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Short communication

Evaluation of *in vivo* mutagenicity of hydroquinone in Muta™ mice

Mariko Matsumoto[a,d], Shoji Masumori[b], Mutsuko Hirata-Koizumi[a], Atsushi Ono[a,*], Masamitsu Honma[c], Kazuhito Yokoyama[d], Akihiko Hirose[a]

[a] Division of Risk Assessment, National Institute of Health Sciences, Tokyo, Japan
[b] Public Interest Incorporated Foundation Biosafety Research Center, Shizuoka, Japan
[c] Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan
[d] Department of Epidemiology and Environmental Health, Juntendo University Faculty of Medicine, Tokyo, Japan

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ABSTRACT

Hydroquinone (HQ) is used in skin bleaching agents, hair dyes, and finger nail treatments. Many skin-lightening cosmetics that contain HQ are currently marketed in Japan. Concerns have been expressed regarding health risks to the general population because the carcinogenicity of HQ was previously suggested in animal studies. HQ induced hepatocellular adenomas and forestomach hyperplasias in mice and renal tubular cell adenomas in male rats. In the present study, the *lacZ* transgenic mutation assay was conducted according to OECD test guideline 488 to determine whether mutagenic mechanisms were involved in HQ-induced carcinogenesis. Male Muta™ mice were repeatedly administered HQ orally at dosages of 0, 25, 50, 100, or 200 mg/kg bw/day for 28 days. Body weight gain was decreased in all treatment groups. No significant differences were observed in mutant frequencies in the liver, stomach, lung, or kidney between HQ-treated mice and the concurrent negative controls, whereas the significant induction of mutations was noted in the positive control, *N*-ethyl-*N*-nitrosourea. These results suggest that a mutagenic mechanism is not responsible for HQ-induced carcinogenesis.

1. Introduction

Hydroquinone (HQ) is used as an antioxidant in the rubber industry and as a developing agent in photography [1]. It is also used in skin bleaching agents, hair dyes, and finger nail treatments [1]. Many kinds of HQ skin-lightening cosmetics are currently available in Japan, up to 10% of which contain >2% HQ. HQ is not listed as a prohibited or limited ingredient for cosmetic use in Japan [2]. However, the cosmetic use of HQ for skin-lightening has been banned in the UK and EU due to the potential carcinogenic risk of HQ [3]. Approximately 200 different types of skin-lightening products contained 0.4–5.0% HQ in the US in 2006, whereas only prescription skin-lightening products can now contain >2–4% HQ and 2% or less is allowed for cosmetic use [1]. The prolonged use of HQ products (1–2%) has been associated with exogenous ochronosis, which was first reported by Findlay et al. [4], and a worldwide total of 789 cases of exogenous ochronosis had been reported by 2007 [5]. In addition to this topical local effect, concerns have been raised regarding the carcinogenic potential of HQ.

Two previous studies examined the carcinogenicity of HQ in rats and mice by oral administration [6,7]. HQ induced hepatocellular adenomas in female mice in one study [6] and in male mice in the other study [7]. Although epithelial hyperplasia of the forestomach was observed in both sexes in these two studies, tumors did not develop. Furthermore, HQ induced renal tubule adenomas in male rats in both of these studies. Increased rates of leukemia were observed in female rats, but the kidneys remained unaffected. A subsequent histopathological evaluation suggested that the interaction between the development of renal tumors and HQ enhanced chronic progressive nephropathy [8], and the relevance of renal carcinogenic effects in male rats to humans was reported to be questionable based on strain- and sex-specific metabolic pathways [9,10].

The initiating and/or promoting activity of HQ was examined in assays for thyroid, bladder, stomach, liver, lung, esophagus, and kidney carcinogenesis in rats [11–17]. The initiating activity of HQ was not observed in any of these studies, and promoting activity was absent in most assays; an increase in the multiplicity of esophageal tumors was reported in one study [12] while that of renal cell tumors was described in another [17]. No initiating effect was observed on skin tumors in a study using mice [18], and no

* Corresponding author at: Division of Risk Assessment, Biological Safety Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel.: +81 33700 9878; fax: +81 33700 1408.

E-mail address: atsushi@nihs.go.jp (A. Ono).

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promoting effect was found on pancreatic carcinogenesis in a study with hamsters [19].

Humans are exposed to HQ by oral, dermal, and inhalation routes. The primary route of exposure to HQ during its manufacture is considered to be the inhalation route. The highest average estimated inhalation dose of HQ during manufacturing is 0.0363 mg/kg bw/day for loader packagers. HQ occurs naturally in plants, and humans can consume it *via* foods or cigarette smoking [20]. HQ was previously shown to be dermally absorbed in humans with a bioavailability of 45.3 ± 11.2% for a 24-h application [21]; therefore, it can be absorbed through cosmetic use. Limited information is currently available on the carcinogenic potential of HQ in humans. A mortality study of 879 workers involved in the manufacture and use of HQ in the US reported no significant increases in death due to kidney cancer, liver cancer, or leukemia [22]. Another cohort study in Denmark found that a total of 24 cancer cases among 837 lithographers, and five cases of malignant melanoma were identified with a relative risk of 3.4. Two of five lithographers developed malignant melanoma following exposure to HQ [23].

HQ showed positive results in chromosomal aberration tests and micronuclei tests both *in vivo* (intraperitoneal or subcutaneous injection) and *in vitro* [24–28]. Ciranni et al. reported that the positive result was observed for micronuclei tests after intraperitoneal administration but not after oral administration [25], indicating routs of administration can affect genotoxic responses of HQ. Oxidative stress associated with HQ was shown to induce cytotoxicity [29] and has also been implicated in DNA damage [30]. A comet assay revealed DNA damage in human embryo lung fibroblasts treated with HQ [31]. Two out of three *in vitro* reverse mutation studies with *Salmonella typhimurium* strains were negative with and without metabolic activation [32,33], while one study showed a positive result in S. *typhimurium* TA104 (−S9) and negative results in another 4 strains of *S. typhimurium* (+/−S9). Mutagenic carcinogens are generally considered to have irreversible effects. If HQ carcinogenesis is related to mutagenic events, the no-threshold concept should be applied for risk assessment. However, no information is currently available for the *in vivo* mutagenicity of HQ. A transgenic mouse mutation assay in the target organs of carcinogenicity is useful for determining whether carcinogenesis is related to mutagenic events. In the present study, we evaluated the *in vivo* mutagenicity of HQ using a transgenic mouse mutation assay.

2. Materials and methods

This study was performed at the BioSafety Research Center (BSRC; Shizuoka, Japan) in accordance with "the Act on Welfare and Management of Animals", "the standards relating to the care and management of laboratory animals and relief of pain" and "Guideline for Animal Experimentation, BSRC". Animals were treated in accordance with "Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms", and "Safety Management Regulations for Recombinant DNA Experiment, BSRC". The study was conducted according to OECD TG 488 (28 July 2011: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays).

2.1. Chemicals

HQ (CAS: 123-31-9, Lot no. WEJ0292, purity: 99.3%) was purchased from Wako Pure Chemical Industries, Ltd. A positive control substance, *N*-ethyl-*N*-nitrosourea, was purchased from Toronto Research Chemicals Inc.

2.2. Animals and treatment

Nine-week old male Muta™ mice were purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan), and 33 animals found to be in good health were selected for use after an 8-day acclimation period. These animals were reared on a basal diet, CRF-1 (Oriental yeast) and water *ad libitum*. Animals were maintained at a room temperature of 20–26 °C, relative humidity of 35–70%, 12 h light/dark cycle, and 12 air changes per hour. Groups of five or six mice were administered HQ by gavage once a day for four weeks at a volume of 10 ml/kg, and at levels of 25, 50, 100, and 200 mg/kg. The highest dose level was set based on the results of the NTP fourteen-day gavage study using B6C3F1 mice in which HQ-related deaths (2/5) were observed within 3 days in males receiving 250 mg/kg bw/day [6]. Separate groups (5 animals/group) of the vehicle control (distilled water) were maintained in the same manner. The positive control was treated with *N*-ethyl-*N*-nitrosourea (i.p.) at 100 mg/kg bw/day once a day for 2 days. Animals were observed once a day every day. Body weight was recorded on days 1, 8, 15, and 22 of the administration period, and 1 and 3 days after the last treatment for HQ-treated animals, and one day before the treatment and 10 days after the last treatment for the positive control animals.

2.3. Tissue and DNA isolation

The liver, stomach, kidney, lung, and thyroid were collected 3 days after the last treatment, and a gross pathological examination was conducted. Positive control animals were sacrificed 10 days after the last treatment, and their organs were collected in the same manner. Tissue samples were quickly frozen in liquid nitrogen and then stored at −80 °C until analysis. Genomic DNA was extracted from the liver and stomach at 0, 50, 100, and 200 mg/kg bw/day, and the lung and kidney at 0, and 200 mg/kg bw/day as follows. Frozen tissue was placed into a Dounce homogenizer and homogenized with a pestle. The homogenized tissue fragments were poured into an ice-cooled centrifuge tube containing sucrose solution. After centrifugation by a centrifuge (LC-122, TOMY) at 3000 r/min (1710 G) for 10 min, the organic layer was incubated with RNase and proteinase K at 50 °C for 3 h. A mixture of phenol and chloroform (1:1) was added, and the water layer was separated after centrifugation at 2500 r/min (1190 G) for 10 min; this operation was repeated two times. Chloroform and isoamyl alcohol (24:1) and the water layer were mixed, and similarly centrifuged. The water layer was added in another centrifuging tube, and ethanol was added to precipitate the DNA. DNA was washed by soaking in 70% ethanol for 10 min. The DNA collected following the evaporation of ethanol was dissolved in TE buffer (NIPPON GENE) at room temperature overnight. The DNA solution was stored in a refrigerator.

The DNA of the thyroid was not able to be extracted and, therefore, was excluded from the evaluation.

2.4. In vitro packaging

DNA packaging was performed according to the Instruction Manual of Transpack (Stratagene). The DNA solution (200–600 μg/mL) was gently mixed with the Transpack packaging extract and incubated at 30 °C for 1.5 h twice, and SM buffer (NaCl, $MgSO_4 \cdot 7H_2O$, Tris–HCl [pH: 7.5], and gelatin) was then added.

2.5. Mutant frequency determination

The phage solution absorbed *Escherichia coli* at room temperature for 20–30 min. An appropriately diluted *E. coli* solution was mixed with LB top agar for the titer plates. The remaining phage-*E. coli* solution was mixed with LB top agar containing P-gal (phenyl-β-D-galactoside, Sigma–Aldrich) for the selection plates. These plates were then incubated overnight at 37 °C. Packaging was repeated to reach a total number of 300,000 plaques. The mutant frequency (MF) was calculated by the following formula: MF = total number of plaques on selection plates/total number of plaques on titer plates.

2.6. Statistical analysis

To assess the homogeneity of data, MFs in the treatment and negative control groups were analyzed with Bartlett's test. When homogeneity was recognized, data were analyzed using the Dunnett test. The Steel test was used for non-homogenous data. MFs between the negative and positive controls were compared by the Student's *t*-test or Aspin–Welch's *t*-test. Five percent levels of probability were used as the criterion for significance.

3. Results

No deaths were recorded in any of the treatment groups; therefore, animals at 25 mg/kg bw/day were excluded for the evaluation of mutagenicity. Body weight gain was decreased in all treatment groups (Fig. 1). No clinical signs of toxicity were observed. No abnormal effects were observed in the gross pathological examination. MFs induced by HQ in the liver, stomach, lung, and kidney are shown in Tables 1–4. MFs in the *lacZ* genes of the liver, stomach, lung, and kidney were not significantly higher than those in the respective negative controls. The positive control, *N*-ethyl-*N*-nitrosourea, induced mutations at a frequency that was 2-fold higher in the liver, 11-fold higher in the stomach, 5-fold higher in the lung, and 3-fold higher in the kidney than in their respective negative control organs.

Table 1
Mutation frequencies in the livers of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water (Negative control)	0	1001	666,000	47	70.6	
		1002	348,300	24	68.9	
		1003	722,700	48	66.4	75 ± 11.5
		1004	652,500	62	95	
		1005	673,200	50	74.3	
Hydroquinone	50	1201	734,400	36	49	
		1202	598,500	15	25.1	
		1203	938,700	46	49	42.4 ± 13.3
		1204	722,700	23	31.8	
		1205	719,100	41	57	
	100	1301	1,159,200	45	38.8	
		1302	754,200	29	38.5	
		1303	1,125,000	50	44.4	44.1 ± 8.5
		1304	816,300	48	58.8	
		1305	919,800	37	40.2	
	200	1401	1,036,800	44	42.4	
		1402	1,673,100	232	138.7	
		1403	760,500	42	55.2	69 ± 40.1
		1404	784,800	51	65	
		1405	527,400	23	43.6	
N-ethyl-N-nitrosourea (Positive control[b])	100	1501	596,700	81	135.7	
		1502	611,100	107	175.1	
		1503	640,800	112	174.8	158 ± 27.5[a]
		1504	803,700	147	182.9	
		1505	650,700	79	121.4	

[a] Significantly different from the negative control ($P < 0.05$) by the Student's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

4. Discussion

In the current *in vivo* mutagenicity study, no deaths were recorded in mice treated with HQ up to the highest dose. The highest dose was set as the maximum tolerated dose based on the results of the NTP fourteen-day gavage study using B6C3F1 mice, in which HQ-related deaths (2/5) were observed in males within three days at 250 mg/kg bw/day [6]. In the NTP study, tremors, convulsions, and decreases in body weight (8%) were also observed at 250 mg/kg bw/day. Toxicity in the current study was slightly weaker than expected; however, body weight gain decreased in all treatment groups, indicating that HQ was absorbed and distributed to a sufficient degree to manifest toxicity.

The MF of 138.7 ($\times 10^{-6}$) in one animal (ID number: 1402) at 200 mg/kg bw/day for the liver was higher than the historical negative control data [Mean ± S.D. = 47.6 ± 17.2 ($\times 10^{-6}$); an acceptable range of 0.00–99.2 ($\times 10^{-6}$)] in the facility. However, this change was considered to be spontaneous because the livers of other

Table 2
Mutation frequencies in the stomachs of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water (Negative control)	0	1001	609,300	25	41	
		1002	420,300	21	50	
		1003	831,600	26	31.3	39.6 ± 7.5
		1004	846,900	36	42.5	
		1005	419,400	14	33.4	
Hydroquinone	50	1201	831,600	33	39.7	
		1202	736,200	41	55.7	
		1203	993,600	41	41.3	54.7 ± 14
		1204	588,600	41	69.7	
		1205	761,400	51	67	
	100	1301	741,600	27	36.4	
		1302	651,600	25	38.4	
		1303	914,400	45	49.2	46.7 ± 9
		1304	805,500	46	57.1	
		1305	763,200	40	52.4	
	200	1401	855,900	43	50.2	
		1402	721,800	40	55.4	
		1403	943,200	73	77.4	55.9 ± 12.3
		1404	1,445,400	70	48.4	
		1405	434,700	21	48.3	
N-ethyl-N-nitrosourea (Positive control[b])	100	1501	621,900	321	516.2	
		1502	327,600	150	457.9	
		1503	745,200	369	495.2	473 ± 31.3[a]
		1504	882,900	399	451.9	
		1505	582,300	258	443.1	

[a] Significantly different from the negative control ($P < 0.05$) by Aspin–Welch's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

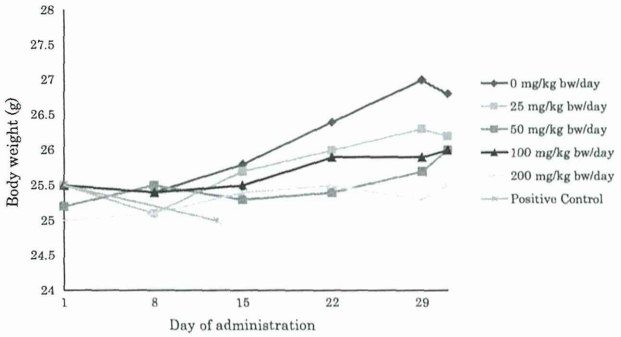

Fig. 1. Body weights of transgenic mice treated with hydroquinone for 28 days. Positive control: *N*-ethyl-*N*-nitrosourea was dosed once a day for 2 days (i.p) and tissues were collected 10 days after the last treatment.

animals at 200 mg/kg bw/day were not similarly affected. This kind of increase in MF could have occurred from a single mutation and clonal expansion [34].

HQ is one of the principal metabolites of benzene. The formation of DNA adducts in the bone marrow was previously reported in male mice exposed to benzene [35], and the same DNA adducts were detected in HL-60 cells or bone marrow treated with HQ *in vitro* [36,37]. However, no DNA adducts were observed in the bone marrow, Zymbal gland, liver, or spleen of female rats given HQ with phenol by gavage [38]. NTP carcinogenicity studies in mice and rats showed different carcinogenic properties between HQ and benzene; benzene showed clearer carcinogenicity in various organs [6,39]. Benzene is known to be leukemogenic in animals and humans [39,40], but no clear evidence has yet been reported to show that HQ induces leukemia in laboratory animals. In the NTP study, female rats showed increased rates of leukemia [6], but these were not significantly higher than those in the historical controls. Leukemogenic effects were not detected in humans who were occupationally exposed to HQ [22,23]. Therefore, HQ itself does not appear to be responsible for the carcinogenicity of benzene.

The main purpose of this study was investigating mutagenicity of HQ responsible to the carcinogenic effects caused by the oral administration. Our current study demonstrated that a mutagenic mechanism was not responsible for the carcinogenesis of HQ, suggesting that HQ can be a threshold carcinogen. Because orally administrated HQ is well absorbed [41], findings of the current study will be applicable for risk assessment for systemic effects of HQ despite of routes of administration. The lowest LOAEL (lowest observed adverse effect level) of a repeated dose was previously reported to be 17.9 mg/kg bw/day (25 mg/kg bw, 5 days/week for 103 weeks) for general toxicity due to lowered body weight and carcinogenicity due to renal tubule adenomas in rats given HQ by gavage [6]. This value can be used

Table 3
Mutation frequencies in the lungs of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water (Negative control)	0	1001	824,400	50	60.7	
		1002	501,300	31	61.8	
		1003	936,000	43	45.9	56.3 ± 10.9
		1004	709,200	49	69.1	
		1005	682,200	30	44.0	
Hydroquinone	200	1401	1,115,100	68	61.0	
		1402	631,800	49	77.6	
		1403	715,500	47	65.7	61.4 ± 26.1
		1404	684,900	58	84.7	
		1405	334,800	6	17.9	
N-ethyl-N-nitrosourea (Positive control[b])	100	1501	681,300	141	207.0	
		1502	458,100	151	329.6	
		1503	848,700	178	209.7	260.2 ± 67.8[a]
		1504	613,800	208	338.9	
		1505	959,400	207	215.8	

[a] Significantly different from the negative control ($p < 0.05$) by Aspin–Welch's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

Table 4
Mutation frequencies in the kidneys of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water (Negative control)	0	1001	572,400	24	41.9	
		1002	512,100	36	70.3	
		1003	753,300	52	69.0	53.4 ± 14.9
		1004	558,000	24	43.0	
		1005	633,600	27	42.6	
Hydroquinone	200	1401	551,700	25	45.3	
		1402	681,300	31	45.5	
		1403	475,200	33	69.4	47.0 ± 13.8
		1404	666,000	29	43.5	
		1405	540,000	17	31.5	
N-ethyl-N-nitrosourea (Positive control[b])	100	1501	431,100	87	201.8	
		1502	305,100	39	127.8	
		1503	543,600	92	169.2	147.8 ± 37.7[a]
		1504	590,400	79	133.8	
		1505	479,700	51	106.3	

[a] Significantly different from the negative control ($p < 0.05$) by the Student's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

as a starting point for risk assessments of the cosmetic use of HQ in humans. However, uncertainty may remain for local effects, because routs of administration may affect genotoxic outcomes.

In conclusion, HQ is considered to be a threshold carcinogen because mutagenic activity was not observed in the liver, stomach, lung, or kidney of HQ-treated mice.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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ORIGINAL ARTICLE

Historical control data on developmental toxicity studies in rodents

Makoto Ema, Katsumi Endoh, Ryou Fukushima, Sakiko Fujii, Hiroaki Hara, Mutsuko Hirata-Koizumi, Akihiko Hirose, Hitoshi Hojo, Masao Horimoto, Nobuhito Hoshino, Yoshinori Hosokawa, Yukari Imai, Hiroshi Inada, Kunifumi Inawaka, Keiichi Itoh, Yoshihiro Katsumata, Hiroyuki Izumi, Hirohito Kato, Maki Maeda, Kiyoshi Matsumoto, Seiki Matsuo, Toshiki Matsuoka, Ikuo Matsuura, Hiroshi Mineshima, Yoji Miwa, Nao Nakano, Masato Naya, Hiroko Noyori, Takafumi Ohta, Harutaka Oku, Atsushi Ono, Tatsuya Shimizu, Kazuhiro Shimomura, Ikuro Takakura, Ryota Tanaka, Taishi Tateishi, Yuko Tominaga, Tohru Uesugi, Chizuru Urakawa, Kaoru Yabe, Akihito Yamashita, Toshiaki Yamauchi, and Ryohei Yokoi
A Study Group for Historical Control Data on Prenatal Developmental Toxicity Studies in Rodents

ABSTRACT **Historical control data on rodent developmental toxicity studies, performed between 1994 and 2010, were obtained from 19 laboratories in Japan, including 10 pharmaceutical and chemical companies and nine contract research organizations. Rats, mice, and hamsters were used for developmental toxicity studies. Data included maternal reproductive findings at terminal cesarean sections and fetal findings including the spontaneous incidences of external, visceral, and skeletal anomalies. No noticeable differences were observed in maternal reproductive data between laboratories. Inter-laboratory variations in the incidences of fetuses with anomalies appeared to be due to differences in the selection of observation parameters, observation criteria, classification of the findings, and terminology of fetal alterations. Historical control data are useful for the appropriate interpretation of experimental results and evaluation of the effects of chemical on reproductive and developmental toxicities.**

Key Words: developmental toxicity, fetal malformation, historical control data, reproductive toxicity, rodent

INTRODUCTION

The availability of comprehensive historical control data is of importance because a comparison of data from study controls with historical control data may be beneficial to evaluate toxicity. Historical control data on reproductive and developmental toxicity studies may be useful for the adequate interpretation of experimental results and evaluation of reproductive and developmental toxicity. Historical control data may help to distinguish treatment-induced changes from spontaneously occurring background changes specific to the species/strains.

Rodents have been widely used in toxicological studies of pharmaceuticals, crop protection compounds, and industrial chemicals, while rats, mice, and rabbits are the more universally accepted laboratory animal species for standardized developmental toxicity testing (Wilson 1973; Schardein 2000; Barrow 2009). Historical control data on reproductive and developmental toxicity studies in laboratory animals have been previously reported in Japan by Kameyama et al. (1980), Morita et al. (1987) (Japanese Pharmaceutical Manufacturer's Association [JPMA] survey, data between 1980 and 1985), and Nakatsuka et al. (1997) (JPMA survey, data between 1986 and 1993). Historical control data on reproductive and developmental toxicity studies using rodents have been extensively reported in abstracts; however, detailed information can not be obtained from these abstracts. Reproductive data can be obtained from a website for rats (CLEA Japan, Inc. 2007) and mice (Giknis and Clifford 2007). Detailed information on reproductive and developmental toxicity studies including spontaneous fetal malformations is available from a website for rats (CD[SD]IGS Study Group 1998, 1999, 2000, 2001, 2003) and a chapter of a book for rats and mice (Kimmel and Price 1990; Tyl and Marr 2006). Only a few peer-reviewed studies are available for Wistar Hannover rats (Aoyama et al. 2002; Liberati et al. 2002; Takeuchi et al. 2011). A retrospective analysis of multi-generation studies using rats has also been performed (Marty et al. 2009).

However, no historical control data have been published on reproductive and developmental toxicity studies of rodents, except for Wistar Hannover rats, over the last decade in Japan. Subtle changes may occur with time due to genetic alterations in the strain or stock of the species used and changes in environmental conditions both in the breeding colony of the supplier and in the laboratory (Kimmel and Price 1990). Therefore, examining changes in data over time within historical control data and comparing study control data with recent as well as cumulative historical control data are of importance. We previously reported historical control data between 1994 and 2010 for developmental toxicity studies of rabbits (Ema et al. 2012). Recent historical control data for rodents between 1994 and 2010 were collected and summarized in this paper.

MATERIALS AND METHODS

The participating laboratories in pharmaceutical and chemical companies and contract research organizations are shown in Table 1. Data were obtained from 19 laboratories in Japan, including 10 pharmaceutical and chemical companies and nine contract research organizations. Data regarding terminal cesarean sections, fetal external anomalies, and visceral and skeletal anomalies and variations in rodents were collected from developmental toxicity studies conducted between 1994 and 2010. Data from range-finding studies that utilized a small number of dams per group (less than 16 litters) were not included in this dataset. Data were summarized separately between 1994 and 2000 and between 2001 and 2010. The animal strain was expressed as a brand name. Data were incorporated from

Correspondence: Makoto Ema, DVM, PhD, Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan. Email: ema-makoto@aist.go.jp
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Table 1 Participating laboratories and researchers

Laboratory	Researcher
Astellas Pharma Inc., Drug Safety Research Laboratories	Seiki Matsuo
	Hiroko Noyori
Public Interest Incorporated Foundation Biosafety Research Center, Foods, Drugs and Pesticides (BSRC)	Keiichi Itoh
	Ryota Tanaka
Bozo Research Center Inc.	Yoshihiro Katsumata
Chiba Institute of Science, Faculty of Risk and Crisis Management	Masao Horimoto
Daiichi Sankyo Co., Ltd., Medicinal Safety Research Laboratories	Toshiki Matsuoka
	Kazuhiro Shimomura
Dainippon Sumitomo Pharma Co., Ltd., Preclinical Research Laboratories	Akihito Yamashita
	Hiroshi Inada
Eisai Co., Ltd., Tsukuba Drug Safety/Sunplanet Co., Ltd., Preclinical Safety Research Laboratories	Maki Maeda
	Hiroshi Mineshima
Ina Research Inc.	Hiroaki Hara
	Tatsuya Shimizu
Institute of Environmental Toxicology, Toxicology Division	Hitoshi Hojo
	Chizuru Urakawa
Kissei Pharmaceutical Co., Ltd.	Ikuro Takakura
	Ryohei Yokoi
Mitsubishi Chemical Medience Corporation, Kashima Laboratory	Ikuo Matsuura
Mitsubishi Chemical Medience Corporation, Kumamoto Laboratory	Nobuhito Hoshino
	Hiroyuki Izumi
	Takafumi Ohta
National Institute of Advanced Industrial Science and Technology (AIST), Research Institute of Science for Safety and Sustainability	Makoto Ema
	Masato Naya
National Institute of Health Sciences, Division of Risk Assessment	Akihiko Hirose
	Mutsuko Hirata-Koizumi
	Atsushi Ono
Nihon Bioresearch Inc.	Katsumi Endoh
	Yoji Miwa
Ono Pharmaceutical Co., Ltd., Safety Research Laboratories, Department of Biology & Pharmacology	Yukari Imai
	Harutaka Oku
Otsuka Pharmaceutical Co., Ltd., Tokushima Research Institute	Yuko Tominaga
	Tohru Uesugi
Safety Research Institute for Chemical Compounds Co., Ltd.	Sakiko Fujii
	Kaoru Yabe
Shin Nippon Biomedical Laboratories (SNBL), Ltd., Drug Safety Research Laboratories	Hirohito Kato
	Taishi Tateishi
Shionogi & Co., Ltd., Drug Developmental Research Laboratories	Nao Nakano
	Ryou Fukushima
Sumitomo Chemical Co., Ltd., Environmental Health Science Laboratory	Yoshinori Hosokawa
	Kunifumi Inawaka
Takeda Pharmaceutical Co. Ltd., Drug Safety Research Laboratories	Kiyoshi Matsumoto
	Toshiaki Yamauchi

a laboratory if the information was based on four studies or more for Crlj:CD(SD) (former name: Crj:CD[SD]) rats, and three studies or more for Crl:CD(SD) (former name: Crj:CD[SD]IGS) rats between 1994 and 2000, and 10 studies or more for Crl:CD(SD) rats between 2001 and 2010. Data were incorporated if there was one study or more for SD rats from other breeders, other strains of rats, mice, and hamsters between 1994 and 2000 and between 2001 and 2010; however, these data were not sufficient for a definitive analysis.

The day of detection of copulation was designated as gestational day (GD) 0. The category of fetal mortality included early resorptions and late fetal deaths. Incidence data for fetal alterations were based on the number of alterations observed in each category as a percentage of the total number of live fetuses examined. If more than one alteration was observed in a fetus, each was reported individually. The incidence of fetuses with malformations or variations was expressed as a proportion of the total number of fetuses with malformations or variations to the total number of fetuses examined. The terminology used for fetal external, visceral, and skeletal alterations was principally based on Horimoto et al. (1998) and Makris et al. (2009).

RESULTS AND DISCUSSION

Mating and cesarean section data

All pregnant dams were prepared by natural mating in rats, mice, and hamsters. Cesarean sections were performed on GD 20 or GD 21 in rat dams, on GD 17 or GD 18 in mouse dams, or on GD 14 in hamster dams.

Mating and cesarean section data from Crlj:CD(SD) rats between 1994 and 2000 and from Crl:CD(SD) rats between 1994 and 2000 and between 2001 and 2010 are shown in Tables 2, 3 and 4, respectively. The average pregnancy rate, number of corpora lutea, number of implantations, number of live fetuses, and fetal mortality were summarized in Table 5. Whereas the average values of fetal mortality in Crlj:CD(SD) rats and Crl:CD(SD) rats were similar, the average numbers of corpora lutea, implantations, and live fetuses in Crl:CD(SD) rats were slightly smaller than those in Crlj:CD(SD) rats. These phenomena were also observed in some surveys of the CD(SD)IGS Study Group (1998, 2000). The values of reproductive parameters of Crlj:CD(SD) were not clearly different from those of the same rat strain previously surveyed in Japan (Morita et al. 1987; Nakatsuka et al. 1997). No noticeable variation was observed in the reproductive parameters of Crl:CD(SD) rats between the two intervals evaluated (1994–2000 and 2001–2010).

The data from Jcl:SD, Slc:SD, and Wistar rats between 1994 and 2000 and between 2001 and 2010 are presented in Tables 6 and 7, respectively. The data from mice and hamsters between 1994 and 2000 and between 2001 and 2010 are shown in Table 8. Although the pregnancy rates of SD rats from other breeders (Jcl:SD and Slc:SD), other strains of rats (Wistar rats), and mice were similar to those of Crlj:SD(CD) and Crl:CD(SD) rats in most laboratories, a relatively low pregnancy rate was noted in Wistar Hannover rats, mice, and hamsters in remaining laboratories. The average numbers of corpora lutea, implantations, and live fetuses of SD rats from other breeders and Wistar rats (Crlj:WI and Jcl:Wistar) were similar to those in Crlj:CD(SD) and/or Crl:CD(SD) rats. The value of fetal mortality in SD rats from other breeders and Wistar rats, including Wistar Hannover rats, was similar to that in Crlj:CD(SD) and/or Crl:CD(SD) rats. The numbers of corpora lutea, implantations, and live fetuses in Hannover Wistar rats were slightly smaller than those in Crlj:SD(CD) and Crl:CD(SD) rats. These findings were consistent with previous surveys, in which reproductive parameters, such as the numbers of corpora lutea, implantations, and live fetuses, were similar among three stocks of Wistar Hannover rats (Takeuchi et al. 2011) and were smaller than those in SD rats (Aoyama et al. 2002; Liberati et al. 2002). More data are required for a definitive analysis of historical control data in these animals.

One laboratory determined fetal body weight with males and females combined, and the remaining laboratories evaluated fetal body weight for each sex separately. Male and female fetal weights should be determined separately because males are heavier than females. The fetal weight varied with each laboratory and in general, roughly related the time and GD of cesarean sections of the dams. The rearing environment may have also had an impact on fetal weight.

External anomalies

Table S1 shows data on external anomalies in Crlj:CD(SD) rats between 1994 and 2000. The incidence of fetuses with external malformations ranged from 0.04 to 0.53% between 1994 and 2000, which was comparable to that of Crlj:CD(SD) rats in previous surveys (0–1.33% in Morita et al. 1987; 0–0.51% in Nakatsuka et al. 1997). In the previous survey (Nakatsuka et al. 1997), a few cases of conjoined twins, but not conjoined triplets, were reported in this rat strain. In the present survey, one case of conjoined triplets was observed in one laboratory. However, no noticeable difference was observed in the types of external anomalies reported 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 S2 and S4, respectively. The incidence of fetuses with external malformations ranged from 0 to 0.36% between 1994 and 2000 and 0.05 to 0.18% between 2001 and 2010, which was comparable to that of Crl:CD(SD) rats in previous surveys (0–0.34%) (CD(SD)IGS Study Group 1998, 1999, 2000, 2001, 2003). The incidence of fetuses with external malformations in Crl:CD(SD) rats was slightly lower than that in Crlj:CD(SD) rats. No noticeable variability was observed in the types of external anomalies between Crl:CD(SD) and Crlj:CD(SD) rats or in the incidence of fetuses with external malformations in Crl:CD(SD) rats between the two intervals evaluated (1994–2000 and 2001–2010).

Visceral anomalies

Data on visceral anomalies in Crlj:CD(SD) rats between 1994 and 2000 are presented in Table S7. The incidence of fetuses with visceral malformations ranged from 0.45 to 16.57% between 1994 and 2000. This incidence was within the range of previous surveys of this rat strain (0–17.59% in Morita et al. 1987; 0.24–34.83% in Nakatsuka et al. 1997). No noticeable difference was found in 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 S8 and S10, respectively. The incidence of fetuses with visceral malformations ranged from 0 to 11.09% between 1994 and 2000 and 0.32 to 8.27% between 2001 and 2010. These incidences were within the ranges of those in the previous surveys on this rat strain (0–26.3%) (CD(SD)IGS Study Group 1998, 1999, 2000, 2001, 2003). No clear difference was noted in the types of anomalies between the previous and present surveys.No noticeable difference was found in the types of visceral anomalies between the two intervals evaluated (1994–2000 and 2001–2010).

Large variations were noted in the incidences of visceral malformations among laboratories between the previous (Morita et al. 1987; Nakatsuka et al. 1997; CD(SD)IGS Study Group 1998, 1999, 2000, 2001, 2003) and present surveys. This phenomenon appeared to be due to differences in the classification of visceral anomalies among laboratories. Visceral anomalies such as thymic cord and some anomalies of the vessels were classified as malformations by some laboratories, but as variations by other laboratories.

Table 2 Mating and cesarean section data from Crlj:CD(SD) [former Crj:CD(SD)] rats between 1994 and 2000

Year	1994–2000	1994–2000	1994–2000	1995–2000	1995–2000	1994–1996	1994–2000	1994–2000
Treatment†	V	V	V	V	V	V	V	V
Feed	NMF	CRF-1	CRF-1	NMF	NMF	MF	CRF-1	CRF-1
No. dams	721	302	201	264	180	120	78	73
No. experiments	35	14	11	12	8	5	4	4
No. dams/experiment	16–24	19–25	17–20	20–24	21–25	24	19–20	17–19
Pregnancy rate (%)	96.2 (80.0–100)	98.0 (95.0–100)	92.2 (81.8–100)	98.5 (90.9–100)	98.3 (95.5–100)	95.8 (91.7–100)	97.5 (95–100)	91.3 (85.0–95.0)
Gestation day (hour) of the cesarean section	20 (13:30–16:00)	20 (9:00–11:00)	20 (10:00–12:00)	20 (13:00–16:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (13:00–16:00)	20 (9:00–12:00)
No. corpora lutea	17.0 (15.7–18.7)	16.4 (14.4–17.6)	17.0 (15.2–19.4)	18.7 (17.7–19.6)	18.0 (16.6–19.9)	17.0 (16.4–17.2)	17.4 (16.7–17.9)	17.4 (16.7–18.1)
No. implantations	15.9 (13.2–18.1)	15.5 (13.1–16.8)	14.7 (12.9–16.8)	16.8 (15.7–17.7)	15.5 (15.0–16.5)	15.8 (14.8–16.8)	16.5 (15.6–17.3)	15.4 (14.9–15.7)
No. live fetuses	15.0 (12.4–17.2)	14.6 (12.6–13.8)	13.9 (11.8–15.8)	15.8 (14.9–16.6)	14.6 (14.2–15.4)	14.4 (13.7–15.0)	15.8 (15.2–16.4)	14.7 (14.3–15.2)
Fetal mortality (%)‡	6.0 (2.5–12.0)	6.1 (2.2–13.8)	5.5 (3.1–9.1)	6.5 (4.4–8.5)	6.0 (3.3–8.9)	8.1 (5.9–9.9)	4.3 (2.7–5.3)	4.3 (2.6–7.0)
Body weight (g)								
All fetuses						3.44 (3.23–3.58)	3.46 (3.45–3.48)	3.44 (3.36–3.48)
Male	3.73 (3.41–4.04)	3.63 (3.48–3.87)	3.35 (3.14–3.78)	3.76 (3.59–3.88)	3.40 (3.35–3.48)	3.53 (3.42–3.58)	3.54 (3.51–3.57)	3.52 (3.45–3.55)
Female	3.55 (3.32–3.83)	3.45 (3.31–3.70)	3.19 (2.97–3.58)	3.56 (3.44–3.66)	3.23 (3.17–3.31)	3.34 (3.23–3.42)	3.38 (3.35–3.42)	3.38 (3.29–3.45)

†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.

Table 3 Mating and cesarean section data from Crl:CD(SD) [former Crj:CD(SD)IGS] rats between 1994 and 2000

Year	1994–2000	1996–2000	1997–2000	1999–2000	1997–2000	1998–2000	1996–2000	1999–2000	2000	1994–2000	1997	1994–2000
Treatment†	V	V	V	V	V	V	V	V	V	V	N/V	V
Feed	CR-LPF	CRF-1/CR-LPF	CRF-1	NMF	CRF-1	CRF-1	NMF	NMF	CRF-LPF	CRF-1	CRF-1	CRF-1
No. dams	393	217	147	125	113	94	99	90	80	77	60	58
No. experiments	20	11	7	6	6	5	4	4	4	4	3	3
No. dams/experiment	19–20	19–22	19–24	20–22	16–20	18–20	19–36	21–25	18–20	18–20	20	18–21
Pregnancy rate (%)	98.3	97.3 (95.0–100)	97.5 (95.0–100)	99.2 (95.5–100)	97.1 (94.2–100)	93.0 (90.0–100)	98.8 (95.0–100)	98.9 (95.5–100)	93.8 (90.6–98.4)	96.3 (90.0–100)	100	93.5 (90.0–95.5)
Gestation day (hour) of the cesarean section	20 (9:00–12:00)	20 (8:00–11:00)	20 (9:00–12:00)	20 (13:00–16:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (13:30–16:00)	20 (9:00–12:00)	21 (9:00–12:00)	20 (13:00–16:00)	20 (9:00–11:00)	20 (9:00–12:00)
No. corpora lutea	15.7 (14.9–16.4)	16.1 (15.1–17.3)	16.0 (15.5–16.9)	17.4 (16.6–18.0-)	16.2 (15.6–16.5)	15.3 (14.9–15.7)	16.1 (15.7–16.3)	16.5 (15.6–17.9)	14.6 (13.7–15.7)	15.9 (15.6–16.1)	15.5 (15.1–15.9)	15.9 (15.7–16.2)
No. implantations	14.8 (13.3–15.8)	14.8 (13.7–16.0)	14.6 (13.7–15.1)	15.6 (14.7–16.2)	15.5 (15.1–16.2)	13.7 (11.8–14.6)	15.5 (14.9–15.9)	14.9 (14.5–15.3)	13.8 (12.8–14.8)	15.1 (14.3–15.5)	15.0 (14.5–15.4)	14.2 (14.0–14.3)
No. live fetuses	14.1 (12.9–15.0)	14.2 (13.3–15.3)	14.0 (13.5–14.6)	14.9 (14.1–15.6)	14.8 (14.3–15.5)	12.9 (11.6–13.5)	14.7 (14.3–15.2)	13.9 (13.6–14.2)	13.3 (12.4–14.1)	14.2 (12.7–15.0)	14.2 (13.8–14.6)	13.4 (13.2–13.7)
Fetal mortality (%)‡	4.8 (1.9–10.8)	4.2 (2.2–7.3)	3.8 (0.4–5.7)	4.8 (3.6–6.3)	4.7 (3.4–5.8)	5.2 (2.8–7.6)	4.9 (4.0–6.8)	6.4 (5.6–7.1)	4.9 (2.1–7.5)	7.2 (3.1–15.4)	5.6 (4.7–6.8)	5.0 (3.7–6.0)
Body weight (g)												
All fetuses						3.97 (3.84–4.10)				3.85 (3.75–3.98)		3.66 (3.64–3.70)
Male	3.47 (3.34–3.61)	3.88 (3.60–4.01)	3.52 (3.33–3.63)	4.20 (4.07–4.31)	3.62 (3.55–3.70)	4.07 (3.93–4.18)	4.11 (4.00–4.19)	3.62 (3.45–3.77)	5.39 (5.29–5.49)	3.96 (3.83–4.09)	3.81 (3.80–3.83)	3.76 (3.72–3.81)
Female	3.30 (3.13–3.42)	3.67 (3.46–3.77)	3.34 (3.19–3.43)	3.98 (3.89–4.07)	3.44 (3.37–3.50)	3.83 (3.73–3.97)	3.89 (3.82–3.96)	3.43 (3.31–3.58)	5.11 (5.03–5.25)	3.75 (3.67–3.86)	3.62 (3.60–3.65)	3.56 (3.54–3.61)

†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 4 Mating and cesarean section data from Crl:CD(SD) [former Crj:CD(SD)IGS] rats between 2001 and 2010

Year	2001–2010	2001–2010	2001–2010	2001–2010	2001–2010	2001–2010	2001–2009	2001–2010	2001–2010	2001–2010	2004–2010
Treatment†	V	V	V	V	V	V	V	V	V	V	N/V
Feed	CE-2/CRF-1	NMF	NMF	CRF-1	CRF-1	CRF-1	CRF-1	CRF-1	CR-LPF	CRF-1	CRF-1
No. dams	1064	934	717	565	567	479	346	332	290	279	192
No. experiments	55	47	36	29	28	25	17	16	15	12	10
No. dams/ experiment	17–24	17–22	19–24	18–20	19–24	16–20	19–22	18–22	17–20	20–25	17–20
Pregnancy rate (%)	96.0 (85.0–100)	98.1 (85.0–100)	98.5 (95.0–100)	97.4 (90.0–100)	99.2 (95.0–100)	95.8 (80.0–100)	98.8 (95.0–100)	97.9 (90.0–100)	96.7	100	96.0 (85.0–100)
Gestation day (hour) of the cesarean section	20 (9:00–12:00)	20 (13:00–16:00)	20 (13:30–16:00)	20 (8:00–11:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (9:00–12:00)	20 (13:00–16:00)	20 (9:00–12:00)	20 (9:00–16:00)	20 (9:00–11:00)
No. corpora lutea	15.7 (13.8–17.6)	16.6 (14.6–18.4)	15.6 (14.1–16.3)	15.1 (13.9–16.2)	15.3 (14.4–16.5)	15.3 (14.3–16.1)	15.9 (15.0–16.8)	15.8 (15.1–17.1)	14.9 (14.4–15.9)	15.4 (14.7–16.3)	15.4 (14.9–16.2)
No. implantations	14.8 (13.1–16.4)	14.9 (13.0–16.2)	14.7 (13.1–15.5)	14.2 (12.5–15.2)	14.6 (13.3–14.8)	14.2 (13.3–15.5)	15.0 (13.5–16.2)	14.8 (14.1–15.4)	14.1 (13.5–14.8)	14.8 (13.8–15.8)	14.5 (13.9–15.1)
No. live fetuses	14.1 (12.4–15.4)	14.2 (12.5–15.3)	13.2 (12.3–15.1)	13.5 (11.8–14.6)	13.8 (12.5–14.8)	13.4 (12.4–14.8)	14.2 (12.8–15.5)	14.3 (13.6–15.0)	13.4 (12.9–14.0)	14.0 (13.0–14.9)	13.6 (12.7–14.3)
Fetal mortality (%)‡	4.8 (2.0–9.3)	4.8 (0.8–8.6)	5.1 (2.6–9.0)	4.5 (2.0–8.0)	5.5 (2.3–10.2)	5.5 (2.5–9.1)	6.2 (3.1–9.9)	3.6 (2.1–6.4)	5.3 (1.8–8.0)	5.9 (3.5–8.0)	6.0 (3.3–9.4)
Body weight (g)											
All fetuses						4.07 (3.93–4.20)					
Male	3.81 (3.58–4.01)	4.18 (3.99–4.36)	4.11 (3.95–4.25)	3.94 (3.85–4.09)	3.71 (3.52–3.91)	4.06 (3.85–4.29)	3.53 (3.29–3.86)	4.06 (3.96–4.21)	3.63 (3.51–3.82)	3.73 (3.64–3.81)	3.77 (3.60–4.03)
Female	3.62 (3.38–3.81)	3.96 (3.76–4.16)	3.90 (3.72–4.05)	3.72 (3.63–3.85)	3.52 (3.33–3.64)	3.84 (3.62–4.11)	3.33 (3.13–3.60)	3.85 (3.76–3.97)	3.44 (3.33–3.58)	3.54 (3.42–3.65)	3.56 (3.40–3.81)

†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 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.

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.

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.
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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.
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Table S19. Skeletal Variations in Crlj:CD(SD) [former Crj:CD(SD)] rats between 1994 and 2000.
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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

Mika Takahashi[1], Shigeru Ishida[2], Mutsuko Hirata-Koizumi[1], Atsushi Ono[1] and Akihiko Hirose[1]

[1]Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[2]Gotemba Laboratory, Bozo Research Center Inc., 1284 Kamado, Gotemba-shi, Shizuoka 412-0039, Japan

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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

Correspondence: Akihiko Hirose (E-mail: hirose@nihs.go.jp)

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