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Table 5 Summary of historical control data on developmental toxicity studies in rodents

Animals	Crlj:CD(SD) rats	Crl:CD(SD) rats		Jcl:SD rats		Slc:SD rats	Crlj:WI rats		Jcl:Wistar rats	Wistar Hannover rats	Crlj:CD1(ICR) mice	Slc:Syrian hamsters
Year	1994–2000	1994–2000	2001–2010	1994–2000	2001–2005	1995–1997	1997–1999	2002–2009	2001	2001–2010	2000–2009	1999
Pregnancy rate (%)	91.3–98.5	93.0–100	95.8–100	90.6–100	95.8–97.5	95.0	95.0–95.5	95.0–98.3	91.7	87.2–100	72.7–100	88.9
No. corpora lutea	16.4–18.7	14.6–17.4	14.9–16.6	16.7–18.4	17.1–19.9	15.7	16.4–17.9	17.3–17.6	15.9	11.5–14.1	13.1–15.6	15.6
No. implantations	14.7–16.8	13.7–15.6	14.1–15.0	16.1–16.8	16.2–17.0	14.7	15.7–16.4	16.1–16.4	14.9	9.6–12.7	11.6–14.6	14.7
No. live fetuses	13.9–15.8	12.9–14.9	13.2–14.3	14.9–15.6	15.3–15.7	13.3	15.2–15.5	15.2–15.3	13.5	9.0–12.2	11.2–14.1	12.1
Fetal mortality (%)	4.3–8.1	3.8–7.2	3.6–6.2	4.9–7.7	5.8–7.1	5.6	3.5–5.4	5.7–7.2	9.4	4.2–7.9	3.2–9.3	17.6
Incidence of fetuses with external malformations (%)†	0.04–0.53	0–0.36	0.05–0.18	0–0.27	0–0.16	0.13	0–0.34	0–0.11	0	0–0.59	0–0.36	1.44
Incidence of fetuses with visceral malformations (%)†	0.45–16.57	0–11.09	0.32–8.27	0–11.93	0.58–5.05	20.16	1.45–15.09	0.71–8.88	0	0–19.28	0–15.17	2.27
Incidence of fetuses with skeletal malformations (%)†	0–3.97	0–8.02	0.10–0.56	0–1.07	0–1.12	0.49	0–4.00	0	1.29	0–24.49	0–2.02	4.79
Incidence of fetuses with skeletal variations ('%)†	3.60–8.36	6.98–22.98	9.42–17.63	30.60–62.37	38.55–43.45	6.85	18.00–43.11	13.91–36.99	11.61	31.56–67.35	33.16–64.71	78.77

Data are expressed as minimum and maximum values.

†The incidence of fetuses with malformations is expressed as a proportion of the total number of fetuses with malformations to the total number of fetuses examined.

Table 6 Mating and cesarean section data from Jcl:SD, Slc:SD, and Crlj:WI rats between 1994 and 2000

Strain	Jcl:SD	Jcl:SD	Jcl:SD	Jcl:SD	Jcl:SD	Slc:SD	Crlj:WI	Crlj:WI
Year	1994–2000	1994–1997	1997–2000	1994	1998	1995–1997	1999	1998
Treatment†	V	V	V	V	V	V	V	V
Feed	CA-1	CE-2	MF	NMF	CRF-1	NMF	NMF	CRF-1
No. dams	216	76	48	24	19	57	21	19
No. experiments	11	4	2	1	1	3	1	1
No. dams/experiment	18–23	16–21	24	24	19	19–19	21	19
Pregnancy rate (%)	96.8 (90.0–100)	90.6 (80.0–100)	100	100	95.0	95.0	95.5	95.0
Gestation day (hour) of the cesarean section	21 (9:00–12:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (13:30–16:00)	21 (8:00–11:00)	20 (13:30:16:00)	20 (13:00–16:00)	20 (8:00–11:00)
No. corpora lutea	18.4 (17.2–19.5)	17.9 (16.5–18.8)	16.7 (16.6–16.8)	17.3	18.3	15.7 (15.1–16.7)	17.9	16.4
No. implantations	16.8 (15.8–17.9)	16.5 (15.3–17.5)	16.2 (15.9–16.4)	16.8	16.1	14.7 (14.6–14.9)	16.4	15.7
No. live fetuses	15.5 (14.4–16.4)	15.3 (14.4–16.0)	14.9 (14.5–15.3)	15.6	15.2	13.3 (13.3–14.5)	15.5	15.2
Fetal mortality (%)‡	7.7 (4.0–11.0)	7.0 (5.3–8.5)	7.6 (6.7–8.5)	6.7	4.9	5.6 (2.9–8.9)	5.4	3.5
Body weight (g)								
All fetuses	5.17 (5.06–5.33)	4.11 (4.04–4.19)	3.94 (3.79–4.07)					
Male	5.30 (5.17–5.51)	4.20 (4.13–4.24)	4.07 (4.07–4.07)	4.13	5.77	3.98 (3.92–4.03)	4.20	3.95
Female	5.03 (4.90–5.16)	4.00 (3.93–4.08)	3.80 (3.79–3.81)	3.91	5.40	3.79 (3.73–3.85)	4.00	3.77

†V, Vehicle-treated.
‡(Number of early resorptions and late fetal deaths/number of implantations) × 100.
Minimum and maximum values from independent experiments are given in parentheses.

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Table 7 Mating and cesarean section data from Jcl:SD, Crlj:WI, Jcl:Wistar, and Wistar Hannover rats between 2001 and 2010

Strain	Jcl:SD	Jcl:SD	Crlj:WI	Crlj :WI	Jcl:Wistar	BrHan: WIST@Jcl (GALAS)	BrlHan: Wist@Jcl (GALAS)	BrlHan: WIST@Jcl (GALAS)	BrlHan: WIST@Jcl (GALAS)	Crl:WI(Han)	Crl:WI(Han)	RccHan: WIST
Year	2001–2003	2005	2002–2009	2007	2001	2002–2010	2001–2010	2004	2009	2010	2001	2010
Treatment†	V	V	V	V	V	V	V	V	V	V	V	N
Feed	NMF	MF	NMF	CE-2	MF	MF	CRF-1	CE-2	CE-2	CRF-1	CE-2	NMF
No. dams	39	24	59	19	24	191	134	20	19	41	19	79
No. experiments	2	1	3	1	1	8	6	1	1	1	1	1
No. dams/experiment	19–20	24	19–20	19	24	23–24	21–23	20	19	41	19	79
Pregnancy rate (%)	97.5 (95.0–100)	95.8	98.3 (95.0–100)	95.0	91.7	97.4 (95.8–100)	95.8 (91.7–100)	100	95.0	87.2	95.0	98.8
Gestation day (hour) of the cesarean section	20 (13:00–16:00)	20 (9:00–12:00)	20 (13:00–16:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (9:00–16:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (9:00–11:30)	20 (9:00–12:00)	20 (13:00–16:00)
No. corpora lutea	19.9 (18.9–20.8)	17.1	17.3 (16.4–18.1)	17.6	15.9	13.8 (13.3–14.4)	13.2 (12.6–14.1)	13.6	14.1	11.5	13.4	13.8
No. implantations	17.0 (16.4–17.5)	16.2	16.1 (15.9–16.5)	16.4	14.9	12.7 (12.0–13.3)	12.3 (11.7–13.0)	12.2	12.7	9.6	12.2	12.1
No. live fetuses	15.7 (15.0–16.4)	15.3	15.2 (14.9–15.4)	15.3	13.5	11.9 (11.4–12.2)	11.3 (10.9–12.1)	11.7	12.2	9.0	11.7	11.3
Fetal mortality (%)‡	7.1 (6.0–8.2)	5.8	5.7 (4.6–6.3)	7.2	9.4	5.8 (3.7–8.9)	7.9 (4.9–14.0)	4.5	4.7	6.3	4.2	6.3
Body weight (g)												
All fetuses		3.99			3.15	3.48 (3.35–3.62)		3.37		3.51		
Male	4.43 (4.40–4.45)	4.06	4.29 (4.21–4.35)	3.83	3.25	3.57 (3.52–3.62)	3.40 (3.31–3.52)	3.45	3.49		3.79	3.89
Female	4.15 (4.07–4.23)	3.91	4.02 (3.91–4.09)	3.59	3.05	3.39 (3.35–3.47)	3.22 (3.12–3.35)	3.23	3.34		3.61	3.71

†V, Vehicle-treated; N, Non-treated.
‡(Number of early resorptions and late fetal deaths/number of implantations) × 100.
Minimum and maximum values from independent experiments are given in parentheses.

Table 8 Mating and cesarean section data from mice and hamsters

Species	Mice	Mice	Mice	Mice	Mice	Hamsters
Strain	Crlj:CD1(ICR)	Crlj:CD1(ICR)	Crlj:CD1(ICR)	Crlj:CD1(ICR)	Crlj:CD1(ICR)	Slc:Syrian
Year	2000	2001–2009	2002–2009	2004	2002	1999
Treatment†	V	V	V	V	V	V
Feed	CE-2	CRF-1	CE-2	NMF	CRF-1	CRF-1
No. dams	16	254	98	21	20	23
No. experiments	1	13	5	1	1	1
No. dams/experiment	16	16–23	16–23	21	20	23
Pregnancy rate (%)	72.7	82.7 (72.0–92.0)	78.9 (68.0–92.0)	95.5	100	88.9
Gestation day (hour) of the cesarean section	18 (9:00–12:00)	18 (7:00–10:00)	18 (9:00–12:00)	17 (13:00–16:00)	17 (9:00–12:00)	14 (9:00–11:00)
No. corpora lutea	13.1	14.0 (11.7–16.2)	14.0 (13.3–14.8)	15.6	14.7	15.6
No. implantations	11.6	12.4 (9.9–14.3)	12.6 (12.1–13.5)	14.4	14.6	14.7
No. live fetuses	11.2	11.5 (9.0–13.1)	11.7 (11.3–12.3)	13.4	14.1	12.1
Fetal mortality (%)‡	3.2	7.7 (4.6–9.4)	9.3 (6.2–14.0)	7.7	3.0	17.6
Body weight (g)						
All fetuses					1.02	
Male	1.51	1.45 (1.39–1.50)	1.45 (1.39–1.51)	1.19	1.05	1.60
Female	1.44	1.39 (1.35–1.43)	1.39 (1.32–1.41)	1.13	0.99	1.50

†V, Vehicle-treated.
‡(Number of early resorptions and late fetal deaths/number of implantations) × 100.
Minimum and maximum values from independent experiments are given in parentheses.

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Skeletal anomalies

Table S13 shows data on skeletal anomalies in Crlj:CD(SD) rats between 1994 and 2000. The incidence of fetuses with skeletal malformations ranged from 0 to 3.97% and was slightly over the range reported previously in this rat strain (0–0.85% in Morita et al. 1987; 0–2.74% in Nakatsuka et al. 1997). This appears to be due to the higher incidence of cleft sternebrae detected in one laboratory. This anomaly was also observed in a previous survey (Nakatsuka et al. 1997).

Data for Crl:CD(SD) rats between 1994 and 2000 and between 2001 and 2010 are presented in Tables S14 and S16, respectively. The incidence of fetuses with skeletal malformations ranged from 0 to 8.02% between 1994 and 2000 and 0.10 to 0.56% between 2001 and 2010. Although this incidence between 2001 and 2010 was within the ranges of that previously reported in this rat strain (0–5.2%) (CD(SD)IGS Study Group 1998, 1999, 2000, 2001, 2003), the incidence between 1994 and 2000 was slightly over the ranges previously reported. This appears to be due to the higher incidence of split costal cartilage and cleft sternebrae (5.28%) found in one laboratory. These anomalies were also observed in previous surveys (Morita et al. 1987; Nakatsuka et al. 1997). No clear difference was observed in the types of anomalies between the previous and present surveys.

Skeletal variations

Data on skeletal variations in Crlj:CD(SD) rats between 1994 and 2000 are presented in Table S19. The incidence of fetuses with skeletal variations ranged from 3.60 to 8.36%. This incidence was within the range of a previous survey of this rat strain (1.82–28.13%) (Nakatsuka et al. 1997). No noticeable difference was found in the types of anomalies between the previous (Morita et al. 1987; Nakatsuka et al. 1997) and present surveys.

Data for Crl:CD(SD) rats between 1994 and 2000 and between 2001 and 2010 are presented in Tables S20 and S22, respectively. The incidence of fetuses with skeletal variations ranged from 6.98 to 22.98% between 1994 and 2000 and 9.42 to 17.63% between 2001 and 2010. These incidences were within the ranges of those in previous surveys of this rat strain (6.8–35.7%) (CD(SD)IGS Study Group 1998, 1999, 2000, 2001, 2003). No clear difference was observed in the types of variations between the previous and present surveys. No noticeable difference was found in the types of skeletal variations between the two intervals evaluated (1994–2000 and 2001–2010).

CONCLUSION

Historical control data on rodent developmental toxicity studies, which were performed between 1994 and 2010, were obtained from 19 laboratories in Japan. Summary of historical control data on developmental toxicity studies in rodents was shown in Table 5. Inter-laboratory variations in the incidences of fetuses with alterations appear to be due to differences in the selection of observation parameters, observation criteria, classification, and terminology of fetal alterations. This survey provides information on historical control data of Crlj:CD(SD), which was completely withdrawn from the Japanese market in 2007, and Crl:CD(SD) rats, which have been developed and completely replaced Crlj:CD(SD) in 2007. Initial information on Wistar Hannover rats, which have been recently introduced into Japan, mice, and hamsters has also been provided in this survey. These historical control data may be helpful in interpreting the effect of chemicals in reproductive and developmental toxicity studies. However, the continuous accumulation of historical control data is needed for an adequate evaluation of reproductive and developmental toxicity data. To further interpret this data and its assessment for human health, it is necessary to harmonize the classification and terminology of fetal alterations.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. External anomalies in Crlj:CD(SD) [former Crj:CD(SD)] rats between 1994 and 2000.
Table S2. External anomalies in Crl:CD(SD) [former Crj:CD(SD)IGS] between 1994 and 2000.
Table S3. External anomalies in Jcl:SD, Slc:SD, and Crlj:WI rats between 1994 and 2000.
Table S4. External anomalies in Crl:CD(SD) [former Crj:CD(SD)IGS] rats between 2001 and 2010.
Table S5. External anomalies in Jcl:SD, Crlj:WI, Jcl:Wistar, and Wistar Hannover rats between 2001 and 2010.
Table S6. External anomalies in mice and hamsters.
Table S7. Visceral anomalies in Crlj:CD(SD) [former Crj:CD(SD)] rats between 1994 and 2000.
Table S8. Visceral anomalies in Crl:CD(SD) [former Crj:CD(SD)IGS] between 1994and 2000.
Table S9. Visceral anomalies in Jcl:SD, Slc:SD, and Crlj:WI rats between 1994 and 2000.
Table S10. Visceral anomalies in Crl:CD(SD) [former Crj:CD(SD)IGS] rats between 2001 and 2010.
Table S11. Visceral anomalies in Jcl:SD, Crlj:WI, Jcl:Wistar, and Wistar Hannover rats between 2001 and 2010.
Table S12. Visceral anomalies in mice and hamsters.
Table S13. Skeletal anomalies in Crlj:CD(SD) [former Crj:CD(SD)] rats between 1994 and 2000.
Table S14. Skeletal anomalies in Crl:CD(SD) [former Crj:CD(SD)IGS] between 1994 and 2000.
Table S15. Skeletal anomalies in Jcl:SD, Slc:SD, and Crlj:WI rats between 1994 and 2000.
Table S16. Skeletal anomalies in Crl:CD(SD) [former Crj:CD(SD)IGS] rats between 2001 and 2010.
Table S17. Skeletal anomalies in Jcl:SD, Crlj:WI, Jcl:Wistar, and Wistar Hannover rats between 2001 and 2010.
Table S18. Skeletal anomalies in mice and hamsters.
Table S19. Skeletal Variations in Crlj:CD(SD) [former Crj:CD(SD)] rats between 1994 and 2000.
Table S20. Skeletal variations in Crl:CD(SD) [former Crj:CD(SD)IGS] between 1994 and 2000.
Table S21. Skeletal variations in Jcl:SD, Slc:SD, and Crlj:WI rats between 1994 and 2000.
Table S22. Skeletal variations in Crl:CD(SD) [former Crj:CD(SD)IGS] rats between 2001 and 2010
Table S23. Skeletal variations in Jcl:SD, Crlj:WI, Jcl:Wistar, and Wistar Hannover rats between 2001 and 2010.
Table S24. Skeletal variations in mice and hamsters.

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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.

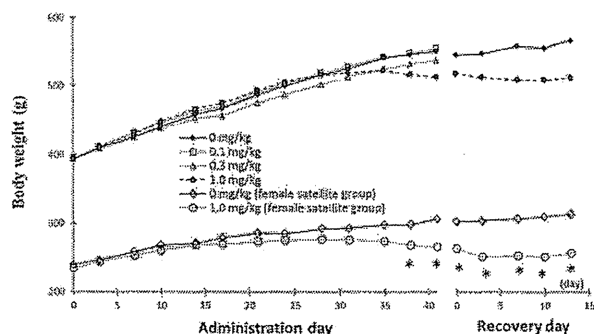

Fig. 1. Body weight of males in main groups and satellite groups for recovery period and females in satellite groups.
*: Significantly different from the control, $p \leq 0.05$.

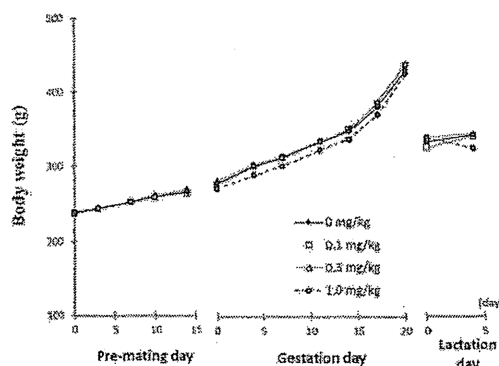

Fig. 2. Body weight of females in main groups.

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

Group		Main group 0 mg/kg/day	Main group 0.1 mg/kg/day	Main group 0.3 mg/kg/day	Main group 1.0 mg/kg/day	Satellite group 0 mg/kg/day	Satellite group 1.0 mg/kg/day
Males							
Number of animals		5	5	5	5	5	5
WBC	($10^2/\mu l$)	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	($10^4/\mu l$)	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	($10^4/\mu l$)	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	($10^2/\mu l$)	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	($10^4/\mu l$)	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	($10^4/\mu l$)	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, $p \leq 0.05$. **: Significantly different from the control, $p \leq 0.01$.

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 0 (control)	0.1	0.3	1.0	Satellite Group 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, $p \leq 0.01$.

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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Short communication

Evaluation of Rats' *In Vivo* Genotoxicity Induced by *N*-ethyl-*N*-nitrosourea in the RBC *Pig-a*, PIGRET, and *gpt* Assays

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The emerging *Pig-a* gene mutation assay, a powerful and promising tool for evaluating *in vivo* genotoxicity, is based on flow cytometric enumeration of red blood cells (RBCs), which are deficient in glycosylphosphatidylinositol anchored protein. Various approaches for measuring *Pig-a* mutant cells have been developed, particularly those focused on peripheral RBCs and reticulocytes (RETs). Previously, it had been reported that *Pig-a* and *gpt* mutant frequencies were relatively increased in *N*-ethyl-*N*-nitrosourea (ENU)- and benzo[*a*]pyrene (BP)-treated mice. The capacity and characteristics of the *Pig-a* assay relative to transgenic rodent (TGR) mutation assays, however, are unclear in rats. Here, using transgenic *gpt* delta rats, we compared the *in vivo* genotoxicity of single oral doses of ENU (40 mg/kg) in the *gpt* gene mutation assay in bone marrow and liver, and *Pig-a* gene mutation assays on RBCs and RETs in the same animals. The *Pig-a* gene mutation assays were conducted at 1, 2, and 4 weeks after treatment, whereas *gpt* assays were conducted on tissues collected at the 4-week terminal sacrifice. Consequently, we detected that *Pig-a* and *gpt* mutant frequencies were clearly increased in ENU-treated rats, indicating that both the *Pig-a* and TGR gene mutation assays can detect *in vivo* ENU genotoxicity equally.

Key words: transgenic rodent mutation assays; glycosylphosphatidylinositol anchor; red blood cells; reticulocyte

Introduction

Because gene mutations are implicated in the etiology of cancer and other human diseases, *in vivo* genotoxicity tests are important as public health management tools. One such tool is the transgenic rodent (TGR) mutation assay, which permits quantitative and accumulative evaluation of genotoxicity in all organs (1). The TGR mutation assay fulfills a need for a practical and widely available *in vivo* test for assessing gene mutation, and this assay has been recommended by regulatory authorities for safety evaluations (2,3) and international guidelines have been published describing the conduct of the assay (4).

The emerging *Pig-a* gene mutation assay, a powerful and promising tool for the evaluation of *in vivo* genotoxicity, was recently developed (5–7). Because the *Pig-a* gene is X-chromosome linked and involves the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, the forward mutation can result in the loss of GPI-anchored protein expression (7). Additionally, *Pig-a* mutation appears to function in a neutral manner whereby the accumulated effects of repeat exposures can be evaluated.

Various approaches for measuring *Pig-a* mutant cells have specifically focused on peripheral red blood cells (RBCs) and reticulocytes (RETs) (8–18). In these reports, although SYTO 13 dye or an antibody against the rat erythroid marker, HIS49, was used to label RETs in whole bloods, there was a limited capacity for counting RETs by flow cytometer. Conversely, a recently developed assay for measuring *Pig-a* mutant RETs, that is the PIGRET assay, is capable of flow cytometric cell counting $>1\times10^6$ RETs for the *Pig-a* mutant by concentrating RETs in whole bloods (19) and the approaches can be technically transferred between laboratories (20).

In this study, we performed the *gpt* and *Pig-a* gene mutation assays on RBCs (RBC *Pig-a* assay) and RETs (PIGRET assay) in the same animals, and we compared their performance in detecting ENU genotoxicity. This report describes the performance, effectiveness, and advantages of the RBC *Pig-a* and PIGRET assays in comparison with the *gpt* assay.

Materials and Methods

Preparation of chemicals: We dissolved ENU (Sigma-Aldrich Japan, Tokyo) in phosphate-buffered saline (PBS; pH 6.0) at 10 mg/mL.

Antibodies: We obtained anti-rat CD59 (clone

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TH9, FITC-conjugated), anti-rat CD71 (clone OX-26, PE-conjugated), and anti-rat erythroid marker (clone HIS49, APC-conjugated) antibodies from BD Biosciences (Tokyo, Japan).

Treatment of rats: Animal experiments were conducted humanely according to the regulations of the Animal Care and Use Committee of the National Institute of Health Sciences, Tokyo, and with their permission. *gpt* delta Wistar Hannover transgenic male rats were obtained from Japan SLC (Shizuoka, Japan). They were housed individually under specific pathogen-free conditions with a 12-h light-dark cycle and given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., Ltd., Tokyo) *ad libitum*. At 8 weeks of age, 5 rats per group were given a single oral administration of ENU (40 mg/kg) or PBS (negative control). Peripheral blood (120 μL) was withdrawn from a tail vein 1, 2, and 4 weeks after the treatments and used for the RBC *Pig-a* and PIGRET assays. At 4 weeks, all rats were sacrificed and the bone marrow and liver samples were collected for the *gpt* assay.

***Pig-a* mutation assays:** The RBC *Pig-a* and PIGRET assays were performed as previously described (5,19,20). Peripheral blood was withdrawn and immediately transferred into EDTA (dipotassium salt)-coated Microtainer® Tubes (BD Biosciences). For the RBC *Pig-a* assay, 3 μL of blood was suspended in 0.2 mL PBS and labeled with anti-rat CD59 (1 μg) and anti-rat erythroid marker (0.133 μg) antibodies. The cells were incubated for 1 h in the dark at room temperature, centrifuged (1,680 × g, 5 min), resuspended in 1 mL PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences). After gating for single cells, approximately 1×10^6 erythroid marker-positive cells were analyzed for the presence of surface CD59 and the *Pig-a* mutant frequency (MF) was calculated as previously described (19,20). For the PIGRET assay, 80 μL of blood was suspended in 0.2 mL PBS and labeled with 1 μg of PE-conjugated anti-rat CD71 antibody. The cells were incubated for 15 min in the dark on ice. After washing with 2 mL of 1 × IMag Buffer (BD Biosciences) and centrifuged (1680 × g, 5 min), the cells were mixed with 50 μL of BD IMag PE Particles Plus-DM (BD Biosciences) and incubated for 15 min in a refrigerator (4°C). The samples were enriched for CD71-positive cells by processing with a BD IMagnet magnetic stand (BD Biosciences) according to the manufacturer's instructions. The enriched samples were labeled with HIS49 and anti-CD59 antibodies as indicated for total RBC labeling, with the exception that the incubation time for labeling enriched RETs was half that for the total RBCs. The final cell suspension volume was 500 μL. *Pig-a* MF of CD71-positive RETs was examined using a FACS Canto II flow cytometer (BD Biosciences) as previously described (19,20).

***gpt* mutation assay:** We extracted high molecular weight genomic DNA from the liver and bone marrow samples using a Recover Ease DNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA), rescued lambda EG10 phages using Transpack Packaging Extract (Agilent Technologies), and conducted the *gpt* mutation assay as previously described (1). *gpt* Mutant frequencies (MFs) were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of colonies with rescued plasmids (1).

Statistical Analyses: The Mann-Whitney U-test was used for comparisons between PBS- and ENU-treated groups at each time point. Statistical analyses were performed using Prism 6 for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA). For these analyses, a p-value of <0.05 was considered significant and one-tailed tests were performed.

Results

The *Pig-a* assay: *Pig-a* MFs of whole RBCs (RBC *Pig-a* MF) in the ENU-treated rats were significantly increased, and the increase was modestly time-dependent (mean ± SD for rats treated with PBS: pre-treatment, $3.20 \pm 1.79 \times 10^{-6}$; 1 week after treatment, $1.80 \pm 1.30 \times 10^{-6}$; 2 weeks after treatment, $0.80 \pm 0.45 \times 10^{-6}$; and 4 weeks after treatment, $2.20 \pm 2.28 \times 10^{-6}$; mean ± SD for rats treated with 40 mg/kg ENU: pre-treatment, $2.80 \pm 2.28 \times 10^{-6}$; 1 week after treatment, $4.60 \pm 1.67 \times 10^{-6}$; 2 weeks after treatment, $31.4 \pm 5.86 \times 10^{-6}$; and 4 weeks after treatment, $52.80 \pm 8.84 \times 10^{-6}$) (Fig. 1A).

In the case of *Pig-a* MFs of RETs (RET *Pig-a* MF), significant increases were detected 1 week after treatment (mean ± SD for rats treated with PBS: pre-treatment, $3.00 \pm 4.81 \times 10^{-6}$; 1 week after treatment, $1.80 \pm 1.92 \times 10^{-6}$; 2 weeks after treatment, $2.80 \pm 1.64 \times 10^{-6}$; and 4 weeks after treatment, $0.80 \pm 0.84 \times 10^{-6}$; mean ± SD for rats treated with 40 mg/kg ENU: pre-treatment, $3.60 \pm 3.21 \times 10^{-6}$; 1 week after treatment, $107.6 \pm 10.5 \times 10^{-6}$; 2 weeks after treatment, $126.6 \pm 15.6 \times 10^{-6}$; and 4 weeks after treatment, $162.6 \pm 54.89 \times 10^{-6}$) (Fig. 1B).

The *gpt* assay on the bone marrow and liver samples

Compared with the solvent control animals (MF for PBS control group, $17.05 \pm 12.10 \times 10^{-6}$), significant increases in bone marrow *gpt* MFs were observed in ENU-treated rats [$87.39 \pm 60.55 \times 10^{-6}$ (Fig. 2)]. *gpt* MFs were also increased in the liver samples (MF for PBS control group, $7.56 \pm 7.97 \times 10^{-6}$; ENU, $79.04 \pm 31.62 \times 10^{-6}$) (Fig. 2).

Discussion

Here, we showed ENU genotoxicity using three different methods: the RBC *Pig-a*, PIGRET, and TGR