most recent body weight. Body weights on day 6 and day 20 of gestation are 300–320 g and 400–420 g, respectively, in SD rats. When the dosage volume is calculated based on the recent body weight, dams will be exposed to overdose (approximately 1.3 times) and excess toxicity to dams must be noted.

17. Paragraph 21
A marginal note * is to be incorporated into the text because this is an important item.

18. Paragraph 24
Delete 'secretion and' in line 3 (duplicated).

19. Paragraph 27
PND 21 is to be PND 22.

Measurement of food consumption is recommended at administration via other routes than diet since food consumption is an important indicator of maternal general toxicity.

20. Paragraph 31
The headline 'Developmental landmarks' is to be 'Physical and developmental landmarks' since body weight, described in paragraph 31, is certainly an indicator of physical development.

'Pinna reflex' is to be 'Pinna detachment'.

Add eye opening since it is an important index related to motor activity.

21. Paragraph 32
The following reference is to be cited in explanation of the usefulness of postcoital age: Tachibana T., Narita H., Ogawa T., Tanimura T. (1998) Using postnatal age to determine test dates leads to misinterpretation when treatments alter gestation length: Results from a collaborative behavioral teratology study in Japan. Neurotoxicol Teratol. 20: 449–457.

Table 3 should be carefully revised since neuropathological examination on PND 11 is no longer routinely recommended. 'Age Period' is to be 'Age period'. [Before PND 21] is to be [At and before PND 21] since PND 21 is the last day of the preweaning period. [PND 21–59(a)] is [PND 22–59(a)]. In the row of physical development, 'weekly' is to be at the level of Body weight (one line downward). In the row of Brain weight and Neuropathology, delete 'at PND 22' in the column of Preweaning since preweaning ends at PND 21. Only a remark (b) may remain in this place (for examination on PND 11). Delete 'optional' in the column of Adolescence. In Note (a), weaning (generally PND 21) is weaning (generally PND 22), and (PND 23–24) should be (PND 24–25).

22. Paragraph 33
Delete the heading 'Physical development'. The reason is given in comment 19.

It is suggested that this paragraph is moved before paragraphs 31 and 32, since the counting and sexing of live pups are the first steps for offspring observation.

23. Paragraph 34
Surface righting, cliff avoidance and swimming development should be added as examples. Also, give pertinent literature on these tests. Swimming is an especially good indicator of behavioral ontogeny.

24. Paragraph 35
The phrase 'preweaning and adult age' in line 1 should be 'preweaning, adolescence and young adult age', according to Table 3.

It is important to minimize maternal stress at the test of motor activity. Practically, the manipulation of separating the pups from the mother and returning them to the cage should be performed as gently as possible. This caution may be applied at other preweaning tests such as body weight measurement.

The description of 'Among the variables . . .' in lines 16–18 may be also applied to tests other than motor activity. Therefore, these statements should be placed in the appropriate earlier paragraphs as a general caution.

An explanation regarding the phrase '1–3 times' is needed (third line from the bottom, second column in Table 3).

25. Paragraph 36
Rotarod, open field and olfactory orientation tests are to be added as examples. As for a reference of olfactory orientation, Gregory EH, Pfaff DW. (1971) Development of olfactory guided behavior in infant rat. Physiol Behav. 6 : 573–576, is suggested.

References should be separately given for each test for the readers' convenience.

26. Paragraph 37
The headline 'Learning and memory tests' should be 'Learning and memory tests (Cognitive function tests)' or 'Cognitive function tests' (Refer to Tables 1–3).

The Biel maze (multiple T-water maze) should be added as an example. The shuttle box avoidance test (active avoidance) may be also added. Pertinent literature on these tests is also to be described.

Two or more different categories of learning and memory tests may be planned to reveal the nature of disturbances of learning and memory.

27. Paragraph 38
PND 21 is to be PND 22.

28. Paragraph 41
Some explanation of GFAP is necessary, together with references, or '(e.g. GFAP)' should be deleted.

29. Paragraph 43
The phrase '(tectum, tegmentum, and cerebral peduncles)' should be deleted.

30. Paragraph 44
The phrase 'typical of the adult brain' is not understandable. Are some words are missing?

31. Paragraph 46
The sentence 'While the use . . .' in lines 7–9 can be rewritten more simply. For instance, 'It is preferable that a pathologist who is unaware of the treatment information scores the slides to substantiate the dose–response relationship'.

32. Paragraph 48
Delete 'perinatal' in line 1. The name of this guideline is simply developmental neurotoxicity study.

The phrase 'human studies, case reports', is to be changed to 'human epidemiological studies or case reports', since case report is one of the categories of human studies.

33. Paragraph 47 after Test report
47 should be 51.

Insert water after diet in the 4th item of Test animals.

The phrase 'reflex ontogeny' in the 9th item of Results must be 'behavioral ontogeny'.

34. Literature
Try to unify the style of the reference presentation. In particular, the writing of journal titles should be uniform (e.g. compare 5 and 7 for Environ Health Perspect and italic presentations such as 28 and 32). It is recommended that the abbreviation of journal titles follows the PubMed, NLM style.

The presentation of the authors' names is also confusing (e.g. 5 vs. 9).

The placement of the published year is also variable (e.g. 3, 5 and 12).

Put a space between 18 and 19. Delete one space after 67.

Some good references as background information can be found in Massaro EJ. (2002) Handbook of neurotoxicology. Vols I and II. Humana Press, Totowa. The four papers in vol II (Henck JW, Rice SA, Cappon GD and Stump DG, and Tilson HA) are very valuable.

35. Appendix A
Totally redraw Fig. 1 according to the description in Tables 2 and 3, and also clarify in the figure legend that this scheme is based on Tables 2 and 3. A suggestion is attached.

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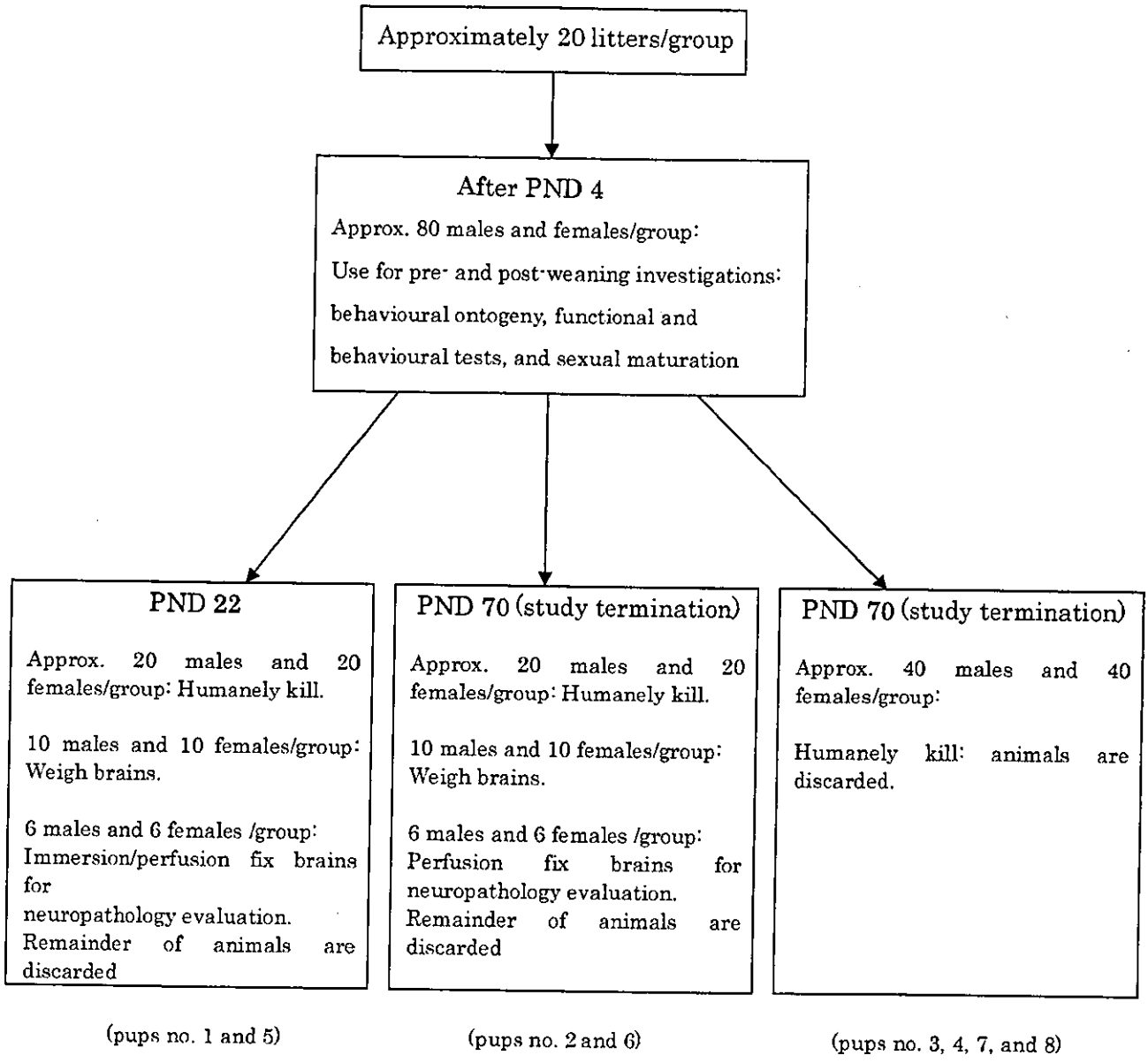
APPENDIX A

Fig. 1 Example of the testing scheme for assignment of animals for functional/behavioral tests, neuropathology evaluation, and brain weights, as described in paragraphs 13, 14, and 15. This diagram is based on the description in Tables 2 and 3. (PND = postnatal day).



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REVISION AND ESTABLISHMENT OF JAPANESE DRINKING WATER QUALITY GUIDELINES FOR DI(2-ETHYLHEXYL) PHTHALATE, TOLUENE AND VINYL CHLORIDE – DIFFERENCES FROM THE LATEST WHO GUIDELINE DRAFTS –

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ABSTRACT — The revision of the Japanese drinking water quality guidelines was established in May 2003. The WHO drinking water quality guidelines for the 3rd edition were also revised and the draft has been open to the public since last year. Most guideline values of each chemical in both Japan and WHO were quite similar; however, there are different overt values for three chemicals. In this short communication, we describe them and discuss the reason for taking the different toxicity endpoints and derivation method for these three chemicals, di(2-ethylhexyl) phthalate, toluene and vinyl chloride.

KEY WORDS: Drinking water quality guidelines, Di(2-ethylhexyl) phthalate, Toluene, Vinyl chloride

INTRODUCTION

The revision of the Japanese drinking water quality guideline was established in May 2003 and implemented on May 2004. In this revising, regulated chemical lists were modified because of the past detection trend or exposure prospect. The chemicals already listed in the previous version were reevaluated and chemicals newly listed in this revision were assessed with the latest toxicity information. The Japanese guidelines derivation has referred to the concurrent WHO revision, and both of the general principles for the guidelines (GD) derivation are almost the same. Although most guideline values of chemicals in Japan were similar to those of WHO, some minor differences between WHO and Japan exist because of different default body weight application for the guideline calculation (50 kg/Japan vs. 60 kg/WHO). Furthermore, in some cases, different drinking water contribution ratios (allocation) to total exposure media were used for the guideline values calculation from tolerable daily intake (TDI) on account of the regional chemical exposure assessment. These differences were not owing to the difference of health risk assessment per se. However, the different guideline values for di(2-ethylhexyl) phthalate (DEHP), toluene and vinyl chloride between the Japanese guidelines revision (2003) and the latest rolling revision of WHO drinking water quality guideline were mainly caused by the health risk assessment variation. In this short communication, we describe the reason for taking the different toxicity endpoints or derivation method of the guidelines. Table 1 shows the guideline values for three chemicals of the WHO 2nd edition (WHO, 1996) established in 1994 and rolling revision in 2003, and previous and present Japanese versions.

DERIVATION OF GUIDELINE VALUES

Di(2-ethylhexyl) phthalate (DEHP)

As the guideline value of DEHP by the WHO 2nd edition, 0.008 mg/L was derived from a no observed

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adverse effect level (NOAEL) of 2.5 mg/kg/day in a rat feeding study (Morton, 1979) for 7 days according to no induction of hepatic peroxisome proliferation. The hepatic tumors were considered to be the most critical endpoint and hepatic peroxisome proliferation to be closely related to the carcinogenic mechanism. An uncertainty factor of 100 was applied only because of the animal most sensitive to peroxisome proliferation, and the allocation of 1% that was used as DEHP is generally not contained in food (WHO, 1996). For the latest WHO assessment, the guideline value of DEHP was not changed from the 2nd edition, because it was not listed for the detailed reevaluation.

In 1994, the Japanese government decided to use the same data and derivation method for domestic drinking water guidelines except for 10% allocation and 50 kg instead of 60 kg for human body weight. The guideline value was 0.06 mg/L.

However, the Japanese government established a TDI for DEHP in 2001 when high contamination was found in some specific foods and the health risk was deeply concerned (Koizumi *et al.*, 2001). In this assessment, TDI ranging from 40 to 140 μg/kg/day was established from a NOAEL of 3.7 mg/kg/day for testicular toxicity in a rat study (Poon *et al.*, 1997) and 14 mg/kg/day for reproductive toxicity in a mouse study (Lamb *et al.*, 1987), respectively, applying an uncertainty factor of 100 for intra- and interspecies differences. As for hepatic peroxisome proliferation, it was taken out for extrapolation to humans because IARC (2000) concluded that the hepatic tumor due to DEHP in rodents (in association with peroxisome proliferation) is not relevant to other animal species including humans (Group 3). Although it is clearly shown that there are strong species differences in testicular toxicity such as severely toxic in rats and guinea pigs, weakly in mice but not in hamsters, marmosets and cynomolgus monkeys, the potential of testicular toxicity in humans cannot be excluded at this moment. Therefore, the guideline of 0.1 mg/L was derived from 40 μg/kg/day of TDI using 10% of allocation, and 2 L of daily water intake for 50 kg body weight of the Japanese population.

Toluene

In 1994, WHO tried to re-assess the toxicity data of toluene and made the same conclusion as the previous value, 0.7 mg/L. A TDI of 0.223 mg/kg/day was derived using the lowest observed adverse effect level (LOAEL) for marginal hepatotoxicity in mice of 312 mg/kg/day (equivalent to 223 mg/kg/day, as there were 5 days per week) (NTP, 1990) and applying an uncertainty factor of 1,000 (100 for inter- and intra-species variation and 10 for the short duration of the study and use of a LOAEL instead of a NOAEL). This TDI yields a guideline value of 0.7 mg/L (rounded figure), allocating 10% of the TDI to drinking-water (WHO, 1996).

The Japanese government used the same data and derivation method for the domestic drinking water guideline except for 50 kg instead of 60 kg for human body weight. The guideline value was established as 0.6 mg/L in 1994.

For the new revision, the Japanese Government used a different toxicity endpoint, neurotoxicity, which is the most typical toxicity for toluene. In the case of neurotoxicity with histopathological changes as well as carcinogenicity and developmental toxicity without maternal toxicity, some additional uncertainty factors should be considered to derive a TDI. Toluene showed neuropathological effects in the brain consisting of neuronal cell necrosis in the dentate gyrus and Ammon's horn of the hippocampus at 1250 and 2500 mg/kg/day. NOAEL for neurotoxicity was 625 mg/kg/day (equivalent to 446 mg/kg/day, as there were 5 days per week) and a TDI of 0.0892 mg/kg/day was derived by application of an uncertainty factor of 5,000 including additional uncertainty factors of 5 for short exposure duration and 10 for neuropathological changes. This TDI yields a guideline value of 0.2 mg/L (rounded figure), allocating 10% of the TDI to drinking-water.

Table 1. Comparison of three guideline values (mg/L) between WHO and Japanese drinking water.

	WHO Guideline		Japanese Guideline	
	1994 (2nd ed.)	Revising 2003 (3rd ed.)	1994	2003
DEHP	0.008	0.008*	0.06	0.1
Toluene	0.7	0.7	0.6	0.2
Vinyl chloride	0.005	0.0003	No setting	0.002

*: No detailed reevaluation draft.

Vinyl chloride

It has been generally accepted that a mathematical model such as a linearized multistage is appropriate to estimate a low-dose cancer risk of a genotoxic carcinogen. There is sufficient evidence showing that vinyl chloride is a multiple site carcinogen and its metabolites are genotoxicants. Table 2 shows the incidences of hepatic tumor-related lesions in studies reported by Feron *et al.* (1981) and Til *et al.* (1991).

In the WHO 2nd edition, a linearized multistage model was applied to the incidence of angiosarcomas in female rats which was reported by Feron *et al.* (1981) only because of a good relationship with the human incidence at that time. An excess cancer risk at 10^{-5} was 0.010 mg/L. The guideline value was 0.005 mg/L, applying an uncertainty factor of 2 for double risk by exposure from birth (WHO, 1996).

On the other hand, in the WHO rolling revision, total liver tumors (angiosarcomas, hepatocellular carcinomas and neoplastic nodules) from the same study are incorporated to derive the guideline value including conversion to human equivalent doses (using the physiologically based pharmacokinetic (PBPK) model of U.S. EPA, 2000, Clewell *et al.*, 2001). A linear low-dose extrapolation was conducted by drawing a straight line between 10% of the low estimate dose (Benchmark dose approach) and the origin (zero dose). The results were nearly identical with those derived using the linearized multistage model. The concentrations in drinking-water of 0.0005 mg/L were calculated as being associated with excess risks of liver tumors of 10^{-5} for lifetime exposure beginning at adulthood. Exposure from birth would double this risk (U.S. EPA, 2000). This would result in a rounded guideline value of 0.0003 mg/L for a theoretical risk of 10^{-5}.

The guideline for vinyl chloride was not set in the previous Japanese guideline.

As described in Table 2, Feron *et al.* (1981) obtained clear evidence of carcinogenicity in rat liver in a three-dose setting study but the low dose of 1.7 mg/kg/day was still carcinogenic in female rats. The same group (Til *et al.*, 1991) conducted a further study up to 0.014 mg/kg/day and showed that the middle dose of 0.13 mg/kg/day was a non-carcinogenic dose. As both studies had been conducted under mostly the same experimental conditions, these data would be considered from a single study with doses ranging 1,000 times. For derivation of the newly established

Table 2. Summary incidence of hepatic tumor-related leisions for two rat carcinogenicity studies conducted by the same group.

	Til *et al.*, 1991				Feron *et al.*, 1981			
mg/kg/day	0	0.014	0.13	1.3	0	1.7	5.0	14.1
Male								
Neoplastic	0/99[a]	0/99	0/99	1/49	0/55	1/58	7*/56	23*/59
nodules	(0)[b]	(0)	(0)	(2.0)	(0)	(1.7)	(12.5)	(39.0)
Hepatocellular	0/99	0/99	0/99	3*/49	0/55	1/58	7*/56	23*/59
carcinoma	(0)	(0)	(0)	(6.1)	(0)	(1.7)	(12.5)	(39.0)
Angiosarcomas	0/99	0/99	0/99	1/49	0/55	1/58	2/56	8*/59
	(0)	(0)	(0)	(2.0)	(0)	(1.7)	(3.6)	(13.6)
Female								
Neoplastic	0/98	0/100	1/96	9*/49	2/57	26**/58	39*/59	44*/57
nodules	(0)	(0)	(1.0)	(18.4)	(8.8)	(44.8)	(66.1)	(77.2)
Hepatocellular	1/98	0/100	1/96	3/49	0/57	4*/58	19*/59	29*/57
carcinoma	(1.0)	(0)	(1.0)	(6.1)	(0)	(6.9)	(33.2)	(50.9)
Angiosarcomas	0/98	0/100	0/96	2/49	0/57	0/58	2/59	9*/57
	(0)	(0)	(0)	(4.1)	(0)	(0)	(3.4)	(15.8)
Total liver					2/57	28/58	49/59	56/57
tumors[c]					(8.8)	(48.2)	(83.1)	(98.2)

[a]: Number of lesion-bearing animals / number of analyzed animals.

[b]: Percentages of incidences.

[c]: The total number of animals with tumors derived from US IRIS(2000) / number of analyzed animals.

Statistically significant compared to the controls with *p<0.05 or **p<0.01 was reported in the original articles.

Japanese guideline value, the neoplastic nodules were not taken into account for the following reasons. As there was no diagnosis of nodular hyperplasia in those reports, there is a possibility that the neoplastic nodules may include not only hepatocellular adenoma but also nodular hyperplasia, which is not considered to be a neoplastic leision. The high incidence of neoplastic nodules at 1.7 mg/kg/day in females quickly dropped to less than half at 1.3 mg/kg/day and virtually no incidence at 0.13 mg/kg/day. This dose-response may not be appropriate for extrapolation to low doses. The incidence slope of total liver tumors mostly reflected the high incidence of neoplastic nodules rather than the real cancer incidence. In addition, because hepatocellular carcinomas and angiosarcomas originate from different cells, liver and vascular cells respectively, the evaluation of combined incidences may draw a conflicting conclusion. Therefore, the dose-response incidences of hepatocellular carcinoma in female rats were considered to be most appropriate for application to dose-response analysis, in view of data from the two reports. After dose conversion based on the PBPK model, an excess risk of 10^{-5} by the multistage model was calculated to be 0.0875 mg/kg/day as a virtual safety dose (VSD). The guideline of 0.002 mg/L was derived using 2 L of daily water intake for 50 kg body weight of the Japanese population. The allocation factor was not applied for the mathematical model approach because of large uncertainty caused by highly lower dose extrapolation.

DISCUSSION

Table 3 summarizes the derivation processes of all three chemicals. Although the detailed reevaluation draft for DEHP has not been published in the 3rd WHO water quality guideline, it was presumed that the derivation process would be same as the 2nd edition because were no changed guideline values. The general principle for the derivation of TDI and VSD is the same between Japan and WHO; however, the difference in the choice of critical endpoints leads to varied guideline values. In the Japanese assessment, testicular toxicity of DEHP and neurotoxicity of toluene were used to derive a TDI instead of their hepatotoxicity adopted by WHO. In the case of vinyl chloride, the same critical study was used for the guideline derivation, but the adopted neoplastic endpoints were differ-

Table 3. Summary of guideline value derivation in WHO (3rd ed.) and Japan (2003).

endpoint	NOAEL (mg/kg/day)	uncertainty factor					TDI or VSD* (mg/kg/day)	allocation (%)	body weight (kg)	water consump. (L)	guideline value (mg/L)
		inter-species	intra-species	use of LOAEL	study period	nature of toxicity					
DEHP(WHO)[a]											
hepatic peroxisome proliferation	2.5	10	10				0.025	1	60	2	0.008
DEHP(Japan)											
testicular toxicity	3.7	10	10				0.04	10	50	2	0.1
Toluene(WHO)											
hepatotoxicity	223	10	10	10			0.223	10	60	2	0.7
Toluene(Japan)											
neurotoxicity	446	10	10		5	10	0.0892	10	50	2	0.2
Vinyl chloride(WHO)											
total liver tumors (angiosarcoma, hepatocellular carcinoma and neoplastic nodules)											0.0003¶
Vinyl chloride(Japan)											
hepatocellular carcinoma							0.0875*		50	2	0.002

[a]: Derived from the 2nd edition.

¶: At the initial calculation from experimental animal data, the guideline concentration of 0.0005 mg/L was derived as 10^{-5} excess risk concentration during adulthood. Then the concentration was decreased to half because of doubled risk for exposure from birth.

*: Virtual safety dose corresponding to an excess cancer risk of 10^{-5}.

ent from each other because of the different interpretation on the cancer risk assessment. The adverse effects in experimental animals for the human health assessment are chosen by consideration of appropriate extrapolation to humans, which is expected from the nature of the toxicity, toxicity mechanism, etc. With regard to taking appropriate toxicity endpoints for derivation, the latest Japanese decision is considered to be more suitable on the basis of recent scientific consideration as described before. Because the revisions for the 3rd edition of water quality guidelines in the WHO are still ongoing, the assessment and the guideline value may be changed until the fixed version is published.

As for the derivation of the guideline value from the TDI, the estimation of the exposure contribution ratio (the allocation) is another important issue. In the case of DEHP, both levels of TDIs or NOAELs estimated in Japan and WHO are similar, although the critical endpoints are different. The guideline values were different at one order of degree from each other, because the allocation factor for drinking water of the TDI estimated in WHO was one-tenth of that in Japan. The allocation depends on environmental circumstances as well as chemical physical properties, and local exposure assessment is necessary for the estimation of the allocation factor of the respective chemical. Although the DEHP exposure contribution for drinking water in the WHO 2nd edition was estimated to be considerably lower, the allocation of 10% was applied in Japan as the default value when the exposure assessment was not elucidated.

Given the risk management of drinking water supplied by the Waterworks, the derivation of the guideline values of chemicals may be a regional issue. However, a large amount of drinking water bottled as mineral water has been circulating worldwide and the regulated values of chemicals will also be based on the drinking water guideline. Therefore the need for the international harmonization of chemical risk assessment will be required even more in the future.

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ORIGINAL ARTICLE

Comparative toxicity study of 2,4,6-trinitrophenol (picric acid) in newborn and young rats

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ABSTRACT **The toxicity of oral 2,4,6-trinitrophenol (TNP) was determined in newborn rats, and compared with that in young rats. In newborn rats, males and females were given TNP at 0, 16.3, 81.4 or 407 mg/kg per day on postnatal days (PND) 4–17 for the dose-finding study, and at 0, 4.1, 16.3 or 65.1 mg/kg per day on PND 4–21 for the main study. Deaths, lower body weight (BW) and behavioral changes were found at 81.4 and 407 mg/kg per day in the dose-finding study, and lower BW was observed in males at 65.1 mg/kg per day during the dosing period of the main study. In young rats, 5-week-old males and females were given TNP at 0, 20, 100 or 500 mg/kg per day for 14 days as the dose-finding study and at 0, 4, 20 or 100 mg/kg per day for 28 days as the main study. Deaths were observed at 500 mg/kg per day in the dose-finding study. Deaths or changes in BW were not found at 100 mg/kg per day or less. At 100 mg/kg per day, hemolytic anemia and testicular toxicity were found. In conclusion, toxicity profiles induced by TNP were markedly different between newborn and young rats.**

Key Words: 2, 4, 6-trinitrophenol, newborn rats, picric acid, repeated-dose toxicity, young rats

INTRODUCTION

The adverse effects of environmental chemicals including endocrine disruptors on not only contemporary but also future generations are causing increasing concern. The possible toxic effect of chemicals on fetuses and newborns has aroused great concern among the public, and the protection of fetuses and newborns has become a major scientific and political issue.

Comprehensive statements for children's health, considering their special vulnerability to certain toxic substances, are shown in the US Environmental Protection Agency Children's Environmental Health Yearbook (US EPA 1998). Infants and young children have greater respiratory and circulatory flow rates, as well as energy and fluid requirements than adults, giving rise to a greater potential for respiratory and intestinal exposure to chemicals per unit body weight (BW) (WHO 1986). Children live close to the ground because of their behavioral patterns of play and their height and perform hand-to-mouth activities, which would expose them to much larger amounts of pollutants in dust and soil (US EPA 1998). However, children could be less sensitive than adults to some chemicals (NRC 1993) because infants have more extracellular water that is the only avenue connecting cells with the outside world (Fomon *et al.* 1982), enough amounts of toxic metabolites are not produced in infants due to their immature metabolic capacities (Kearns & Reed 1989), or the developing brain has increased plasticity.

Because of these unique characteristics, children react differently from adults. Differences in susceptibility to toxicants between children and adults may result from a combination of toxicokinetic, toxicodynamic and exposure factors (Schwenk *et al.* 2002). The potential toxic effects of chemicals on children cannot be anticipated using data on adults, and a data set on exposed children is essential for the assessment of children's health. Although gathering information on the toxicity of chemicals in newborns is very important to evaluate children's health, toxicity data on chemical compounds in newborns are limited.

We have already reported the differences in the susceptibility to toxicities of chemicals between newborn and young rats (Koizumi *et al.* 2001, Koizumi 2002, Koizumi 2003;

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Fukuda *et al.* 2004). We demonstrated that the toxic response in newborn rats was at most four times (4-nitrophenol and 2,4-dinitrophenol), approximately three times (3-aminophenol), and three to four times (3-methylphenol) higher than that in young rats. The toxicological profiles of 4-nitrophenol (Koizumi *et al.* 2001), 2,4-dinitrophenol (Koizumi *et al.* 2001), 3-aminophenol(Koizumi *et al.* 2002), and 3-methylphenol (Koizumi *et al.* 2003) were similar in newborn rats and young rats. The nephrotoxicity of tetrabromobisphenol A was specific for newborn rats (Fukuda *et al.* 2004).

2,4,6-Trinitrophenol (TNP) was listed in the Organisation for Economic Co-operation and Development (OECD) High Production Volume Chemical Table in 1999, meaning that it is produced at levels greater than 1000 tonnes per year in at least one OECD member country. TNP is known as picric acid, has a yellow color and is explosive. This compound is used in the production of gunpowder, fireworks, agricultural chemicals and dyes, and is widely used in industry, by the military, and as a research/clinical chemistry reagent. Much of the human toxicity data showed that exposure to picric acid was primarily through inhalation of dust or through skin contact (Wyman *et al.* 1992). This chemical caused irritation of eyes, a transient yellowish appearance, and skin sensitization in humans (Health Council of the Netherlands 2002). Wyman *et al.* (1992) investigated the acute toxicity, distribution, and metabolism of TNP using Fischer 344 rats. The values of oral LD50 in male and female rats were 290 and 200 mg/kg, respectively. TNP was found to bring about severe acidosis during acute intoxication. Recently, a 28-day repeat dose oral toxicity study of this compound in young rats was conducted as part of the Japanese Existing Chemical Safety Program (MHLW 2001), in which the no observed effect level (NOEL) and toxicity profile of chemicals were evaluated.

In the present paper, we re-evaluated the toxicity of TNP in young rats (MHLW 2001), determined the toxicity of TNP in newborn rats, and compared the findings.

MATERIALS AND METHODS

Chemicals

TNP (2,4,6-trinitrophenol, CAS. no. 88-89-1, purity: 81.4%) was obtained from Mitsui Chemicals (Tokyo, Japan) and suspended in a 0.5% CMC-Na (carboxymethyl cellulose sodium salt; Nacalai Tesque, Kyoto, Japan or Iwai Chemicals, Tokyo, Japan) aqueous solution mixed with 0.1% Tween-80 (polyoxyethylene sorbitan monooleate; Nacalai Tesque, Kyoto, Japan or Difco Laboratories, Detroit, USA).

Animals

In the newborn rat study, pregnant SPF Crj:CD(SD)IGS rats (gestation day 13) were purchased from Atsugi Breeding Center, Charles River Japan (Yokohama, Japan) and allowed to deliver spontaneously. The animals were maintained in an environmentally controlled room at 24 ± 2°C with a relative humidity of 55 ± 10% and a 12:12 h light/dark cycle. Newborn rats were separated from dams on postnatal day (PND) 3.

In the young rat study, 4-week-old males and females of the same strain were purchased from the same farm. The animals were maintained in an environmentally controlled room at 22 ± 2°C with a relative humidity of 55 ± 15% and a 12:12 h light/dark cycle.

All animals in the newborn and young rat studies were allowed free access to a sterilized basal diet (MF, Oriental Yeast, Tokyo, Japan) and water. Rats were euthanized by exsanguination under anesthesia using sodium pentobarbital in the newborn rat study and sodium thiopental in the young rat study.

Repeated dose study in newborn rats

Time schedule of the newborn rat studies is shown in Figure 1.

Dose-finding study

Sixteen males and 16 females were randomly selected and assigned to four dose groups, including a control group. Four foster mothers were used. One foster mother suckled the four males and four females. Pups (4/sex per dose) were given TNP by gavage at 0, 16.3, 81.4 or 407 mg (as TNP)/kg per day on PND 4–17 (14 days) and killed on PND 18 after overnight starvation. General condition, BW, hematology, blood biochemistry, necropsy, and organ weights were examined.

Main study

Forty-eight males and 48 females for two autopsy groups (the ends of the dosing period and recovery-maintenance period) were randomly selected and assigned to four dose groups, including a control group. Twelve foster mothers were used. One foster mother suckled the four males and four females up to weaning on PND 22. After weaning, rats of the recovery-maintenance group were individually maintained for 9 weeks. Pups (6/sex per dose) were given TNP by gavage at 0, 4.1, 16.3 or 65.1 mg (as TNP)/kg per day on PND 4–21 (18 days), and killed after overnight starvation following the last treatment. The dosage levels were determined based on the results of the dose-finding study in newborn rats. Recovery-maintenance groups (6/sex per dose) given the same dosages were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks, almost the same age as at the end of the recovery period of the main study of young rats.

General condition was observed two times per day (before and after administration) for pups (separated from each foster mother) and foster mothers during the dosing period, and

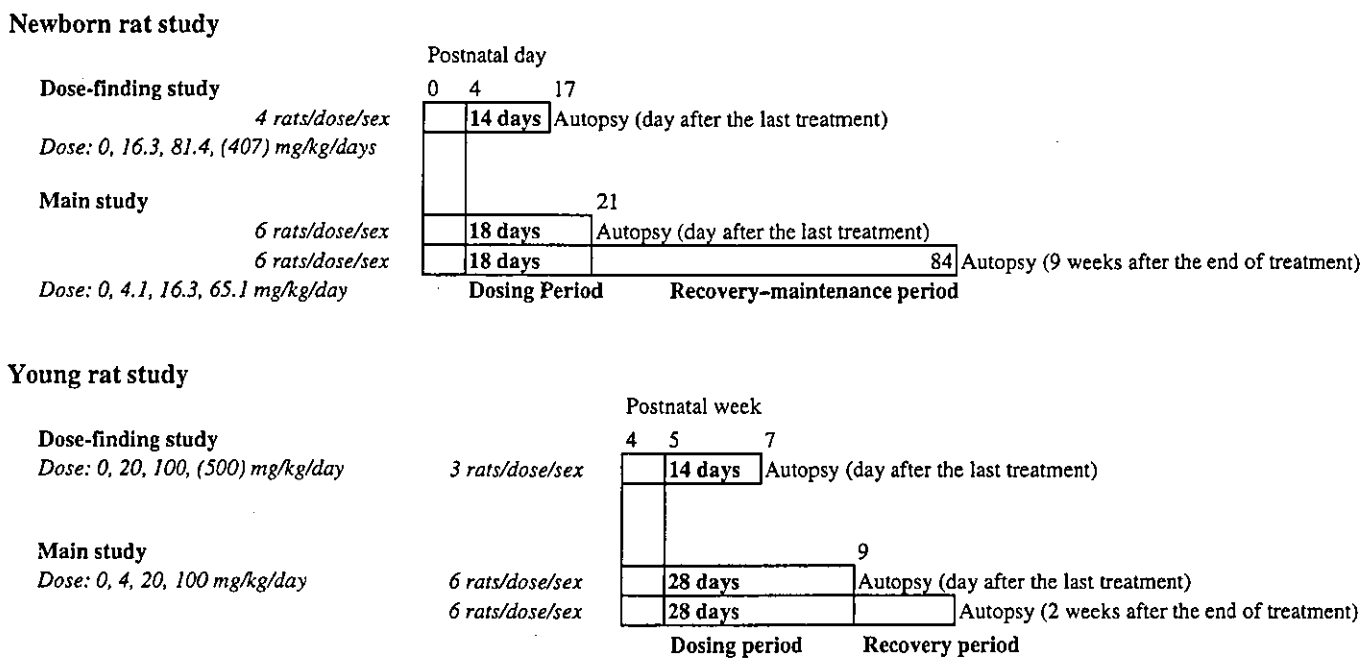

Fig. 1 Time schedule of the newborn and young rat studies.

daily for pups during the recovery-maintenance period. BW and food consumption were measured more than two times per week. All pups were examined for developmental landmarks; pinna detachment on PND 4, piliation on PND 8, incisor eruption on PND 10, gait and eye opening on PND 15, testes descent on PND 21, preputial separation on PND 42, and/or vaginal opening on PND 42. BW was measured on the day of testes descent, preputial separation and/or vaginal opening. All pups were examined for the assessment of reflex ontogeny; surface righting reflex and ipsilateral flexor reflex on PND 5, visual placing response on PND 16, and Preyer's reflex on PND 28.

In urinalysis, color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, urine sediment, specific gravity, osmotic pressure and volume of urine were examined only at the end of the recovery-maintenance period. Rats were killed on PND 22 or PND 85. On the day that the rats were killed, blood was collected from the abdominal vein. Hematological parameters, such as the red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), platelet counts, reticulocyte ratio (Ret), differential leukocyte count, and blood clotting parameters, such as prothrombin time and activated thromboplastin time, were determined. The blood biochemical parameters, such as the total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase, phospholipids, calcium, inorganic phosphorus, sodium, potassium and chloride levels in serum, were also determined. After a gross examination, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenals, thyroids, lungs, testes, epididymides and/or ovaries were weighed. The organs were fixed with 10% buffered formation-phosphate (2.5% glutaraldehyde's prefixation for the eyes, Bouin's prefixation for the testes and epididymis) and paraffin sections were routinely prepared and stained with hematoxylin–eosin for microscopic examination. The study using newborn rats was conducted at Panapharm Laboratories Co., Ltd. (Uto, Japan) under Good Laboratory Practice (GLP) conditions (OECD 1981; MHW 1988).

Repeated dose study in young rats

Time schedule of the young rat studies is shown in Figure 1.

Dose-finding study

Five-week-old rats (3/sex per dose) were given TNP by gavage at 0, 20, 100 or 500 mg (as TNP)/kg per day for 14 days and killed the day following the last administration after overnight starvation. General condition, BW and food consumption, hematology, necropsy, and organ weights were examined.

Main study

Five-week-old rats (6/sex per dose) were given TNP by gavage at 0, 4, 20 or 100 mg (as TNP)/kg per day for 28 days and killed after overnight starvation following the last

treatment. The dosage levels were determined based on the results of the dose-finding study in young rats. Recovery groups (0 or 100 mg/kg per day) (6/sex per dose) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. Rats were examined for general condition, BW, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings. The study using young rats was conducted at Kashima Laboratory, Mitsubishi Chemical Safety Institute Ltd. (Kashima, Japan) under GLP conditions (MHW 1988; OECD 1997).

Statistical analysis

Continuous data were analyzed with Bartlett's test for homogeneity of variance. If the data were homogeneous, Dunnett's test was conducted for group comparisons between control and individual TNP-treated groups. If not homogenous, the data were analyzed using Steel's test. Quantitative data for histopathology were analyzed with Mann–Whitney's *U*-test or Fisher's exact test. In the newborn rat study, the chi-square test was conducted for physical and sexual development and reflex ontogeny. The 0.05 or 0.01 level of probability was used as the criterion for significance.

RESULTS

Repeated dose study in newborn rats (dose-finding study)

Death occurred at 81.4 mg/kg per day in one male on day 3 of the dosing period, two females on days 6 and 7 of the dosing period, and at 407 mg/kg per day in all rats by day 4 of the dosing period. In these dead rats, hypoactivity, bradypnea and hypothermia were observed. Only hypoactivity was found in surviving rats at 81.4 mg/kg per day on days 3, 5, or 8 of the dosing period. Yellowish fur was observed in all TNP-treated rats.

A significantly lower BW (max. 16% decreased) in males, and suppression of weight gain (max. 35% decreased) in females were noted at 81.4 mg/kg per day. The organ weights are summarized in Table 1. At 81.4 mg/kg per day, a significantly higher relative weight of the liver (13% increased) and lower relative weight of the kidney (14% decreased) were observed in males.

No consistent changes related to the administration of TNP in hematological or blood biochemical parameters or necropsy findings were found at any doses.

Repeated dose study in newborn rats (main study)

There were no deaths throughout the experimental period in males and females, even at 65.1 mg/kg per day. Yellowish fur was observed in all TNP-treated rats. A significantly lower BW (max. 7% decreased) was found in males on days 4 and 8 of the dosing period at 65.1 mg/kg per day. During the recovery-maintenance period, no dose-dependent effects on BW and food consumption were observed.

No toxicological effects of TNP on physical development, reflex ontogeny, and sexual maturation were detected at any doses in the newborn rat study.

The organ weights are summarized in Table 1. Significantly higher relative weights of the liver in males and females (13 and 12% increased, respectively) were observed at 65.1 mg/kg per day.

No consistent changes related to the administration of TNP were found in hematological or biochemical parameters, urinalysis or histopathological findings.

Repeated dose study in young rats (dose-finding study)

All male rats and one female rat at 500 mg/kg per day died by day 2 of the dosing period. No death was found at 20 and 100 mg/kg per day. Yellowish fur was observed in all TNP-treated rats. BW of males and females at 20 and 100 mg/kg per day were not significantly different from controls during the dosing period.

The results of hematological examinations are summarized in Table 2. Significantly lower values of Hb and Ht, and a higher value of Ret were detected in females at 100 mg/kg per day.

The organ weights are summarized in Table 3. At 100 mg/kg per day, a significantly higher value of relative spleen weight (14% increased) in males, and a significantly higher value of relative liver weight (18% increased) in females were observed.

Repeated dose study in young rats (main study)

There were no deaths throughout the experimental period even at 100 mg/kg per day. Yellowish fur was observed in all TNP-treated rats. A yellowish color change of urine was also found in all TNP-treated groups during the dosing period and this coloration disappeared during the recovery period. BW of males and females in the TNP-treated groups were not significantly different from controls during the dosing and recovery periods. No consistent changes in food consumption were found in the TNP-treated groups.

The results of hematological examinations are summarized in Table 2. Significantly higher values of WBC and Ret and lower values of RBC and Hb were observed in males at 100 mg/kg per day. At this dose, significantly higher values of WBC, MCV and Ret, and lower values of RBC, Hb and MCHC were also found in females.

The organ weights are summarized in Table 3. Significantly higher values of relative liver weight (12% increased) and relative spleen weight (45% increased) and significantly lower value of relative epididymides weight (21% decreased) were observed in males at 100 mg/kg per day at the end of the dosing period. A Significantly lower value of relative epididymides weight at 100 mg/kg per day was also

Table 1 Organ weights in the newborn rat study of 2,4,6-trinitrophenol

	Dose-finding study†			Main study‡			
Dose (mg/kg per day)	0	16.3	81.4	0	4.1	16.3	65.1
Males							
No. animals	4	4	3	6	6	6	6
Body weight§ (g)	48.9 ± 3.7	47.7 ± 2.6	42.3 ± 2.0*	63.4 ± 4.9	63.0 ± 2.8	63.7 ± 5.7	61.8 ± 4.8
Liver (g)	1.73 ± 0.14	1.67 ± 0.13	1.70 ± 0.13	2.69 ± 0.22	2.74 ± 0.14	2.79 ± 0.24	2.97 ± 0.38
(g/100 g BW)	(3.55 ± 0.10)	(3.49 ± 0.12)	(4.01 ± 0.13)**	(4.25 ± 0.16)	(4.35 ± 0.12)	(4.38 ± 0.08)	(4.79 ± 0.28)**
Spleen (g)	0.21 ± 0.04	0.21 ± 0.02	0.17 ± 0.01	0.34 ± 0.07	0.35 ± 0.06	0.38 ± 0.04	0.37 ± 0.06
(g/100 g BW)	(0.44 ± 0.07)	(0.45 ± 0.05)	(0.40 ± 0.03)	(0.54 ± 0.07)	(0.56 ± 0.08)	(0.60 ± 0.05)	(0.60 ± 0.05)
Kidneys (g)	0.58 ± 0.03	0.56 ± 0.04	0.43 ± 0.05**	0.74 ± 0.12	0.73 ± 0.08	0.77 ± 0.03	0.73 ± 0.12
(g/100 g BW)	(1.18 ± 0.04)	(1.17 ± 0.05)	(1.02 ± 0.08)**	(1.16 ± 0.12)	(1.16 ± 0.09)	(1.21 ± 0.10)	(1.18 ± 0.12)
Epididymides (mg)	–	–	–	57.6 ± 4.6	55.4 ± 6.0	57.6 ± 7.3	50.3 ± 3.7
(mg/100 g BW)	–	–	–	(91.1 ± 6.9)	(87.9 ± 7.2)	(91.3 ± 16.4)	(81.9 ± 7.9)
Testes (mg)	–	–	–	326 ± 47	302 ± 27	319 ± 22	295 ± 20
(mg/100 g BW)	–	–	–	(513 ± 54)	(479 ± 26)	(504 ± 44)	(478 ± 27)
Females							
No. animals	4	4	2	6	6	6	6
Body weight§ (g)	45.2 ± 2.2	47.5 ± 3.1	38.6	59.0 ± 3.3	59.6 ± 2.3	57.0 ± 4.6	58.8 ± 5.3
Liver (g)	1.57 ± 0.08	1.72 ± 0.09	1.64	2.46 ± 0.22	2.44 ± 0.24	2.33 ± 0.25	2.75 ± 0.28
(g/100 g BW)	(3.48 ± 0.25)	(3.62 ± 0.10)	(4.23)	(4.18 ± 0.35)	(4.09 ± 0.29)	(4.09 ± 0.19)	(4.67 ± 0.19)*
Spleen (g)	0.20 ± 0.03	0.20 ± 0.04	0.17	0.32 ± 0.04	0.33 ± 0.04	0.29 ± 0.05	0.37 ± 0.05
(g/100 g BW)	(0.43 ± 0.04)	(0.43 ± 0.06)	(0.44)	(0.54 ± 0.05)	(0.55 ± 0.07)	(0.51 ± 0.08)	(0.62 ± 0.03)
Kidneys (g)	0.55 ± 0.02	0.57 ± 0.05	0.43	0.69 ± 0.05	0.69 ± 0.06	0.66 ± 0.06	0.70 ± 0.05
(g/100 g BW)	(1.22 ± 0.06)	(1.20 ± 0.06)	(1.12)	(1.17 ± 0.09)	(1.16 ± 0.08)	(1.16 ± 0.10)	(1.20 ± 0.06)

†Rats were killed on postnatal day (PND) 18; ‡rats were killed on PND 22; §body weight (BW) after overnight starvation follow the last dosing. Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. –, no data.

Table 2 Hematological parameters in the young rat study of 2,4,6-trinitrophenol

	Dose-finding study†			Main study‡			
Dose (mg/kg per day)	0	20	100	0	4	20	100
Males							
No. animals	3	3	3	6	6	6	6
WBC ($\times 10^2/\mu L$)	117 ± 26	94 ± 20	108 ± 21	93 ± 14	98 ± 14	112 ± 22	146 ± 38**
RBC ($\times 10^4/\mu L$)	682 ± 13	651 ± 24	646 ± 32	720 ± 32	720 ± 13	739 ± 34	661 ± 52*
Hb (g/dL)	14.0 ± 0.6	13.8 ± 0.2	13.8 ± 0.6	14.3 ± 0.3	14.6 ± 0.5	14.8 ± 0.7	13.4 ± 0.7*
Ht (%)	40.9 ± 1.4	41.3 ± 1.5	40.9 ± 2.7	40.9 ± 1.0	41.5 ± 1.8	42.6 ± 1.4	39.1 ± 2.2
MCV (fL)	60.0 ± 3.1	63.4 ± 1.1	63.3 ± 1.7	56.8 ± 1.6	57.7 ± 2.3	57.8 ± 2.3	59.3 ± 2.7
MCHC (%)	34.2 ± 0.3	33.5 ± 1.0	33.7 ± 0.9	35.0 ± 0.7	35.2 ± 0.6	34.8 ± 0.6	34.1 ± 0.5
Ret (‰)	59.8 ± 5.6	61.1 ± 3.7	72.6 ± 8.2	31.4 ± 1.4	29.8 ± 4.1	31.6 ± 3.8	54.7 ± 7.6**
Females							
No. animals	3	3	3	6	6	6	6
WBC ($\times 10^2/\mu L$)	82 ± 7	70 ± 12	98 ± 31	67 ± 18	79 ± 27	73 ± 15	123 ± 33**
RBC ($\times 10^4/\mu L$)	711 ± 6	690 ± 31	639 ± 47	706 ± 30	711 ± 47	713 ± 41	608 ± 19**
Hb (g/dL)	14.6 ± 0.1	14.5 ± 0.3	13.5 ± 0.7*	14.2 ± 0.5	14.3 ± 0.5	14.3 ± 0.6	12.6 ± 0.3**
Ht (%)	42.4 ± 0.3	41.4 ± 0.6	38.5 ± 1.7**	39.3 ± 1.2	40.3 ± 1.9	40.3 ± 1.8	37.3 ± 0.9
MCV (fL)	59.6 ± 0.8	60.0 ± 3.6	60.3 ± 1.8	55.8 ± 0.9	56.9 ± 3.4	56.6 ± 1.7	61.4 ± 2.4**
MCHC (%)	34.5 ± 0.4	35.2 ± 0.3	35.0 ± 0.3	36.2 ± 0.9	35.6 ± 0.6	35.6 ± 0.7	33.9 ± 0.3**
Ret (‰)	37.6 ± 1.5	39.6 ± 6.9	56.3 ± 3.6**	25.5 ± 4.6	25.2 ± 1.0	24.1 ± 3.3	65.5 ± 5.9*

†Rats were killed at 7 weeks of age; ‡rats were killed at 9 weeks of age. Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. Hb, hemoglobin; Ht, hematocrit; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell count; Ret, reticulocyte ratio; WBC, white blood cell count.

Table 3 Organ weights in the young rat study of 2,4,6-trinitrophenol

Dose	Dose-finding study†			Main study‡				Main study (at the end of recovery period)§	
(mg/kg per day)	0	20	100	0	4	20	100	0	100
Males									
No. animals	3	3	3	6	6	6	6	6	6
Body weight¶ (g)	267 ± 15	257 ± 7	276 ± 9	374 ± 12	380 ± 31	384 ± 35	367 ± 27	449 ± 20	529 ± 43
Liver (g)	10.8 ± 0.4	10.9 ± 0.7	12.2 ± 0.2*	14.2 ± 1.3	14.0 ± 0.9	14.4 ± 1.8	15.6 ± 1.1	15.5 ± 1.1	14.8 ± 2.2
(g/100 g BW)	(4.04 ± 0.12)	(4.26 ± 0.39)	(4.43 ± 0.17)	(3.79 ± 0.31)	(3.69 ± 0.19)	(3.73 ± 0.23)	(4.24 ± 0.24)*	(3.46 ± 0.22)	(3.45 ± 0.20)
Spleen (g)	0.77 ± 0.10	0.75 ± 0.03	0.91 ± 0.07	0.82 ± 0.08	0.76 ± 0.08	0.89 ± 0.19	1.18 ± 0.16**	0.86 ± 0.09	0.84 ± 0.07
(g/100 g BW)	(0.29 ± 0.03)	(0.29 ± 0.02)	(0.33 ± 0.02)*	(0.22 ± 0.02)	(0.20 ± 0.02)	(0.23 ± 0.03)	(0.32 ± 0.03)**	(0.19 ± 0.02)	(0.20 ± 0.01)
Kidneys (g)	2.29 ± 0.25	2.12 ± 0.16	2.39 ± 0.12	2.62 ± 0.13	2.57 ± 0.13	2.81 ± 0.33	2.72 ± 0.13	2.85 ± 0.23	2.92 ± 0.31
(g/100 g BW)	(0.86 ± 0.06)	(0.83 ± 0.04)	(0.87 ± 0.02)	(0.70 ± 0.03)	(0.68 ± 0.05)	(0.73 ± 0.06)	(0.74 ± 0.03)	(0.64 ± 0.05)	(0.68 ± 0.04)
Testes (g)	–	–	–	3.08 ± 0.32	3.09 ± 0.19	3.13 ± 0.25	3.29 ± 0.35	3.30 ± 0.09	2.64 ± 1.07
(g/100 g BW)	–	–	–	(0.82 ± 0.09)	(0.82 ± 0.06)	(0.82 ± 0.05)	(0.90 ± 0.05)	(0.74 ± 0.03)	(0.61 ± 0.22)
Epididymides (g)	–	–	–	0.82 ± 0.06	0.78 ± 0.06	0.78 ± 0.07	0.63 ± 0.10**	1.10 ± 0.07	0.82 ± 0.11**
(g/100 g BW)	–	–	–	(0.22 ± 0.02)	(0.21 ± 0.02)	(0.20 ± 0.01)	(0.17 ± 0.03)**	(0.24 ± 0.01)	(0.20 ± 0.03)**
Female									
No. animals	3	3	3	6	6	6	6	6	6
Body weight¶ (g)	165 ± 9	172 ± 4	175 ± 8	242 ± 19	241 ± 17	237 ± 29	233 ± 14	283 ± 18	270 ± 19
Liver (g)	6.4 ± 0.8	6.7 ± 0.1	8.0 ± 0.6*	8.2 ± 0.7	8.0 ± 0.8	8.2 ± 1.5	9.7 ± 1.2	9.3 ± 0.7	9.3 ± 1.1
(g/100 g BW)	(3.85 ± 0.28)	(3.90 ± 0.07)	(4.54 ± 0.23)*	(3.38 ± 0.11)	(3.32 ± 0.15)	(3.45 ± 0.19)	(4.16 ± 0.27)**	(3.27 ± 0.15)	(3.43 ± 0.27)
Spleen (g)	0.49 ± 0.13	0.45 ± 0.13	0.56 ± 0.05	0.51 ± 0.08	0.58 ± 0.05	0.54 ± 0.08	0.98 ± 0.12**	0.60 ± 0.10	0.63 ± 0.09
(g/100 g BW)	(0.30 ± 0.06)	(0.26 ± 0.08)	(0.32 ± 0.01)	(0.21 ± 0.04)	(0.24 ± 0.02)	(0.23 ± 0.20)	(0.42 ± 0.05)**	(0.21 ± 0.02)	(0.23 ± 0.02)
Kidneys (g)	1.40 ± 0.03	1.45 ± 0.13	1.52 ± 0.17	1.77 ± 0.16	1.73 ± 0.20	1.67 ± 0.20	1.86 ± 0.17	1.82 ± 0.12	1.86 ± 0.10
(g/100 g BW)	(0.85 ± 0.03)	(0.84 ± 0.07)	(0.87 ± 0.11)	(0.74 ± 0.07)	(0.71 ± 0.04)	(0.71 ± 0.05)	(0.80 ± 0.06)	(0.65 ± 0.06)	(0.69 ± 0.06)

†Rats were killed at 7 weeks of age; ‡rats were killed at 9 weeks of age; §rats were killed at 11 weeks of age; ¶body weight (BW) after overnight starvation following the last dosing. Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. –, no data.

noted at the end of the recovery period. At this dose in females, significantly higher values of relative liver weight (23% increased) and relative spleen weight (100% increased) were noted. No other changes related to the administration of TNP were found.

At the end of the dosing period, enlargement of the spleen and erosion or ulcers in the cecum were observed in males and females at 100 mg/kg per day. Small testes were found at 100 mg/kg per day at the end of the recovery period.

The histopathological findings are summarized in Table 4. Significant changes were noted at 100 mg/kg per day. Spleens with the development of a germinal center and extramedullary hematopoiesis were observed in males and females at 100 mg/kg per day at the end of the dosing period. Hemosiderin deposition in the spleen was found in males and females at 100 mg/kg per day at the end of the dosing and recovery periods. Centrilobular hypertrophy of hepatocytes in the liver and ulcers in the cecum were observed in males and females at 100 mg/kg per day at the end of the dosing period. Testes with diffuse atrophy of seminiferous tubules were noted at 100 mg/kg per day at the end of the dosing period, and severe atrophy was observed at the end of the recovery period. A decreased number of sperm and lumen with cell debris were observed in the epididymides at 100 mg/kg per day at the end of the dosing and recovery periods.

There were no consistent changes related to the administration of TNP in biochemical parameters for blood or urine.

DISCUSSION

In the present study, we re-evaluated the toxicity of TNP in young rats in terms of the NOAEL and toxicity profile, and determined the toxicity of this chemical in newborn rats, then compared the toxicity in newborn and young rats. We showed here that TNP had a markedly different toxicity profile between newborn and young rats.

As for the yellowish fur in all newborn and young rats treated with TNP, their hair roots and skin showed no anomalies therefore it does not seem to be an adverse effect of TNP.

In the newborn rat study, the major toxicity was death and low BW without any other toxicologically significant changes at 81.4 mg/kg per day in the dose-finding study. Deaths occurred in days 3–7 after dosing onset. At the lower dose, 65.1 mg/kg per day in the main study, a slightly low BW in males was observed only at 4 and 8 days after dosing onset. This slight and transient loss of BW might be accepted as having no toxicological significance in general, but we considered it to be closely related to the death that occurred at the higher dose, 81.4 mg/kg per day, because death and low BW were observed on the same days after dosing onset (late in the first week). Slight changes in relative liver and kidney weights were observed but not considered toxicologically significant because there were no changes in biochemical and urinary parameters, or histopathological findings. Based on low BW at 65.1 mg/kg per day in males, the NOAEL for newborn rats was considered 16.3 mg/kg per day.

In the MHLW (2001) report, the NOEL was concluded 4 mg/kg per day based on yellowish fur and decreased level of urine potassium in young rats. The major adverse effects of TNP were hemolytic anemia and testicular toxicity without death or changes of BW at 100 mg/kg per day in the main study with young rats. No toxic effects were detected at 20 mg/kg per day or less after administration of TNP in the dose-finding or main study with young rats. Based on these findings, we re-evaluated that the NOAEL for young rats was considered 20 mg/kg per day.

TNP, at 81.4 mg/kg per day or more, caused behavioral changes in the newborn rat study but not in the young rat study at 100 mg/kg per day. The immature blood–brain barrier in newborn rats may explain these phenomena. The diffusional resistance is primarily the result of tight junctions between endothelial cells, the absence of pores within the cells and a thicker, more developed basement membrane surrounding each cell (Reese & Karnovsky 1967; Scheuplein *et al.* 2002). In rats, capillary diffusion decreases during postnatal weeks 3–4 (Bär & Wolff 1972).

Histopathological and hematological examinations revealed hemolytic anemia as evidenced by reductions of RBC and Hb and hemosiderin deposition and extramedullary hematopoiesis in spleen at 100 mg/kg per day in the young rat study, but not in the newborn rat study at 81.4 mg/kg per day. Hemolytic anemia can be induced by various kinds of medicines and chemicals including some aromatic amines due to oxidation (Bloom & Brandt 2001). TNP may not be the causal substance because it occurred in young rats but not in newborn rats whose metabolic capacity is immature, such as lower total cytochrome P-450 levels (Imaoka *et al.* 1991). Thus, TNP metabolites might be the cause. As for absorption and excretion of TNP in rats, Wyman *et al.* (1992) reported that fasted rats would absorb about 60% of orally treated TNP in 24 h and the main metabolite was picramic acid following oral dosing in rats. Picramic acid, a type of aromatic amine, would be the most likely candidate, although there is no evidence of hemolytic anemia caused by picramic acid. The information together suggests that the absence of hemolytic anemia in newborn rats may be due to insufficient amounts of picramic acid produced as a metabolite of TNP.

As for the testicular toxicity, degenerating primary spermatocytes and alterations in Sertoli cells were caused by di(2-ethylhexyl) phthalate in 5-week-old, but not 3-week-old, rats (Sjöberg *et al.* 1985). TNP also had toxic effects on the testes and epididymides in young rats, but not in

Table 4 Histopathological findings at the end of dosing and recovery periods in the young rat main study of 2,4,6-trinitrophenol

		Dosing period†					Recovery period‡		
Dose (mg/kg per day)		0	4	20	100		0	100	
Males									
No. animals examined		6	6	6	6		6	6	
Spleen									
Development, germinal center	+	0	0	0	5	*	0	0	
Extramedullary hematopoiesis, erythrocyte	+	0	0	0	6	**	0	0	
Hemosiderin deposition	Total	0	0	0	4		0	6	**
	+	0	0	0	3		0	6	
	++	0	0	0	1		0	0	
Cecum									
Ulcer	Total	0	0	0	4		0	0	
	+	0	0	0	1		0	0	
	++	0	0	0	2		0	0	
	+++	0	0	0	1		0	0	
Liver									
Hypertrophy, hepatocytes, centrilobular	+	0	0	0	4		0	0	
Testis									
Atrophy, seminiferous tubules, diffuse	Total	0	0	0	6	**	0	5	*
	+	0	0	0	6		0	2	
	++	0	0	0	0		0	3	
Epididymis									
Cell debris, lumen	Total	0	0	0	4		0	1	
	+	0	0	0	3		0	1	
	++	0	0	0	1		0	0	
Decrease in sperm	Total	0	0	0	6	**	0	3	
	+	0	0	0	5		0	0	
	++	0	0	0	1		0	1	
	+++	0	0	0	0		0	2	
Females									
No. animals examined		6	6	6	6		6	6	
Spleen									
Development, germinal center	+	0	0	0	5	*	0	0	
Extramedullary hematopoiesis, erythrocyte	+	0	0	0	6	**	0	0	
Hemosiderin deposition	Total	0	0	0	6	**	0	6	**
	+	0	0	0	3		0	6	
	++	0	0	0	3		0	0	
Cecum									
Ulcer	++	0	0	0	3		0	0	
Liver									
Hypertrophy, hepatocytes, centrilobular	+	0	0	0	3		0	0	

Grade sign: +, mild; ++, moderate; +++, marked. †Rats were killed at 7 weeks of age; ‡rats were killed at 9 weeks of age. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group.

newborn rats. The Sertoli cells play an important role in the establishment and maintenance of the specific microenvironment of the adluminal compartment of the seminiferous epithelium and this is a prerequisite for normal spermatogenesis (Sjöberg *et al.* 1986). In rats, Sertoli cells proliferate rapidly from day 19 of gestation to PND 15, then slow down and cease multiplying on approximately PND 20 (Orth 1982, Orth 1984; Toppari *et al.* 1996). The dosing periods were PND 4–21 and postnatal weeks 5–8 in the newborn and young rat studies, respectively. Therefore, TNP seems unlikely to affect the differentiation and proliferation of Sertoli cells, and seems likely to affect the maturation of spermatids, although it remains to be elucidated whether this is a direct effect of TNP or some kind of TNP metabolite.

In conclusion, in the newborn rat study, the NOAEL for TNP were 16.3 mg/kg per day, low BW at 65.1 mg/kg per day or more, and death at 81.4 mg/kg per day were observed. In the young rat study, the NOAEL for TNP were 20 mg/kg per day and hemolytic anemia and testicular toxicity were found at 100 mg/kg per day.

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Bull.Natl.Inst.Health Sci.,122, 56-61 (2004) **Technical Data**

生殖発生毒性を指標としたダイオキシンの耐容1日摂取量（TDI）算定の考え方について

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The recent TDI derivation of the dioxin based on the reproductive and developmental toxicity.

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SUMMARY

In 1998, WHO-IPCS re-assessed the TDI of dioxin, which was derived from the body burdens of TCDD exposed to the experimental animals. Then, the international assessment agencies and governmental assessment agencies have conducted the dioxin fs health assessment using the similar method to the WHO-IPCS approach. The key endpoints were reproductive and developmental toxicity caused by *in utero* and lactational exposure. Each assessment agencies used the similar data set of the toxicity studies, however, there are some differences about the TDI derivation method and the selection of adverse endpoints. This report reviewed the recent reproductive and developmental toxicity studies of the dioxins and summarized the health assessment in the international or governmental agencies, and discussed the appropriate TDI derivation.

Key Word: dioxin, tolerable daily intake, reproductive and developmental toxicity

はじめに

1998年にWHO-IPCSが，ダイオキシンの体内蓄積性を考慮して体内負荷量という概念を用いて，耐容1日摂取量（TDI）の再評価を行った[1]．我が国でも1999年に，体内負荷量を基にしてTDIの設定を行っている[2]．これ以前は，発がん性を感受性の高いエンドポイントとしてTDIを設定していたが，この体内負荷量という物差しを用いることにより，胎児期及び授乳暴露による次世代への影響がより感受性の高い毒性指標となることが明らかになった．その結果，これ以降はヨーロッパ各国やJECFAなどの評価機関では，この評価法に従い耐用摂取量評価を算定してきている．本研究では，この評価法の重要なエンドポイントであるダイオキシン類による生殖発生毒性に関して，1998年以降の新しい知見と，各国および国際評価機関でのTDI算定経過をまとめると共に，現時点での適切なTDIのあり方について考察した．

体内負荷量とTDI

まず，体内負荷量を用いたTDIの算定法について概略を示す．一般に化学物質の耐用摂取量は，最も感受性の高い毒性学的エンドポイントを基に算定される．ヒトを対象とした定量的で信頼性の高い疫学研究などの知見がある場合はそれを用いるが，通常は疫学上の交絡因子を完全に排除することは難しく，動物実験における化学物質の投与用量を基に，ヒトにおける耐用摂取量を算出している．1990年代の前半頃まではダイオキシンに関して最も感受性の高い毒性は，げっ歯類に対する発がん性であると考えられており，評価機関の多くは，ラットを用いた2年間の長期投与試験における無毒性量（NOAEL）：1ng/kg/dayを基に，不確実係数（多くは100）を適用して耐容1日摂取量を設定していた．しかし，1998年のWHO-IPCS1)での評価以後は，ダイオキシン類のように脂溶性が高く，排泄が遅い物質は，長い時間をかけて徐々に体内に化学物質が蓄積していくことや，ヒトとラットでは数百倍も排泄速度が異なることから，投与量と蓄積濃度（＝体内負荷量）と関係はヒトとラットで著しく異なることになり，投与量をベースにして毒性発現を比較するのは適当ではないと判断された．また，ダイオキシン類による毒性発現は蓄積量である体内負荷量に依存して発現していることが示され，近年は，この体内負荷量を基に定量的な毒性評価を行うようになった．この体内負荷量という物差しを用いることにより，従来の発がん性よりも，胎児期及び授乳期暴露による次世代への影響がより感受性の高い毒性指標となることが明らかになった．この際，摂取量と体内負荷量との換算は，1コンパートメントモデルの定状状態における，以下の近似式で示すことができる．

摂取量(ng/kg/day)＝[体内負荷量(ng/kg) X ln(2)]／[半減期(day) X吸収率]

また，ヒトや実験動物に関する様々な知見より，ヒトと実