also the status of the p53 gene can affect sensitivity/specificity[4]. The currently employed *in vitro* assay systems have high performance and relatively low cost, and have proven valuable as reliable screening methods to detect mutation.

There is a current trend toward more extensive use of exposure-related risk assessment of genotoxic agents, and therefore more emphasis is being placed on results from the *in vivo* assay systems. The rodent micronucleus assay to detect chromosomal aberrations is well-established in the field of regulatory science[5,6] and has been incorporated into the standard battery for assessing chemical mutagenicity. Although the micronucleus assay has been widely used, the target organ has been limited exclusively to hematopoietic cells; *e.g.*, bone marrow polychromatic erythrocytes and peripheral reticulocytes. The most important role of genotoxicity evaluation is to identify the mechanism of carcinogenicity and to determine whether it is dependent on genotoxicity or not. Accordingly, the use of only hematopoietic cells is insufficient to answer this question. The transgenic animal models (OECD TG488)[7] and the single cell gel electrophoresis assay (comet assay)(OECD TG in preparation) have become well recognized and are widely used because these assays can evaluate essentially any tissue of interest. Recently, the micronucleus assay has been extended to many organs (*e.g.*, testes, skin, liver, digestive tract)[8]. Thus three important endpoints of genotoxicity can be covered by *in vivo* assay systems: the comet assay detects DNA damage, the transgenic animal model detects gene mutation, and the micronucleus assay detects chromosomal aberrations. Because these assays can be applied to the organ that is the target for carcinogenicity, the genotoxicity assay can play an important role for chemical safety assessment for humans.

Although the importance of the *in vivo* assay systems is recognized, another important issue is animal welfare. Therefore, it is important to seek a balance between these two important issues, and attention has been given to minimizing animal use while at the same time obtaining the data necessary for risk assessment[6]. Examples include: 1) The micronucleus assay using a tiny amount of peripheral blood without killing experimental animals can provide time-course information after treatment[9]. This method can reduce animal usage. 2) Omission of the concurrently treated positive control animals in the assay is appropriate when a laboratory has demonstrated experience and pre-prepared scoring controls are included. 3) Several endpoints may often be combined into one assay; for example, the micronucleus assay and comet assay sharing animals for both endpoints[10]. This also reduces animal use. 4) The possible ultimate method is the integration of genotoxic endpoints into the general toxicological study[11]. This not only reduces the number of animals to be used but improves the overall assessment and interpretation of chemical safety to humans by providing toxicity, exposure, and metabolic information in the context of the genotoxicity assay.

Assay systems used for regulatory safety assessments should be well validated and widely used for a long period. On the one hand, standardization of methods for research oriented studies is not critical. On the other hand, for regulatory science, the assay methods to be used must be well-characterized and standardized, including characterization of limitations and the possibility of false-positive/false-negative results. Validation studies should be designed based on the established strategy and should evaluate the technical aspects of the method, intra- and inter-laboratory reproducibility, and performance to detect genotoxic chemicals and also not to detect non-genotoxic chemicals[12,13].

Strategy of Assessment and Interpretation

1) Data Quality

At the FSC, risk assessment of food and food related chemicals, including agricultural chemicals, is routinely undertaken. One of the most important criteria is that the assessment should be done using high quality test data, especially when the data are obtained from the public literature. Adherence to test guideline (TG) and good laboratory practice (GLP) cannot fully guarantee the quality of data, but these are minimum requirements of qualified data. In the case of assessment of new agricultural chemicals, the majority of data are obtained using TG and done under conditions of GLP compliance, while in the case of food additives, it is often necessary to use published data obtained without consideration of TG nor GLP. Moreover, some chemicals may need to be assessed without any final reports of tests on the chemicals, using only the evaluation reports conducted by overseas authorities.

2) Expert Judgment and Low-dose Assessment

Some low level food additive chemicals, especially flavoring chemicals that have been evaluated and used in overseas countries, require the use of a strategy that uses only information about structural alerts, mode of action (MOA), and overall weight of evidence (WOE)[14] together with an established threshold of toxicological concern (TTC)[15–17]. It is best to conduct risk assessments that consider mechanisms of all toxicological endpoints, referred to as mechanistic

risk characterization. It is, however, usually difficult to explain all toxicological effects by credible mechanisms. It is also difficult to assess weak/marginal effects biologically, although statistical evaluation can be done especially at very high dose levels. For risk assessment, exposure analysis is important especially at low dose levels, to establish an acceptable Margin of Exposure (MOE)[18].

3) Threshold

One important consideration in genotoxicity risk assessment is the theoretical lack of threshold for DNA-reactive chemicals. Theoretically, reactivity is based on the probability of collision between DNA and the chemical substance. This cannot result in a "0" probability of introducing a lesion that could theoretically cause, for example, a cancer or birth defect. Organisms, however, have many defense mechanisms against genotoxic events. For example, DNA repair mechanisms rapidly remove lesions on the DNA to restore normal DNA. Such protective mechanisms suggest that even agents that directly introduce DNA lesions may have a threshold of exposure below which the probability of producing a health effect is insignificantly small.

Kirsch-Volders, *et al.* (2000)[19] proposed simple definitions for "real" threshold and "alleged" threshold as follows:

"Real'' threshold: a concentration/dose below which the measured effect does not occur.

"Alleged" threshold: a concentration/dose below which the measured effect does occur, but cannot be detected, because the system is not sufficiently sensitive to discriminate it from spontaneous events.

Now it is widely accepted that the genotoxic chemicals that do not target DNA directly have thresholds, at least a practical threshold[20]. For example, spindle poisons, which induce numerical chromosomal aberration, target mitotic apparatus[21], topoisomerase family, which disturbs the fidelity of DNA unwinding and resulting in infidelity of DNA duplication[22,23], the imbalance of nucleotides in cells which also disturbs the fidelity of DNA duplication[24,25], *etc.*, are thought to have such thresholds. Whenever the mechanism of mutagenesis is based on lesions in biomolecules other than DNA, it is generally accepted that a threshold can be identified.

For risk characterization, it is important how the threshold of genotoxicity is considered, especially in the case of direct-acting mutagens that interact with DNA. As mentioned above, mutagens that target macromolecules other than DNA can be assessed by considering exposure level relative to a defined threshold. Although international worldwide agreement has not yet been obtained to accept a threshold concept for direct DNA-reactive mutagens, appropriate approaches for estimating acceptable exposure levels to such mutagens are being discussed by international expert committees. As mentioned in the former section, there is already substantial evidence to demonstrate practical thresholds for at least some directly DNA-reactive mutagens[26,27]. We think it is possible to assess such mutagens in the same way as other toxicological effects that exhibit thresholds[28,29], by determining a virtually safe dose (VSD), ensuring levels As Low As Reasonably Achievable (ALARA) in most cases, and/or establishing appropriate TTCs.

There are several cases in which evidence has shown a threshold concentration/dose, at least a practical threshold, below which the measured effect does not occur although the exposure is enough for some other effects occur[26,27]. These cases include observations of DNA damage, bacterial gene mutation, and chromosomal aberrations. The *rec* assay provides a good example (**Fig. 1**). The test chemical is spotted on the filter paper (black circle on the right) and diffuses into the plate making a concentration gradient. Repair deficient *Bacillus subtilis* strain (M45) cannot survive in an area close to the test chemical spotted site where the concentration is high. In contrast, the repair proficient strain (H17) survives at the area even close to the spotted site where the chemical exists at higher concentration. This is evidence to show that there are protective mechanisms that would be expected to result in a threshold at low exposure levels.

Kirsch-Volders, *et al.*[19] discussed the alleged threshold because any assay system may not be sufficiently sensitive to detect the endpoint at very low concentration/dose levels. However, Asano, *et al.* showed that increasing the number of cells analyzed in the peripheral blood micronucleus assay up to 1 million cells per animal by flow cytometer on three model chemicals still did not demonstrate an effect at low concentration (**Fig. 2**)[30]. Sensitivity of course depends on the unit evaluated, numbers of cells or animals. Although it may be possible to increase sensitivity using more animals or cells, the important issue is the biological relevance of minimal effects. Animal welfare considerations also place a limit on the extent to which numbers of animals are increased to obtain small, and possibly biologically insignificant, increases in sensitivity.

These apparent threshold phenomena are also observed in the bacterial reverse mutation (**Fig. 3**). **Fig. 3** shows the outcomes of the Ames assay using TA1535 and YG7108, which is an O^6-methylguanine DNA methltransferase-deficient strain. The YG7108 strain induced reverse mutation by *N*-methyl-*N*-nitro-nitrosoguanidine at concentration levels where the TA1535 strain did not respond[26]. Finally, the authors would like to propose the following definitions of threshold, including the case of DNA direct-acting mutagens:

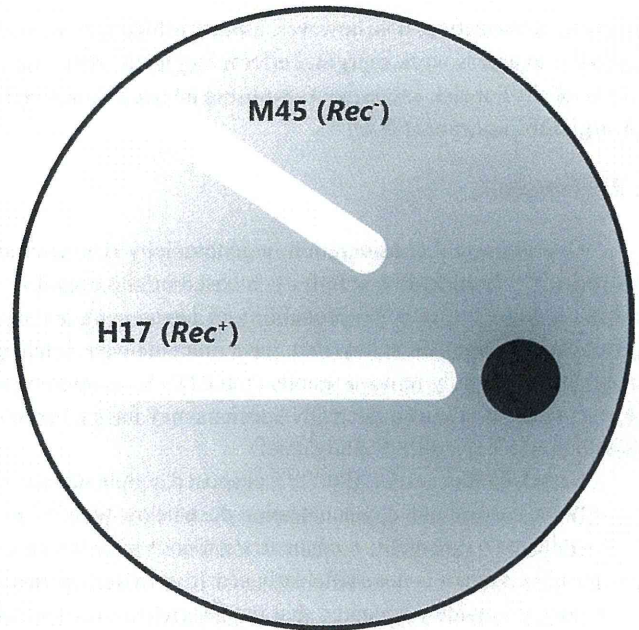

Fig. 1. *rec*-assay has been used widely in Japan to assess DNA damage using *Bacillus subtilis* wild strain H17 with repair capacity and strain M45 deficient DNA repair.

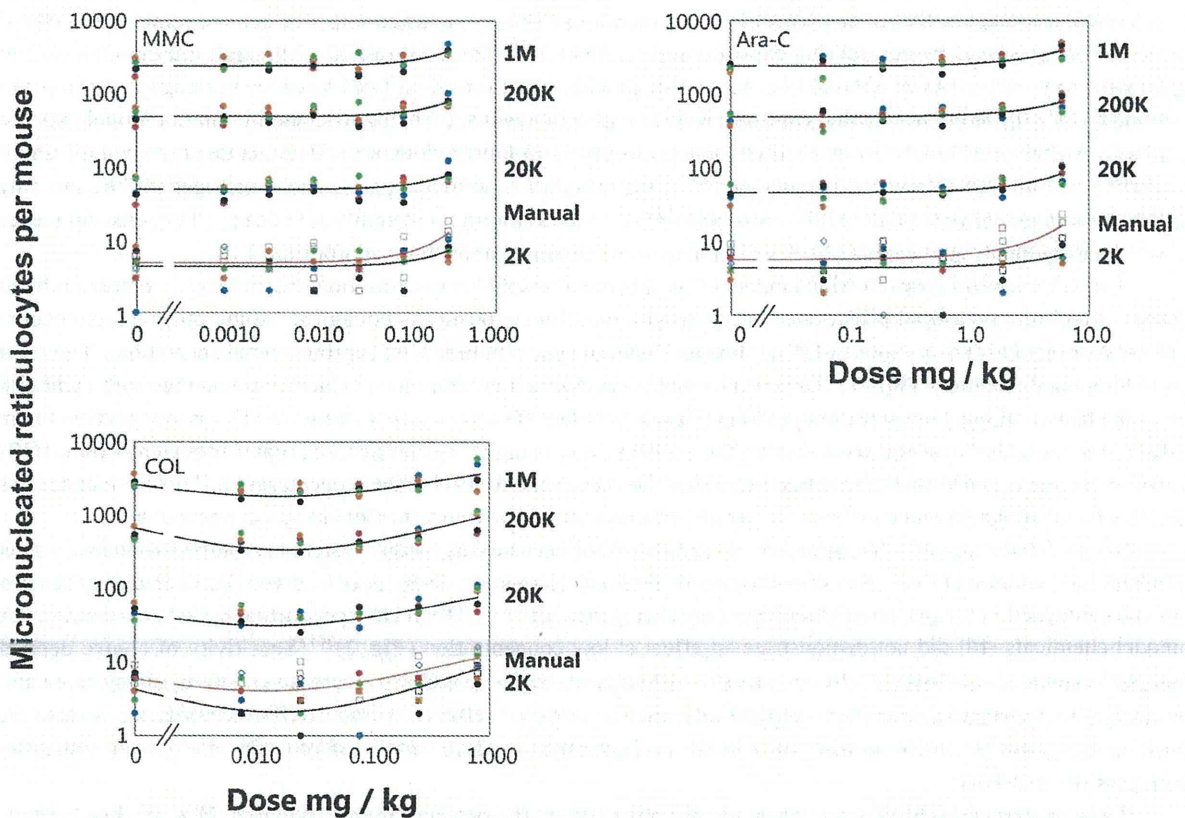

Fig. 2. Mouse micronucleus assay using 3 model chemicals with different mode of action analyzed from 2000 cells per animal manually (red) and flow-cytometry up 1 million cells per animal with flow cytometer (black).

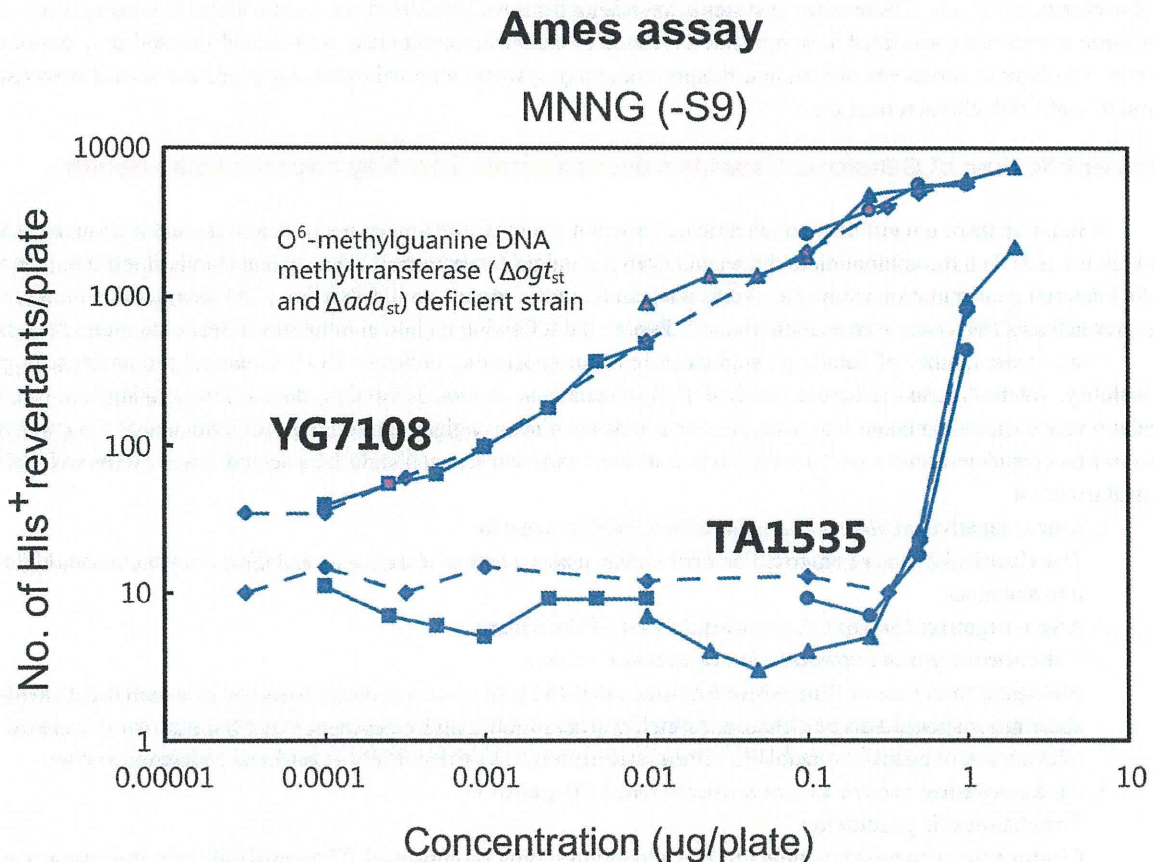

Fig. 3. Dose response curves of reverse gene mutation using TA1535 and O$^{6)}$-methylguanine DNA methltransferase deficient strain YG7108 on *N*-methyl-*N*-nitro-nitrosoguanidine

"Real" threshold: a concentration/dose below which the measured effect does not occur.

"Practical" threshold: a concentration/dose below which the measured effect is not observed (does not occur or cannot be detected by ordinary assay systems) and below which any effect is not considered biologically relevant.

According to the definition, for the purpose of risk characterization, we do not have to consider "no threshold" or "no risk" even for DNA direct acting mutagens. It may be necessary, however, to add larger safety-factors in the case of clearly mutagenic chemicals.

4) Standard Battery and Additional Studies

It is the basic strategy to apply a battery system for identification of hazard of agricultural chemicals. This battery includes a "microbial (*S. typhimurium* and *E. coli*) gene mutation assay (commonly called the Ames test) with and without an exogenous metabolic activation system (OECD TG471)", an "*in vitro* chromosomal aberration assay (OECD TG473)", and an "*in vivo* rodent micronucleus assay (OECD TG474)". The mouse lymphoma TK assay (MLA) (OECD TG476/under preparation) and *in vitro* micronucleus assay (OECD TG478) are alternatives to the *in vitro* chromosomal aberration assay and evaluate equally the induction of structural and numerical chromosomal aberrations by test chemicals. Recently, other *in vivo* assay systems have been, or are being, developed and validated. The transgenic animal model to detect gene mutation (OECD TG488) and the comet assay to detect DNA damages (OECD TG under preparation) have been introduced. Both assays can be applied to any organs of animals, and thus it is possible to assess these endpoints in the organ that is the target of carcinogenicity. Moreover, the development of the micronucleus assay in tissues other than bone marrow has been achieved; moreover it can target tissues other than hematopoietic tissue, *e.g.*, testes, skin, liver, and digestive tracts[8)].

There are many other assay systems to detect genotoxic events. For example, gene mutation assays using insects, the sperm abnormality assay, cytogenetic assays using plants, the gene-conversion assay using yeast, the sister chroma-

tid exchange assay, *etc.* These assay systems have neither been well validated nor used widely. Accordingly, the results of these assays are considered as supportive evidence of chemical genotoxicity and should be used on a case-by-case basis. The basic components of standard battery and assay systems with validated test guideline should be considered first to make risk characterization.

5) Identification of Genotoxic Hazards from Chemicals That May Impact Human Health

Standard hazard identification of chemicals for genotoxicity as the first step of risk assessment is shown in **Table 2**, which is based on the combination of the results from a standard test battery. As the current standard test battery consists of a bacterial gene mutation assay (*i.e.*, Ames test), an *in vitro* chromosomal aberration (CA) assay, and an *in vivo* rodent micronucleus (MN) assay (see section 4) and **Table 1**), the following eight combinations of test outcomes are possible:

In all cases, quality of data (*e.g.*, application of appropriate guidelines or GLP), chemical properties (*e.g.*, purity, solubility, volatility)/class/structure, study design, mechanism or mode of action, dose-effect relationship, or biological relevance should be taken into account. For additional tests, weight and/or strength of evidence of the existing data should be considered on a case by case basis, and the additional test(s) should be selected from within well-validated standard tests.

1. Ames, negative; *in vitro* CA, negative; *in vivo* MN, negative
   The chemical is not genotoxic. For final conclusion, quality of data (*i.e.*, reliability, relevance) should be taken into account.
2. Ames, negative; *in vitro* CA, positive; *in vivo* MN, negative
   The chemical is not considered to be genotoxic *in vivo.*
   Biological relevance of the positive findings should be taken into account. Structural or numerical chromosomal aberrations should also be checked. Actually, there should also be evidence that bone marrow is exposed to the relevant active agent or metabolite if the conclusion is to be that it is not considered genotoxic *in vivo.*
3. Ames, negative; *in vitro* CA, negative; *in vivo* MN, positive
   The chemical is genotoxic.
   Clastogenic or aneugenic effect in MN induction should be considered. The possibility of non-genotoxic mechanism in MN induction is also taken into account on a case by case basis. Expert judgment is needed.
4. Ames, negative; *in vitro* CA, positive; *in vivo* MN, positive
   The chemical is genotoxic.
   Germ cell mutagenicity test might be considered on a case by case basis. Or, evaluation of effect to the germ cells in data from reproductive toxicity studies might be considered. Expert judgment is needed.
5. Ames, positive; *in vitro* CA, negative; *in vivo* MN, negative
   The chemical is concluded to be neither genotoxic nor genotoxic *in vivo.*
   Consider specific bacterial metabolism of the chemical for positive results in the Ames test. The case needs further investigation *in vitro* and/or second *in vivo* test. If a negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic. If positive, the chemical is genotoxic and a germ cell mutagenicity test should be considered. Expert judgment is needed.
6. Ames, positive; *in vitro* CA, positive; *in vivo* MN, negative
   The chemical is concluded to be neither genotoxic nor genotoxic *in vivo.*
   Structural or numerical chromosomal aberrations should be checked. Need second *in vivo* test. If a negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic *in vivo.* If positive, the chemical is genotoxic and a germ cell mutagenicity test should be considered. Expert judgment is needed.
7. Ames, positive; *in vitro* CA, negative; *in vivo* MN, positive
   The chemical is genotoxic.
   A second *in vivo* test is required. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered to cause gene mutation *in vivo.* If positive, a germ cell mutagenicity test should be considered. Expert judgment is needed.
8. Ames, positive; *in vitro* CA, positive; *in vivo* MN, positive
   The chemical is genotoxic, and is a possible germ cell mutagen.
   A germ cell mutagenicity test should be considered. If a positive result is obtained, the chemical is a germ cell mutagen and possible heritable mutagen. Expert judgment is needed.

Table 2. Genotoxic Hazard Categorization of Chemical Based on the Results of the Test Battery

No.	Ames	*In vitro* CA	*In vivo* MN	Judgement	Need for additional test(s)	Explanation
1	−	−	−	Not genotoxic	No	The chemical is not genotoxic. Quality of data (*i.e.*, reliability, relevance) should be taken into account.
2	−	+	−	Not genotoxic	No	The chemical is not considered to be genotoxic *in vivo* . Structural or numerical chromosomal aberrations should be checked. Bone marrow exposure to the chemical or metabolite should also be checked.
3	−	−	+	Genotoxic	No	The chemical is genotoxic. Consider clastogenic or aneugenic effect in MN induction. The possibility of non-genotoxic mechanism in MN induction is taken into account on a case by case basis. Expert judgement is needed
4	−	+	+	Genotoxic	No	The chemical is genotoxic. Germ cell mutagenicity test might be considered on a case by case basis. Or, evaluation of effect to the germ cells in data from reproductive toxicity studies might be considered. Expert judgement is needed.
5	+	−	−	Not concluded yet	Yes	The chemical is not concluded to be either genotoxic or not genotoxic *in vivo* . Consider specific bacterial metabolism of the chemical for positive result in the Ames test. Need further investigation *in vitro* and/or second *in vivo* test. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic. If positive, the chemical is genotoxic and germ cell mutagenicity test should be considered. Expert judgement is needed.
6	+	+	−	Not concluded yet	Yes	The chemical is not concluded to be genotoxic *in vivo* or not. Structural or numerical chromosomal aberrations should bechekced. Need second *in vivo* test. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered genotoxic *in vivo* . If positive, the chemical is genotoxic and germ cell mutagenicity test should be considered. Expert judgement is needed.
7	+	−	+	Genotoxic	Yes	The chemical is genotoxic. Need for second *in vivo* test. If negative result was obtained in the *in vivo* transgenic gene mutation model or *in vivo* comet assay in the suitable organs, the chemical is not considered to cause gene mutation *in vivo* . If positive, germ cell mutagenicity test should be considered. Expert judgement is needed.
8	+	+	+	Genotoxic	Yes	The chemical is genotoxic, and is a possible germ cell mutagen. Germ cell mutagenicity test should be considered. If positive result is obtained, the chemical is a germ cell mutagen and possible heritable mutagen. Expert judgement is needed.

In all cases, quality of data (*e.g.*, application of guideline or GLP), chemical properties (*e.g.*, purity, solubility, volatility)/class/structure, study design, mechanism or mode of action, dose-effect relationship, or biological relevance should be taken into account. The weight and/or strength of evidence of existing data should be considered for futher testing on a case by case basis. Additional test(s) should be selected from among well-validated standard tests.

If the chemical with possible genotoxic hazard (*e.g.*, No. 3 to 8) is carcinogenic, it might be a genotoxic carcinogen. However, evaluation such as carcinogenic mode of action in the target organ or toxicokinetics data will be needed to determine that it is a "genotoxic" carcinogen. If the chemical is not carcinogenic, genotoxic hazrd of the chemical to human health will be low concern. Even if so, effects to germ cells should be considered on a case by case basis.

If a chemical that is a possible genotoxic hazard (*e.g.*, No. 3 to 8) is carcinogenic, it might be a genotoxic carcinogen. However, evaluation such as carcinogenic mode of action in the target organ or toxicokinetics data will be needed to determine that it is a "genotoxic" carcinogen. If the chemical is not carcinogenic, the potential genotoxic hazard of the chemical to human health will be of low concern. Even if so, its effect to germ cells should be considered on a case by case basis.

For risk assessment of genotoxic hazard with respect to human health, exposure assessment and mode of action should be taken into account. Determination of a VSD, the TTC, and MOA or MOE will be considered as appropriate.

Discussion

The risk characterization should include proper hazard identification, dose-response relationship analysis, and consideration of exposure levels. Genotoxicity has been evaluated mainly by hazard identification in the course of chemical risk assessment. However, qualitative assessments should be considered in combination with evaluation of actual exposure levels in the context of the exposure-response relationship and with consideration of the practical threshold concept and agreed-upon exposure thresholds of concern. Generally, there is tendency to detect genotoxicity of chemicals at high concentrations/dose levels. The biological relevance of observed effects and the possibility to extrapolate to the actual exposure levels to humans should also be considered. To detect genotoxicity, there are many *in vitro* assay systems but we do not have good tools to convert from the results in *in vitro* assays to the *in vivo* situation—especially the extrapolations required to estimate the human ADI. *In vitro* assays may play an important role in characterizing hazard and defining mechanisms, thereby supporting the principals of animal welfare, but for accurate risk characterization *in vivo* assay systems play a toxicologically more important role. New *in vivo* assay systems and integration of multiple genetic endpoints into individual assays, including standard toxicological assays, can minimize animal use while providing the information necessary for accurate risk characterization[7,31)]. It is not necessary to discuss the importance of the concept of threshold. It is well accepted that there are thresholds for genotoxicants that target macromolecules other than DNA. While the existence of thresholds for genotoxicants that react directly with DNA is still under discussion, there is evidence showing the existence of thresholds, at least practical thresholds, for some direct acting genotoxic chemicals. In many cases in the field of toxicology, the TTC concept is accepted as a part of chemical safety assessment[20,32,33)]. The TTC concept might also be applicable to the assessment of genotoxicity in the same manner as with other toxicological endpoints. The concept of threshold is also relevant to the strategy of assessing the safety of complex mixtures. People are exposed to many kinds of chemicals concomitantly, but it is general practice to assess each single chemical independently. There is no clear evidence to show that complex mixtures give more potent genotoxicity than additive effects. If we accept the existence of threshold, even practical one, it makes easy to expand the idea to the complex mixture simply based on the exposure levels of the component.

For risk characterization, sound scientific data and reasoning are critical, but fairness and transparency are also important. These factors are especially important for risk communication, which is just as important as risk assessment and risk management. Guidance for risk characterization must include fairness and transparency among the teams that perform risk-based assessments of chemical safety.

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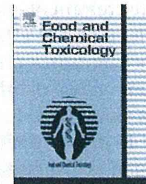

An antioxidant, N,N′-diphenyl-p-phenylenediamine (DPPD), affects labor and delivery in rats: A 28-day repeated dose test and reproduction/developmental toxicity test

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ABSTRACT

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

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1. Introduction

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

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

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

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

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

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

2. Materials and methods

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

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

2.1. Animals

2.1.1. The 28-day repeated dose study

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

2.1.2. The reproduction/developmental study

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

2.2. Chemicals and dosing

2.2.1. The 28-day repeated dose study

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

2.2.2. The reproduction/developmental study

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

2.3. Observations

2.3.1. The 28-day repeated dose study

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

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

2.3.2. The reproduction/developmental study

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

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

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

2.4. Data analysis

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

2.5. Evaluation of bilirubin measurements by the diazo method

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

3. Results

3.1. The 28-day repeated dose study

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

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

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

3.2. The reproduction/developmental study

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conflict of Interest

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

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Original Article

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

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

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

Animals and housing conditions

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

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

Chemicals and dosing

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

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

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

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

Observations

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

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

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

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

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

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

Data analysis

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

RESULTS

Parental toxicity

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

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

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

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

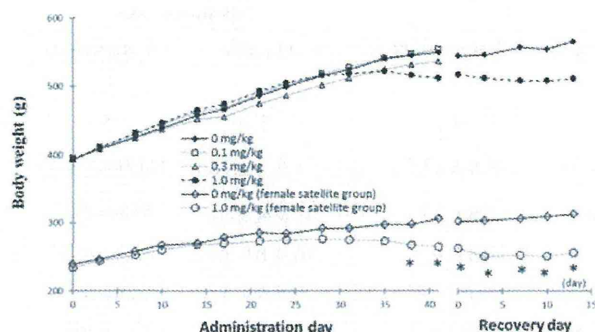

Fig. 1. Body weight of males in main groups and satellite groups for recovery period and females in satellite groups.
*: Significantly different from the control, $p \leqq 0.05$.

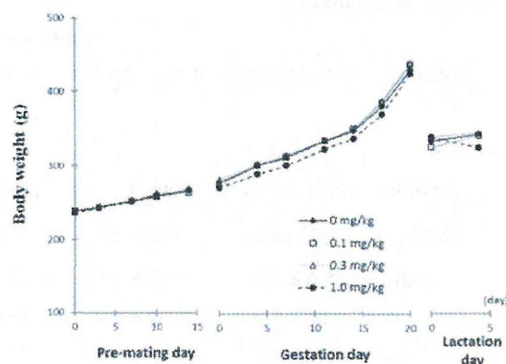

Fig. 2. Body weight of females in main groups.

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

Reproductive and developmental findings

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

DISCUSSION

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