 50 mg/kg bw/day group was sacrificed on day 9 of the administration period for incorrect operation at the time of the dosage. At 300 mg/kg bw/day, two females showed piloerection, hypothermia, and pale skin on day 23 of pregnancy. One of these two females died and the other was sacrificed due to dystocia on day 23 of pregnancy. Another female showing piloerection and pale skin delivered only three live pups. Nesting and nursing were not observed in this female, and this female was sacrificed on day 1 of lactation due to total litter loss. In addition, one female showed piloerection on day 23 of gestation, and another female showed pale skin on day 22 of gestation at 300 mg/kg bw/day. However, no abnormalities were found in their delivery.

No effects were observed in the organ weights of male and female rats given DPPD. The following gross pathological findings were observed in two females who died or were sacrificed on

Table 1
Body weight and food consumption in rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)	Male				Female			
	0	100	300	1000	0	100	300	1000
Body weight (g)								
Day 1	162 ± 6	164 ± 4	163 ± 7	162 ± 6	139 ± 5	138 ± 6	139 ± 7	139 ± 6
Day 7	213 ± 12	211 ± 6	216 ± 10	211 ± 10	162 ± 8	162 ± 5	159 ± 5	161 ± 9
Day 14	272 ± 16	273 ± 8	277 ± 10	268 ± 18	182 ± 11	182 ± 13	180 ± 10	181 ± 10
Day 21	325 ± 21	329 ± 15	329 ± 15	314 ± 24	203 ± 11	213 ± 16	202 ± 13	203 ± 15
Day 28	362 ± 27	368 ± 26	363 ± 14	349 ± 28	223 ± 11	230 ± 23	220 ± 13	220 ± 19
Food consumption (g/rat/day)								
Week 1	30 ± 4	30 ± 2	31 ± 3	29 ± 2	23 ± 3	21 ± 4	23 ± 2	22 ± 3
Week 2	32 ± 3	34 ± 1	33 ± 1	31 ± 3	21 ± 3	24 ± 3	22 ± 4	22 ± 2
Week 3	34 ± 3	36 ± 2	34 ± 2	32 ± 2*	23 ± 2	24 ± 4	22 ± 4	22 ± 3
Week 4	41 ± 4	38 ± 3	35 ± 3*	31 ± 3**	24 ± 3	24 ± 5	24 ± 1	23 ± 2

* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett test).

Table 2
Urinary findings of rats treated with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
No. of animals		5	5	5	5	5	5	5	5
Color	Colorless	1	0	0	0	0	0	0	0
	Pale yellow	4	5	5	5	5	5	5	5
Cloudy	Negligible	5	5	5	5	5	5	5	5
pH	7.0	4	1	2	3	0	0	0	0
	7.5	1	4	3	1	1	2	1	2
	8.0	1	0	0	0	4	1	3	3
	8.5	0	0	0	0	0	2	1	0
Protein[a]	±	0	5**	5**	4*	1	0	1	1
	1+	4	0**	0**	0*	4	4	3	3
	2+	1	0**	0**	1*	0	1	1	1
Occult blood	Negligible	5	5	5	5	5	5	5	5
Urobilinogen	0.1 (ehrlich unit/dL)	5	5	5	5	5	5	5	5
Bilirubin	Negligible	5	5	5	5	5	5	5	5

* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett test).
[a] Protein: ± (15–30 mg/dL), 1+ (30–100 mg/dL), 2+ (100–300 mg/dL).

Table 3
Hematological findings of rats treated with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
RBC	(10^4/μL)	811 ± 37	773 ± 27	773 ± 41	800 ± 70	768 ± 28	739 ± 46	741 ± 57	777 ± 10
Hb	(g/dL)	15.6 ± 0.4	15.2 ± 0.4	15.1 ± 0.4	15.3 ± 1.0	14.9 ± 0.5	14.5 ± 0.5	14.2 ± 0.9	15.0 ± 0.3
Ht	(%)	47.4 ± 1.2	46.4 ± 1.0	46.3 ± 1.6	46.3 ± 2.9	44.4 ± 1.5	43.3 ± 1.4	42.5 ± 2.6	44.6 ± 0.4
MCV	(fL)	58.8 ± 2.4	60.0 ± 1.0	59.8 ± 2.9	58.2 ± 2.4	57.8 ± 1.9	58.6 ± 1.9	57.6 ± 1.9	57.6 ± 1.1
MCH	(pg)	19.3 ± 0.7	19.6 ± 0.3	19.6 ± 0.7	19.1 ± 0.7	19.4 ± 0.6	19.6 ± 0.7	19.2 ± 0.5	19.3 ± 0.3
MCHC	(%)	33.0 ± 0.2	32.7 ± 0.2	32.7 ± 0.5	33.0 ± 0.5	33.6 ± 0.3	33.4 ± 0.5	33.4 ± 0.4	33.6 ± 0.5
Ret.	(‰)	27.7 ± 5.4	28.5 ± 8.2	30.5 ± 6.0	32.4 ± 13.8	19.6 ± 1.8	23.6 ± 3.4	23.1 ± 4.9	19.5 ± 4.4
PT	(s)	13.1 ± 0.5	13.2 ± 0.2	13.1 ± 0.2	13.5 ± 0.4	13.3 ± 0.2	13.1 ± 0.2	13.0 ± 0.4	13.2 ± 0.4
APTT	(s)	20.9 ± 1.8	22.2 ± 1.1	20.5 ± 1.1	22.3 ± 0.6	18.4 ± 1.6	17.6 ± 1.1	17.7 ± 1.1	18.0 ± 0.8
Platelet	(10^4/μL)	141 ± 10	130 ± 8	133 ± 16	150 ± 22	121 ± 16	122 ± 10	124 ± 10	132 ± 15
WBC	(10^2/μL)	76 ± 25	76 ± 21	57 ± 12	69 ± 10	59 ± 23	39 ± 10	38 ± 9	44 ± 8

RBC: Red blood cell; Hb: Hemoglobin; Ht: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; Ret.: Reticulocyte; PT: prothrombin time; APTT: Activated partial thromboplastin time; WBC: White blood cells.

Table 4
Serum biochemistry in rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)		Male				Female			
		0	100	300	1000	0	100	300	1000
No. of animals		5	5	5	5	5	5	5	5
LDH	(IU/L)	358 ± 153	289 ± 92	335 ± 123	349 ± 132	458 ± 119	341 ± 125	463 ± 233	406 ± 120
AST	(IU/L)	71 ± 8	84 ± 23	75 ± 4	72 ± 6	78 ± 8	67 ± 5	77 ± 13	94 ± 57
ALT	(IU/L)	34 ± 1	45 ± 20	38 ± 5	35 ± 2	28 ± 4	29 ± 4	30 ± 4	32 ± 13
ALP	(IU/L)	808 ± 78	819 ± 136	774 ± 52	818 ± 188	579 ± 48	426 ± 62	460 ± 152	452 ± 93
γ-GTP	(IU/L)	0.61 ± 0.27	0.40 ± 0.23	2.34 ± 4.08	0.42 ± 0.10	1.32 ± 0.48	0.81 ± 0.39	1.35 ± 0.08	0.63 ± 0.25*
T. protein	(g/dL)	5.82 ± 0.30	5.76 ± 0.29	5.82 ± 0.21	5.79 ± 0.11	5.96 ± 0.27	5.85 ± 0.10	5.88 ± 0.26	5.90 ± 0.18
Albumin	(g/dL)	2.90 ± 0.30	2.73 ± 0.23	2.86 ± 0.22	2.99 ± 0.17	3.12 ± 0.29	3.00 ± 0.09	3.06 ± 0.28	3.14 ± 0.18
Albumin/Globulin		0.99 ± 0.10	0.90 ± 0.06	0.97 ± 0.09	1.07 ± 0.09	1.11 ± 0.14	1.05 ± 0.06	1.09 ± 0.12	1.13 ± 0.07
T. cholesterol	(mg/dL)	62 ± 10	79 ± 19	81 ± 7	64 ± 13	79 ± 7	91 ± 15	75 ± 14	74 ± 10
Triglycerides	(mg/dL)	68 ± 26	64 ± 8	51 ± 17	56 ± 12	27 ± 11	27 ± 7	24 ± 10	18 ± 3
Glucose	(mg/dL)	152 ± 14	155 ± 11	145 ± 8	146 ± 7	128 ± 8	139 ± 7	133 ± 9	138 ± 22
BUN	(mg/dL)	14.4 ± 1.4	13.3 ± 1.8	12.3 ± 2.3	13.0 ± 1.2	15.5 ± 2.1	13.9 ± 2.2	14.0 ± 2.3	15.6 ± 3.2
Creatinine	(mg/dL)	0.38 ± 0.02	0.37 ± 0.04	0.40 ± 0.02	0.41 ± 0.03	0.46 ± 0.06	0.39 ± 0.05	0.39 ± 0.04	0.41 ± 0.06
T. bilirubin[a]	(mg/dL)	0.33 ± 0.05	0.53 ± 0.05**	0.60 ± 0.10**	0.61 ± 0.09**	0.26 ± 0.05	0.26 ± 0.01	0.29 ± 0.03	0.29 ± 0.04
Calcium	(mg/dL)	9.9 ± 0.5	9.8 ± 0.1	9.8 ± 0.2	9.8 ± 0.3	9.6 ± 0.4	9.5 ± 0.3	9.3 ± 0.2	9.5 ± 0.3
Phosphorus	(mg/dL)	8.2 ± 0.5	8.6 ± 0.4	8.4 ± 0.6	8.5 ± 0.6	7.0 ± 0.9	7.3 ± 0.5	6.8 ± 0.7	7.0 ± 0.5
Sodium	(mEq/L)	146 ± 1	147 ± 1	147 ± 0	146 ± 1	146 ± 1	147 ± 1	148 ± 2	147 ± 2
Potassium	(mEq/L)	4.93 ± 0.46	4.97 ± 0.16	5.15 ± 0.28	5.37 ± 0.41	5.04 ± 0.38	4.96 ± 0.52	4.94 ± 0.33	4.90 ± 0.32
Chloride	(mEq/L)	104 ± 2	105 ± 1	104 ± 2	105 ± 1	108 ± 1	108 ± 2	108 ± 3	108 ± 0

LDH: lactate dehydrogenase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.
* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett type test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett type test).
[a] Interference of DPPD with bilirubin measurements in male rats is described in Sections 3 and 4 (see Table 5 also).

Table 5
Total bilirubin levels in male rat serum with or without the S9 mix measured by the diazo method.

DPPD (mg/mL)	Total bilirubin (mg/dL)	Total bilirubin (mg/dL)
	Without the S9 mix	With the S9 mix[a]
0 (serum)	0.28	0.34
0 (serum and vehicle[b])	0.33	0.35
0.001	0.32	
0.01	0.37	
0.1	1.03	1.04
1	1.17	

[a] Rat liver S9.
[b] Vehicle: acetone and dimethyl sulfoxide (1:1).

day 23 of pregnancy: hemorrhage in the lumen of the uterus, incomplete retention and red color in the lung, and dark red medulla and hardness on the kidney in both animals; hydrothorax in the thoracic cavity, attachment of red content in mucosa of the glandular stomach and recessed area, or red spots in the duodenum in either animal. In the histopathological examination, slight hemorrhage in the endometrium, and very slight edema, very slight foam cell accumulation in alveolus, and very slight capillary fibrinous thromboses in the lung were observed in the two females. The histopathological examination revealed no toxicological effects in other males and females.

Table 7 shows reproductive and developmental findings in rats given DPPD. One female at 8 mg/kg bw/day did not deliver pups by day 25 of gestation. An autopsy on day 26 of gestation revealed no implantations in this female. This female was excluded from the statistical evaluation of pregnant females. No changes attributable to the chemical were noted in the number of mated pairs, number of copulated pairs, copulation index, number of fertile males, fertility index, length of estrus cycle, pairing days until copulation, number of corpora lutea, number of implantations, implantation index, and number of pregnant females. Gestation lengths were significantly longer than the control group at 50 and 300 mg/kg bw/day.

Although no statistical significance was observed, the number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation dose dependently decreased. The number of live pups and viability index were also decreased on day 4 of lactation in treatment groups, especially at 300 mg/kg bw/day. No changes were observed in littef weights and body weights of pups on days 0 and 4 of the lactation period. No gross external or internal abnormalities were observed in pups.

4. Discussion

In the repeated dose study, no deaths were observed in any of the groups; there were no effects on the clinical observation, detailed

Table 6
Incidence of histopathological findings of rats dosed with DPPD by gavage for 28 days.

Dose (mg/kg bw/day)	Grade	Administration period				Recovery period	
		0	100	300	1000	0	1000
Male							
No. of animals		5	0	0	5	0	0
Lung							
Arterial mineralization	+	0	–	–	1	–	–
Foam cell accumulation	+	1	–	–	1	–	–
Heart							
Myocardial degeneration/fibrosis	+	0	–	–	1	–	–
Liver							
Microgramuloma	+	1	–	–	0	–	–
Extramedullary hematopoiesis	+	1	–	–	0	–	–
Kidney							
Hyaline droplet in the proximal tubular epithelium	+	4	–	–	5	–	–
Basophilic tubule	+	1	–	–	2	–	–
Thymus							
Hemorrhage	+	1	–	–	1	–	–
Spleen							
Extramedullary hematopoiesis	+	5	–	–	5	–	–
Deposition of a brown pigment	+	5	–	–	5	–	–
Prostate							
Interstitial lymphocytic infiltration	+	1	–	–	0	–	–
Female							
No. of animals		5	0	0	5	5	5
Lung							
Arterial mineralization	+	1	–	–	2	–	–
Osseous metaplasia	+	1	–	–	0	–	–
Liver							
Microgramuloma	+	2	–	–	1	–	–
Kidney							
Basophilic tubule	+	1	–	–	2	–	–
Solitary cyst	+	1	–	–	1	–	–
	++	1	–	–	0	–	–
Thymus							
Hemorrhage	+	0	–	–	1	–	1 (1)
Spleen							
Extramedullary hematopoiesis	+	5	–	–	5	–	–
Deposition of a brown pigment	+	5	–	–	5	–	–
Pituitary							
Remnant of Rahke's pouch	+	0	–	–	1	–	–
Utreus							
Dilatation of the lumen	+	0	–	1 (1)	0	–	–

Grade (+: slight change; ++: mild change; –: not applicable).
Parentheses indicate the number of rats examined.

Table 7
Reproductive and developmental findings in rats dosed with DPPD by gavage in the reproduction/developmental toxicity study.

Dose (mg/kg bw/day)	0	8	50	300
Number of mated pairs	13	13	12	13
Number of copulated pairs	13	13	12	13
Copulation index	100.0	100.0	100.0	100.0
Number of fertile males	13	12	12	13
Fertility index	100.0	92.3	100.0	100.0
Length of the estrous cycle in the pre-treatment period (days)	4.1 ± 0.3 (13)	4.2 ± 0.4 (13)	4.3 ± 0.5 (12)	4.1 ± 0.3 (13)
Length of the estrous cycle in the treatment period (days)	4.0 ± 0.0 (13)	4.1 ± 0.3 (13)	4.3 ± 0.5 (12)	4.2 ± 0.4 (12)
Pairing days until copulation	2.4 ± 1.3	2.7 ± 1.3	2.8 ± 1.5	2.7 ± 1.3
Number of corpora lutea	17.8 ± 2.2 (13)	18.4 ± 3.3 (12)	17.3 ± 1.3 (12)	16.9 ± 1.3 (11)
Number of implantations	15.9 ± 1.5 (13)	16.3 ± 2.7 (12)	16.2 ± 1.0 (12)	15.8 ± 1.9 (11)
Implantation index	90.7 ± 11.9 (13)	89.6 ± 16.3 (12)	94.0 ± 5.7 (12)	93.5 ± 8.1 (11)
Number of pregnant females	13	12	12	13
Number of pregnant females with live pups	13	12	12	11
Gestation length (days)	22.4 ± 0.5 (13)	22.8 ± 0.5 (12)	23.0 ± 0.0** (12)	23.0 ± 0.4** (11)
Day 0 of lactation				
Number of pups born	14.8 ± 2.1 (13)	14.8 ± 3.1 (12)	14.3 ± 1.5 (12)	13.7 ± 3.1 (11)
Delivery index	92.5 ± 7.5 (13)	90.7 ± 8.2 (12)	88.3 ± 8.7 (12)	86.7 ± 16.1 (11)
Number of live pups	14.7 ± 2.1 (13)	14.4 ± 2.7 (12)	13.8 ± 1.5 (12)	12.8 ± 4.1 (11)
Sex ratio	44.3 ± 18.3 (13)	39.4 ± 12.1 (12)	47.6 ± 14.1 (12)	48.1 ± 13.2 (11)
Birth index	92.1 ± 7.9 (13)	88.4 ± 7.1 (12)	85.8 ± 10.1 (12)	81.2 ± 24.7 (11)
Live birth index	99.5 ± 1.7 (13)	97.7 ± 5.4 (12)	97.2 ± 5.3 (12)	92.0 ± 20.7 (11)
Day 4 of lactation				
Number of live pups	14.5 ± 1.9 (13)	13.9 ± 2.6 (12)	13.8 ± 1.4 (12)	12.2 ± 5.0 (11)
Sex ratio	44.7 ± 18.2 (13)	39.4 ± 12.2 (12)	47.9 ± 14.3 (12)	48.0 ± 14.6 (10)
Viability index	99.1 ± 2.2 (13)	97.0 ± 8.5 (12)	99.5 ± 1.8 (12)	87.5 ± 30.0 (11)

Parentheses indicate the number of dams.
Copulation index = (number of copulated pairs/number of mated pairs) × 100%.
Fertility index = (number of fertile males/number of copulated pairs) × 100%.
Delivery index = (number of pups born/number of implantations) × 100%.
Birth index = (number of live pups on day 0/number of implantations) × 100%.
Live birth index = (number of live pups on day 0/number of pups born) × 100%.
Sex ratio = (number of male live pups/number of live pups) × 100%.
Viability index on day 4 of lactation = (number of live pups on day 4/number of live pups on day 0) × 100%.
* Significantly different from the control group ($p < 0.05$, Kruskal–Wallis followed by the Dunnett type test).
** Significantly different from the control group ($p < 0.01$, Kruskal–Wallis followed by the Dunnett type test).

clinical observation, sensory function, motor activity, body weight, urinary examination, hematological findings, organ weights, or histopathological findings. In the blood chemistry examination, total bilirubin levels significantly increased in all treatment groups at the end of the treatment period in males; however, bilirubin and urobilinogen levels in urine did not increase. In addition, no related effects such as histopathological changes in the liver were observed. Because both bilirubin and DPPD contain –NH substitutes, the interference of DPPD with bilirubin measurements was anticipated. When DPPD was added to rat serum, bilirubin levels measured by the diazo method increased in a concentration-related manner with or without the rat S9 mix. Therefore, increased bilirubin levels in the present study were considered to be due to interference by DPPD. However, it is of interest that these effects were not observed in females.

Information on absorption, distribution, metabolism, and excretion (ADME) of DPPD is available in male rats (Umeniwa et al., 1985). DPPD dosed by an intraduodenal route was rapidly metabolized to DPPD glucuronide and was also suggested to be metabolized to hydroxylated DPPD. After a 6-day oral dosing, the total fecal excretion of DPPD was 55.4% (unchanged) and total urinary excretion of unchanged DPPD and glucuronide DPPD was 0.04%; unchanged DPPD was temporary detected in fat tissues. There is no information available on sex differences for the ADME of DPPD, and it is difficult to predict whether sex differences do indeed exist. Results of the present study may suggest that detectable DPPD or DPPD metabolites by the diazo method were very low in the serum of female rats for some reason.

In the reproduction/developmental toxicity study presented here, no effects were observed in male fertility function. The number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation dose dependently decreased, but they were not significant. No changes were observed in litter weights and body weights of pups on days 0 and 4 of the lactation period. We confirmed that gavage doses of DPPD significantly prolonged the gestation period in rats.

Marois (1998) investigated a possible mechanism of the prolonged gestation period caused by DPPD. When prostaglandin $F_{2\alpha}$, a regulator of uterus contraction, was injected into rats given 40 mg DPPD from the 14th day of pregnancy, observed adverse effects decreased. Prostaglandin E production was markedly inhibited by DPPD in rabbit kidney medulla slices (Fujimoto et al., 1984; Fujita et al., 1982). Prostaglanidins E_2 and $F_{2\alpha}$ induce uterus contraction (Parkington et al., 1999), and the prolonged gestation period was considered to be due to low prostaglandins levels caused by DPPD administration, similar to non-steroidal anti-inflammatory drugs (NSAIDs). If DPPD acts like NSAIDs, adverse effects such as gastrointestinal disturbances, antiplatelet activity, and kidney failure, known side effects of NSAIDs (Ejaz et al., 2004), can be caused by repeated doses of DPPD.

Prostaglandins are synthesized from arachidonic acid by cyclooxygenase (COX). Prostaglandins play an important role in modulating mucosal integrity and various functions of the gastrointestinal tract, and NSAIDs are known to damage the gastrointestinal tract by reducing these functions (Al-Saeed, 2012; Takeuchi et al., 2010). In the reproduction/developmental toxicity study, hemorrhage in the stomach and duodenum were observed in dead or sacrificed dams at 300 mg/kg bw/day, but no toxicologically significant effects were observed in food consumption. In the repeated dose study, food consumption significantly decreased at 300 and 1000 mg/kg bw/day in males; there is a possibility that DPPD affected the gastrointestinal tract in males. However, these changes

were not considered to be toxicologically significant because of high food consumption in the control group, no differences in body weights, and no gross- or histo-pathological effects in the gastrointestinal tract in the repeated dose study. This result was consistent with a 2-year feeding study in which no histopathological effects were found in the gastrointestinal tract (Hasegawa et al., 1989).

Prostaglandins also regulate platelet aggregation, and NSAIDs are known to inhibit platelet aggregation (Fabre et al., 2001). In the reproduction/developmental toxicity study, pale skin and hemorrhage in the uterus, stomach, and duodenum were observed in dead or sacrificed dams at 300 mg/kg bw/day. It is questionable if these observations may suggest inhibitory effects of platelet aggregation. In the repeated dose study, slight hydrometra in the uterus was observed in one female at 300 mg/kg bw/day at the end of administration period, but it was not dose dependent. In addition, heamorrhage in the thymus in one female was observed at 1000 mg/kg bw/day at the end of recovery period in the repeated dose study, but it was not observed at the end of administration period. Therefore, hydrometra in the uterus and heamorrhage in the thymus observed in the repeated dose study were considered to be incidental.

Gavage doses of DPPD showed weaker effects than a previously reported feeding dose study. In a feeding study by Oser and Oser (1956), the mean gestation period was significantly longer [22.9 days (22–24 days), 24.1 days (22–25 days), 25.2 days (23–29 days), and 24.7 (22–27 days) at 0.025, 0.10, 0.40, and 1.60% (7, 28, 113, and 450 mg/kg bw/day: conversion data from RTECS)] than that of the control group [22.1 days (21–23 days)] (Oser and Oser, 1956). An increased gestation length was associated with higher birth weights due to the longer growth period in the uterus and resulted in dystocia and stillbirths in the feeding study. It was considered that feeding doses of DPPD continuously inhibited prostaglandin synthesis, while gavage doses allowed prostaglandin synthesis intermittently.

In the 28-day repeated dose study, neither deaths nor dose-related adverse effects were observed up to 1000 mg/kg bw/day (the highest dose tested) in both sexes. Therefore, the NOAEL of repeated dose toxicity was considered to be 1000 mg/kg bw/day in rats. In the reproduction/developmental toxicity study, no adverse effects were found in male reproduction up to 300 mg/kg bw/day (the highest dose tested). However, significant longer gestation length was observed at 50 and 300 mg/kg bw/day in dams, and the NOAEL of reproduction/developmental toxicity was considered to be 8 mg/kg bw/day in rats.

Although a reproductive toxicity study is important for risk assessment, sometimes it is not conducted by predicting the effects from available repeated dose studies. When low reproductive toxicity is expected from repeated dose studies, only a prenatal developmental toxicity study can be conducted to observe the developmental effects of chemicals, but effects on fertility and parturition are not observed in this study. In case of DPPD, the results of a long term feeding study in rats (Hasegawa et al., 1989), and the current repeated dose study indicated very low toxicity; it could be expected that DPPD is unlikely to cause reproductive effects. However, our reproductive/developmental toxicity study showed a huge discrepancy in NOAEL with these repeated dose studies. Our experience suggests that conducting a reproduction/developmental study, which includes mating and parturition, is important for the risk assessment of reproductive toxicity.

In conclusion, the results of the current study sufficiently provide initial toxicity data for repeated dose and reproduction/developmental toxicities of DPPD. The NOAEL of repeated dose toxicity was considered to be 1000 mg/kg bw/day based on no adverse effects. The NOAEL of reproduction/developmental toxicity was considered to be 8 mg/kg bw/day based on a longer gestation length at 50 and 300 mg/kg bw/day.

Conflict of Interest

None of the authors have any conflicts of interest associated with this study.

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Reproductive and developmental toxicity screening test of 3-cyanopyridine in rats

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ABSTRACT

Crl:CD(SD)rats were given 3-cyanopyridine by gavage at 0, 5, 30 or 180 mg/kg/day. Males were dosed for 42 days beginning 14 days before mating, and females for 40–53 days beginning 14 days before mating to day 3 of lactation, including throughout the mating and gestation periods. General toxicity, mainly liver damage, was observed in males at ≥30 mg/kg/day and in females at ≥5 mg/kg/day. Sertoli cell vacuolation was observed at 180 mg/kg/day, and spermatocyte damages were observed at ≥30 mg/kg/day. Effects on estrous cycles, corpora lutea and implantations, and unsuccessfully mated females, despite additional mating, were observed at 180 mg/kg/day. Delayed initiation of delivery, dystocia, and deaths or moribundities of pregnant females were observed at 180 mg/kg/day, and only two pregnant rats delivered live pups at that dose. The NOAEL for reproductive/developmental toxicity was concluded to be 30 mg/kg/day.

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1. Introduction

3-Cyanopyridine (CAS No. 100-54-9) is a white to yellowish white crystalline solid with a characteristic odor [1]. In Japan, the annual production and import volume of 3-cyanopyridine was reported to be from 100 to 1000 tons in 2009 [2], and probably greater than 2.27 tons in the US [3]. The major uses of this chemical are as raw material for drugs and pesticides [1]. 3-Cyanopyridine's production and use in organic synthesis may result in its release to the environment through various waste streams [1]. There are no data available on the actual exposure levels at present. The possibility of human exposure to 3-cyanopyridine has aroused concern regarding its toxicological potential.

Only limited information is available about the toxicity of 3-cyanopyridine. It was reported that the range of oral LD50 values was 1185–1680 mg/kg in rats [4–6] and 1225 mg/kg in mice [7], and 3-cyanopyridine absorbed through intact skin caused the death of rabbits [6]. 3-Cyanopyridine showed eye irritation [6,8] and irritation of damaged skin [6], but did not irritate intact skin [9] in rabbits. Since there is insufficient information on toxicity, this chemical was selected as an object substance in an existing chemical testing program by the Japanese government [10]. In this program, an acute toxicity test, a repeated dose 28-day oral toxicity study, a bacterial reverse mutation test, and an *in vitro* mammalian chromosome aberration test were performed according to OECD test guidelines [10]. The results are briefly summarized as follows: Oral LD50s were 1475 mg/kg for male rats and 1455 mg/kg for female rats. In the 28-day repeated orally dose toxicity test with rats at doses of 0, 5, 30 and 180 mg/kg/day, the no observed effect level is considered to be 5 mg/kg/day based on increased liver and kidney weights, centrilobular hypertrophy of hepatocytes, and hyaline droplets in proximal tubules observed at 30 mg/kg/day or more. In adding to these tests in the existing chemical testing program by the Japanese government [10], a reproduction/developmental toxicity screening test was performed according to OECD test guideline 421, because the evaluation of reproductive and developmental toxicity is essential in the risk assessment of chemicals. In this paper, we report the data of the reproduction/developmental toxicity screening test of 3-cyanopyridine.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 "Reproduction/Developmental Toxicity Screening Test" [11], and in accordance with the principles for Good Laboratory Practice [12,13] at the Safety Research Institute for Chemical Compounds (Sapporo, Japan). The experiment was approved by the Animal Care and Use Committee of the Safety Research Institute for Chemical Compounds, and was performed in accordance with the ethics criteria contained in the bylaws of the Committee.

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