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# Evaluation of developmental toxicity of β-thujaplicin (hinokitiol) following oral administration during organogenesis in rats

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**Abstract**

The objective of this study was to evaluate the developmental toxicity of β-thujaplicin (TP) in rats. Pregnant rats were given TP by gastric intubation at 15, 45, or 135 mg/kg on days 6–15 of pregnancy. The maternal body weight gain during administration at 45 and 135 mg/kg and after administration at 136 mg/kg and adjusted weight gain at 45 and 135 mg/kg were significantly reduced. A significant decrease in food consumption during and after administration was found at 45 and 135 mg/kg. A significant increase in the incidence of postimplantation loss was found in pregnant rats given TP at 135 mg/kg. A significantly lower weight was found in female fetuses at 45 and 135 mg/kg and in male fetuses at 135 mg/kg. Although a significantly increased incidence of fetuses with skeletal variations and decreased degree of ossification were found at 135 mg/kg, no significant increase in external, skeletal and internal malformations was detected after administration of TP. The data demonstrated that TP had adverse effects on embryonic/fetal survival and growth only at maternal toxic doses. No adverse effects on morphological development were found in rats fetuses. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.


*Keywords:* β-Thujaplicin; Hinokitiol; Developmental toxicity; Teratogenicity; Rat

## 1. Introduction

β-Thujaplicin (TP; CAS No. 499-44-5; Hinokitiol; 4-isopropyltropolone) is a phenolic component of essential oils extracted from cypress trees. TP has been found to act as an antibacterial agent (Saeki et al., 1989; Osawa et al., 1990; Tonari, 1998) and an antitumor agent (Yamato et al., 1984; Inamori et al., 1993). In addition, it possesses phytogrowth-inhibitory effects (Inamori et al., 1991). TP is used as a natural food preservative in Japan.

Several reports on the toxicity of TP are available. In mutagenicity screening tests of TP, positive results were obtained in a Rec-assay with S9 mix at 1.0 mg/disk and chromosome aberration test in vitro at 0.002–0.003 mg/ml, but not in the Ames test or a micronucleus test in mice (Sofuni et al., 1993). The DNA damaging activity of TP was weak in a spore Rec-assay (Ueno and Ishizaki, 1992). The values of LD50 have been reported to be 504 mg/kg in male ddy mice and 469 mg/kg in female ddy mice after oral gavage of TP (Shimizu et al., 1993). Recently, Ogata et al. (1999) reported a significant increase in the incidence of fetuses with malformations after oral administration of TP at 560 mg/kg and higher on day 9 of pregnancy in ICR mice and that TP induced dysmorphogenicity in cultured mouse embryos at concentrations of 6.25 and 12.5 μg/ml. However, there is no information on the developmental toxicity of TP in rats. Therefore, the present study was conducted to evaluate the potential teratogenicity of TP after administration throughout organogenesis in rats.

*Abbreviations:* TP, β-thujaplicin; LOAEL, lowest-observed-adverse-effect level; NOAEL, no-observed-adverse-effect level.

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## 2. Materials and methods

### 2.1. Animals

Wistar rats (Jcl: Wistar, Clea Co., Ltd., Tokyo, Japan) were used throughout this study. Animals were reared on a basal diet (F-1; Funabashi Farm Co., Funabashi, Japan) and tap water ad libitum and maintained in an air-conditioned room at 24±1 °C, with a relative humidity of 55±5%, under a controlled 12-h light/dark cycle. Virgin female rats, weighing 216–244 g, were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats were distributed on a random basis into four groups of 16–17 rats each and housed individually.

### 2.2. Chemicals and dosing

The female rats were dosed once daily by gastric intubation with TP (purity >98%, SEIWA Technological Laboratories Ltd., Tokyo, Japan) at a dose of 0 (control), 15, 45, or 135 mg/kg from day 6 through day 15 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of TP by gastric intubation on days 6–15 of pregnancy caused maternal deaths and decreased maternal body weight gain and caused an increase in postimplantation loss and decrease in fetal weight at 125 mg/kg and higher in rats. TP was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The volume of each dose was adjusted to 5 ml/kg of body weight based on daily body weight. The control rats received olive oil only. The formulations were kept in a cool and dark place for no more than 7 days.

### 2.3. Observations

The maternal body weight and food consumption were recorded daily. The pregnant rats were euthanized by ether overdose on day 20 of pregnancy. The peritoneal cavity and uterus were opened, and the numbers of live and dead fetuses and of resorptions were counted. The gravid uterus was removed and the dams weighed again. The adjusted weight gain, i.e. maternal weight gain throughout pregnancy corrected for gravid uterine weight, was calculated. To confirm the dam's pregnancy status, the uteri were immersed in 2% sodium hydroxide solution for over 1 h. The uteri were cleared and the implantation traces were seen to be stained yellowish-brown (Yamada et al., 1985). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. Approximately one-half of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Kawamura et al., 1990) and examined for skeletal malformations. The remaining live fetuses in each litter were fixed in Bouin's solution prior to dissection. To detect internal malformations, fetal heads were examined by the free-hand razor-blade sectioning method of Barrow and Taylor (1969) and the thoracic areas were examined by Nishimura's micro-dissecting method (1974), a modification of Barrow and Taylor's method.

### 2.4. Data analysis

The litter was considered the experimental unit. The initial body weight, body weight gain and food consumption of pregnant rats, numbers of implantations, postimplantation loss and live fetuses per litter and body weight of live fetuses were evaluated by analysis of variance, followed by Dunnett's multiple comparison test if differences were found. The incidences of post-implantation loss and fetal malformations per litter were analyzed by the Kruskal–Wallis test to assess the overall effects. Whenever a significant trend was noted, pairwise comparisons were made using the Mann–Whitney test. Fisher's exact test was used when the incidence in the control group was zero. The 0.05 level of probability was used as the criterion for significance.

## 3. Results

Table 1 shows the maternal findings in rats given TP during organogenesis. One pregnant rat was dead on day 20 of pregnancy at 135 mg/kg. The body weight gain on days 6–16 at 45 and 135 mg/kg and on days 16–20 at 135 mg/kg was reduced significantly. The adjusted weight gain, which indicates the net weight gain of pregnant rats, was significantly lower in the 45 and 135 mg/kg groups than in the control group. The food consumption on days 6–16 and days 16–20 was significantly lower in the 45 and 135 mg/kg groups than the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for pregnant rats are 45 and 15 mg/kg, respectively.

Pregnancy outcome in rats given TP during organogenesis are presented in Table 2. Litters totally resorbed were found in three of the 16 pregnant rats at 135 mg/kg. A significant increase in the number of resorptions per litter and incidence of postimplantation loss per litter and a significant decrease in the number of live fetuses per litter were also noted at 135 mg/kg. The weights of live fetuses were significantly decreased at 45 mg/kg and higher in females and at 135 mg/kg in males.

A summary of morphological findings in live fetuses of rats given TP during organogenesis is shown in Table 3. No fetus with external malformations was observed in any group. Skeletal examination revealed

Table 1
Maternal findings in rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

| Dose (mg/kg) | 0 (control) | 15 | 45 | 135 |
|---|---|---|---|---|
| No. pregnant rats | 16 | 16 | 16 | 17 |
| No. of dead rats | 0 | 0 | 0 | 1 |
| Initial body weight | 227±8 | 227±7 | 227±6 | 227±6 |
| *Body weight gain during pregnancy (g)*[a] | | | | |
| Days 0–6 | 17±5 | 17±4 | 16±2 | 17±3 |
| Days 6–16 | 45±4 | 39±6 | 32±7* | 13±9* |
| Days 16–20 | 48±6 | 48±5 | 42±6 | 21±12* |
| Adjusted weight gain during pregnancy (g)[a,b] | 39±7 | 36±8 | 28±10* | 24±5* |
| *Food consumption during pregnancy (g)*[a] | | | | |
| Days 0–6 | 105±7 | 101±6 | 98±5* | 101±5 |
| Days 6–16 | 157±12 | 147±13 | 129±12* | 103±11* |
| Days 16–20 | 72±5 | 70±4 | 63±7* | 66±6* |

[a] Values are given as mean±S.D.
[b] Adjusted weight gain refers to maternal body weight gain excluding the gravid uterus.
* Significantly different from the control, $P<0.05$.

Table 2
Reproductive findings in rats givenβ-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

| Dose (mg/kg) | 0 (control) | 15 | 45 | 135 |
|---|---|---|---|---|
| No. litters | 16 | 16 | 16 | 16 |
| No. corpora lutea per litter[a] | 16.3±1.3 | 16.3±1.3 | 15.7±1.4 | 16.3±0.9 |
| No. implantations per litter[a] | 15.4±1.4 | 15.5±1.2 | 14.8±1.7 | 15.6±1.3 |
| No. of litters totally resorbed | 0 | 0 | 0 | 3 |
| No. resorptions per litter[a] | 1.3±1.4 | 1.3±1.2 | 2.0±1.0 | 9.9±4.6* |
| No. dead fetuses per litter[a] | 0.1±0.3 | 0 | 0 | 0 |
| % Postimplantation loss per litter[b] | 8.5 | 8.0 | 13.6 | 63.5* |
| No. live fetuses per litter[a] | 14.1±1.4 | 14.3±1.5 | 12.8±1.8 | 5.7±4.6* |
| Sex ratio of live fetuses (male/female) | 114/111 | 116/112 | 107/97 | 56/35 |
| *Body weight of live fetuses (g)*[a] | | | | |
| Male | 3.39±0.19 | 3.26±0.19 | 3.25±0.18 | 2.71±0.21* |
| Female | 3.19±0.18 | 3.13±0.18 | 3.02±0.19* | 2.62±0.11* |

[a] Values are given as mean±SD.
[b] (No. resorptions and dead fetuses/No. implantations)×100.
* Significantly different from the control, $P<0.05$.

one fetus with sternoschisis at 135 mg/kg. Skeletal variations in the vertebrae, ribs, and/or sternebrae were found in all groups. The incidences of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs were significantly higher in the 135 mg/kg group than the control group. The numbers of ossification centers of the caudal vertebrae and of the sternebrae were significantly decreased at 135 mg/kg. Hypoplasia of the spleen occurred in two fetuses in one dam at 135 mg/kg. A few fetuses with thymic remnant in the nick and/or left umbilical artery were found in the control group and TP-treated groups. However, there was no significant difference in the incidence of fetuses with internal malformations and variations between the TP-treated groups and the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for fetal rats are 45 and 15 mg/kg, respectively.

## 4. Discussion

This study was designed to screen for general developmental toxicity in rats. Doses of TP expected to induce maternal and developmental toxicity, such as a decrease in maternal body weight gain and food consumption and in fetal weight and an increase in postimplantation loss, were given to pregnant rats to characterize the effects of TP on embryonic/fetal development. Maternal toxicity, as evidenced by a significant decrease in body weight gain and food consumption

Table 3
Morphological examinations in fetuses of rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

| Dose (mg/kg) | 0(control) | 15 | 45 | 135 |
|---|---|---|---|---|
| *External examination* | | | | |
| No. fetuses (litters) examined | 225(16) | 228(16) | 204(16) | 91(13) |
| No. fetuses (litters) with malformations | 0 | 0 | 0 | 0 |
| *Skeletal examination* | | | | |
| No. fetuses (litters) examined | 116(16) | 117(16) | 105(16) | 49(13) |
| No. fetuses (litters) with malformations | 0 | 0 | 0 | 1(1) |
| Sternoschisis | 0 | 0 | 0 | 1(1) |
| No. of fetuses (litters) with variations | 11(7) | 8(4) | 10(7) | 21(11)** |
| Cervical rib | 4(2) | 3(1) | 3(2) | 1(1) |
| Splitting of thoracic vertebral bodies | 0 | 1(1) | 0 | 0 |
| 14th ribs | | | | |
| Extra | 0 | 0 | 0 | 4(3) |
| Rudimentary | 2(1) | 1(1) | 2(2) | 9(7)** |
| Bipartite sternebrae | 1(1) | 2(1) | 1(1) | 9(7)** |
| Asymmetry of sternebrae | 5(5) | 1(1) | 4(3) | 3(3) |
| Degree of ossification[a] | | | | |
| No. of ossification centers of caudal vertebrae | 3.3±0.4 | 3.1±0.4 | 3.2±0.4 | 2.8±0.3** |
| No. of sternebrae | 4.9±0.4 | 4.9±0.6 | 4.8±0.5 | 3.9±0.7** |
| *Internal examination* | | | | |
| No. fetuses (litters) examined | 109(16) | 111(16) | 99(16) | 42(12) |
| No. fetuses (litters) with malformations | 0 | 0 | 0 | 2(1) |
| Hypoplasia of spleen | 0 | 0 | 0 | 2(1) |
| No. of fetuses (litters) with variations | 5(3) | 3(3) | 2(2) | 2(2) |
| Thymic remnant in neck | 4(3) | 1(1) | 2(2) | 2(2) |
| Left umbilical artery | 1(1) | 2(2) | 0 | 0 |

[a] Values are given as mean±SD.
* Significantly different from the control, $P<0.05$.

during the administration period was found at 45 mg/kg and higher. Although pregnant rats in the 45 mg/kg group recovered with respect to body weight after cessation of administration of TP, such recovery did not occur in the high dose group. This may be due to a lack of conceptuses at 135 mg/kg. However, a significantly low adjusted weight gain at 45 mg/kg and higher may suggest maternal toxicity. These findings indicate that TP exerts maternal toxicity at 45 mg/kg and higher when administered during organogenesis in rats.

Developmental endpoints should include the number and percent of pre-and postimplantation loss, morphological alterations in fetuses, and decreased fetal weight (Kimmel and Price, 1990; Schardein, 2000; OECD, 2001). Schardein (2000) stated that fetal size is an important in the assessment of potential teratogen as an indicator of developmental toxicity, and reduction in size or growth retardation commonly occurs among fetuses of dams given dosages that are toxic to the dam, to the offspring, or both. In the present study, a significant increase in the incidence of postimplantation loss was found at 135 mg/kg and a significantly decreased weight of female fetuses was found at 45 mg/kg and higher. These findings indicated that TP is embryolethal at 135 mg/kg and toxic to fetal growth at 45 mg/kg and higher when administered during the period of organogenesis.

As for morphological examinations in the fetuses of exposed mother, a few fetuses with skeletal or internal malformations were found in the 135 mg/kg group. The malformations observed in the present study are not thought to be due to the administration of TP, because they occurred at a very low incidence and are of types that occur sporadically among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). Several types of skeletal and internal variations were also found in both the control group and TP-treated groups. These variations are frequently observed in fetuses of rats at term (Kimmel and Wilson, 1973; Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). In the 135 mg/kg group, a significant increase in the incidence of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs, but no extra ribs, and a significant decrease in the degree of ossification were accompanied by a significant decrease in the fetal weight. These findings show a correlation between these morphological alterations and growth retardation in fetuses. Although a skeletal variation, i.e. super-

numerary extra 14th ribs, is a warning sign of possible teratogenicity, the rudimentary 14th ribs, sternebral variations, and bilobed centra of the vertebral column are a normal variation (Kimmel and Wilson, 1973). Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and this might result from a delay in growth or morphologenesis that has otherwise followed a normal pattern of development. Consideration of these findings together suggests that the morphological changes observed in the present study do not indicate a teratogenic response and that TP possesses no teratogenic potential in rats.

In a developmental toxicity study in mice in which a single administration of TP was given at 420, 560, 750, or 1000 mg/kg by gastric intubation on day 9 of pregnancy, maternal deaths, dams with litter totally resorbed, and a significant increase in embryolethality were found at 750 mg/kg and higher (Ogata et al., 1999). A significant increase in the incidence of fetuses with malformations was accompanied by a significant decrease in fetal weight at 560 mg/kg and higher. Two highest doses, 750 and 1000 mg/kg, were maternally lethal, and the dose level of 560 mg/kg was very close to the maternally lethal dose. Thus, fetal malformations occurred after a single administration of TP at high doses in a single species. In other words, TP may be capable to produce fetal malformations under extreme experimental conditions in mice. Studies in additional species would be of great value in evaluating developmental toxicity of TP in conventional experimental conditions. We demonstrated here that TP possesses no adverse effects on morphological development in rat fetuses when administered during the whole period of organogenesis at doses which caused a decreased fetal weight, increased incidence of postimplantation loss, and maternal toxicity.

In conclusion, the administration of TP to pregnant rats throughout organogenesis had adverse effects on maternal rats and embryonic/fetal survival and growth but had no adverse effects on morphological development of fetuses even at maternally toxic and embryolethal doses. The data indicate that TP adversely affected the embryonic/fetal survival and growth only at maternally toxic doses in rats. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.

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COMMENTARY

# Comments from the Behavioral Teratology Committee of the Japanese Teratology Society on OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426, Developmental Neurotoxicity Study, Draft Document (September 2003)

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**ABSTRACT** **In September 2003, a new revision of the draft guideline (Organization for Economic Co-operation and Development [OECD] Guideline for the Testing of Chemicals, Proposal for a New Guideline 426, Developmental Neurotoxicity Study) was distributed. The draft guideline consists of 51 paragraphs and an appendix. The National Coordinators were requested to arrange national expert reviews of the guideline proposal in their member countries. The member of the Behavioral Teratology (BT) Committee of the Japanese Teratology Society (JTS) reviewed, discussed and commented on the draft Test Guideline proposal. The BT Committee of the JTS also commented that the International Collaborative Study to validate this protocol should be definitely performed. These comments were sent to the OECD Secretariat. The BT Committee of the JTS expects that the comments are useful for further discussion.**

Key Words: behavior, developmental neurotoxicity, OECD, test guideline

## INTRODUCTION

The Organization for Economic Co-operation and Development (OECD) Working Group on Reproduction and Developmental Toxicity at Copenhagen in June 1995 (OECD 1995) recommended that a guideline for developmental neurotoxicity should be written. In June 1996 at Copenhagen, an OECD Consultation Meeting on Developmental Neurotoxicity provided the Secretariat with the draft report on the outline of a new guideline (OECD 1996). The Behavioral Teratology (BT) Committee of the Japanese Teratology Society (JTS), in association with the Meeting of Neurobehavioral Toxicology of the Japanese Society of Toxicology, commented on this draft report. After this meeting, a draft

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proposal for Test Guideline 426, Developmental Neurotoxicity Study was developed, and was submitted to the Secretariat in February 1998. The draft guideline was distributed in December 1998. The BT Committee of the JTS commented again on this draft guideline. The draft guideline proposal was extensively revised and distributed in October 1999. General issues regarding the design of developmental neurotoxicity studies were discussed in an OECD Expert Consultation Meeting and International Life Sciences Institute (ILSI) Risk Science Institute Workshop in Washington, DC, USA, in October 2000 (OECD 2003). In September 2003, a new revision of the guideline was distributed. This revised draft Test Guideline proposal is posted on the OECD public web pages of the Test Guidelines Programme at: http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html. The draft guideline consists of 51 paragraphs and an appendix. National Coordinators were requested to arrange national expert reviews of the guideline proposal in their member countries. The deadline for the expert responses to this revised draft Test Guideline was January 16, 2004.

A meeting of the BT Committee (Chairman: Dr Y. Fukui, Professor, University of Tokushima School of Medicine) of the JTS was held on January 11, 2004, in Osaka, and the members of this committee reviewed, discussed and commented on the draft Test Guideline proposal. The BT Committee of the JTS also commented that the International Collaborative Study to validate this protocol as indicated in OECD ENV/EHS/HK/mc/2003.49 should be definitely performed. These comments were sent to the OECD Secretariat through the Japanese National Coordinator (Director of the Office of Chemical Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Japan) on January 16, 2004, but it is to be noted that they are not official comments from Ministry of Health, Labour and Welfare, Japan.

The BT Committee of the JTS expects that the comments are useful for further discussion.

The comments from the BT Committee of the JTS are as follows:

## GENERAL COMMENTS

1 New terms such as behavioral ontogeny, instead of reflex ontogeny in the 1999 draft, are introduced in the 2003 draft, but unification of terms is insufficient in the various parts of the text.
2 The rationale for the weaning day should be stated. Day of weaning is recommended to be PND 22, but PND 21 from the previous draft still appears in some parts of the text. The description day of test performance should be unified throughout the text.
3 More flexibility of the study design must be stressed. The use of 'should' is seen too frequently.
4 Guidance for higher levels of the study, such as social behavior, pharmacologic challenge, and neurochemistry, is insufficient.
5 Examination of maternal toxicity is insufficient except for clinical signs. It is advised that dams are autopsied and examined at least macroscopically.
6 The description of use of species other than rats, such as non-human primates, is scanty.
7 Considerable recent references have been added, but there is more pertinent literature to be cited.
8 The front page (DRAFT DOCUMENT [September 2003]) should be page 1, and the present page 1 is to be changed to page 2, and so on. The final page, Appendix A, would be page 21.

## SPECIFIC COMMENTS

**1. Paragraph 2**

The exposure period is expanded from 'lactation' to 'during early life'. This change is very welcomed, but the following explanation is limited to the exposure until weaning. Some description of administration of the test substances directly to offspring after weaning should be given, since human developmental neurotoxicity of chemicals in early childhood has become a great concern.

The phrase 'during pregnancy or' should be '*in utero* and'. Pregnancy primarily refers to dams, not to fetuses.

**2. Paragraph 3**

The phrase 'developmental toxicity and/or adult neurotoxicity study (e.g. Test Guidelines 415, 416, 424)' is to be changed to 'prenatal developmental toxicity, one- or two-generation study and/or adult neurotoxicity study (e.g. Test Guidelines 414, 415, 416, 424)'.

The phrase 'or as an add-on study' should be concretely explained, since the meaning is not clear.

Does 'other types of toxicity' include developmental (fetal) toxicity or is it limited to adult? It is necessary to specify this.

**3. Paragraph 4**

The phrase 'perinatal' in line 2 is to be 'prenatal', since the latter is the OECD term of Guideline 414.

**4. Paragraph 5**

The word 'and/or' in line 2 is to be 'and'.

The term 'reflex ontogeny' in line 5 is to be 'behavioral ontogeny'.

**5. Paragraph 6**

Since 'stand-alone' is a specific computer term, it is preferable to replace it with a more common word.

**6. Paragraph 7**
The usefulness of other species, especially non-human primates, for higher levels of learning and memory study, may be more circumstantially stated.

**7. Paragraph 9**
The third sentence should be changed to 'After evidence of copulation, individual housing of mated animals is recommended'. The sentence 'If mated animals are caged in small groups, animals should be caged separately in individual cages no later than day 15 of pregnancy' should be inserted following the third sentence.

**8. Paragraph 10**
It may be necessary to describe the males used for mating.

Usually, rats are obtained as a lot that may contain some brothers. Therefore, it is not practical for breeding males to be equalized across a group.

**9. Paragraph 12**
The numbers '8–12' in line three are to be changed to '8–10'. In cases of litter sizes of 12, many litters may be insufficient in number. When the number of pups in a litter is less than the designated number, it is not acceptable to add some pups from different dams for fostering.

Those litters with an insufficient number of pups should not be principally used for the study. These remarks are to be clearly described here.

Identification of individual pups is recommended to be performed at birth or soon after birth when the body weight is measured.

**10. '*Assignment of . . .*' and paragraphs 13–15**
It is recommended that this portion is placed after *Dosage* and *Administration of doses*, since dosage and administration are more directly related to dams than assignment of offspring.

**11. Paragraph 14**
The rationale is not clear why the same pair of male and female littermates is assigned for motor activity testing, while for all the other tests the same or separate pairs may be used.

**12. Paragraph 15**
'Behavioral/functional tests' in Tables 1 and 2 should be 'Functional/behavioral tests', concordant with the description in Table 3. Function is a broader category than behavior.

The contents of 'functional/behavioral test' in Tables 1 and 2 are not clear. In the text, 'functional tests' are listed in line 11. In Table 3, 'functional/behavioral endpoints' consist of three major items, motor activity, motor and sensory function, and learning and memory. Therefore, the major component of 'functional/behavioral tests' in Tables 1 and 2 would be motor and sensory function.

Note (c) to Table 1 is questionable unless the same pups are used to check the changes of findings in adolescent and young adult ages. Moreover, the number of animals tested is recommended to be 20 in Table 2. Therefore, it is generally preferable to adopt the procedures indicated in Table 2 since the offspring tested for cognitive function etc. are examined for neuropathology, and the correlation between behavioral abnormalities and neuropathological changes can be checked. Thus, Table 2 is recommended to be the first choice and treated as Table 1. The total sentences in this paragraph should be rewritten according to this consideration. Optional and Neuropathology in Tables 1 and 2 should be optional and neuropathology (small letters).

Pups no. of the female in the preweaning investigation in Table 2 is 5, not 2.

**13. Paragraph 16**
The phrase 'maternal or developmental toxicity or neurotoxicity' in line 10 is to be changed to 'maternal or developmental toxicity' or 'maternal or developmental toxicity including neurotoxicity', since neurotoxicity is a part of toxicity and is related to both dams and offspring.

In some cases, a high dose can not be chosen to induce maternal toxicity. Thus, it is highly recommended to add a sentence to explain the rationale in cases where no maternal toxic dose level is selected for the high dose.

A description regarding limit dose should be added.

**14. Paragraph 17**
The word 'should' should be changed to 'may' (lines 1 and 4).

The sentence 'However, an evaluation of direct dosing to pups has not been established yet.' should be inserted following the last sentence.

**15. Paragraph 19**
In case of dietary or via drinking water administration, due consideration should be taken that pups receive the test substances not only from milk but also considerably from diet or water in the later period of lactation.

The phrase 'except for the day of parturition' and the sentence 'The test substance should be administered after completion of parturition.' should be inserted following the end of the last sentence.

**16. Paragraph 20**
The first sentence should be deleted. In reproductive and developmental studies including teratological study and pre- and postnatal study, the dosage volume in each dam is practically calculated by two different methods: (a) based only on body weight on day 6 of gestation or (b) based on the

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.

## REFERENCES

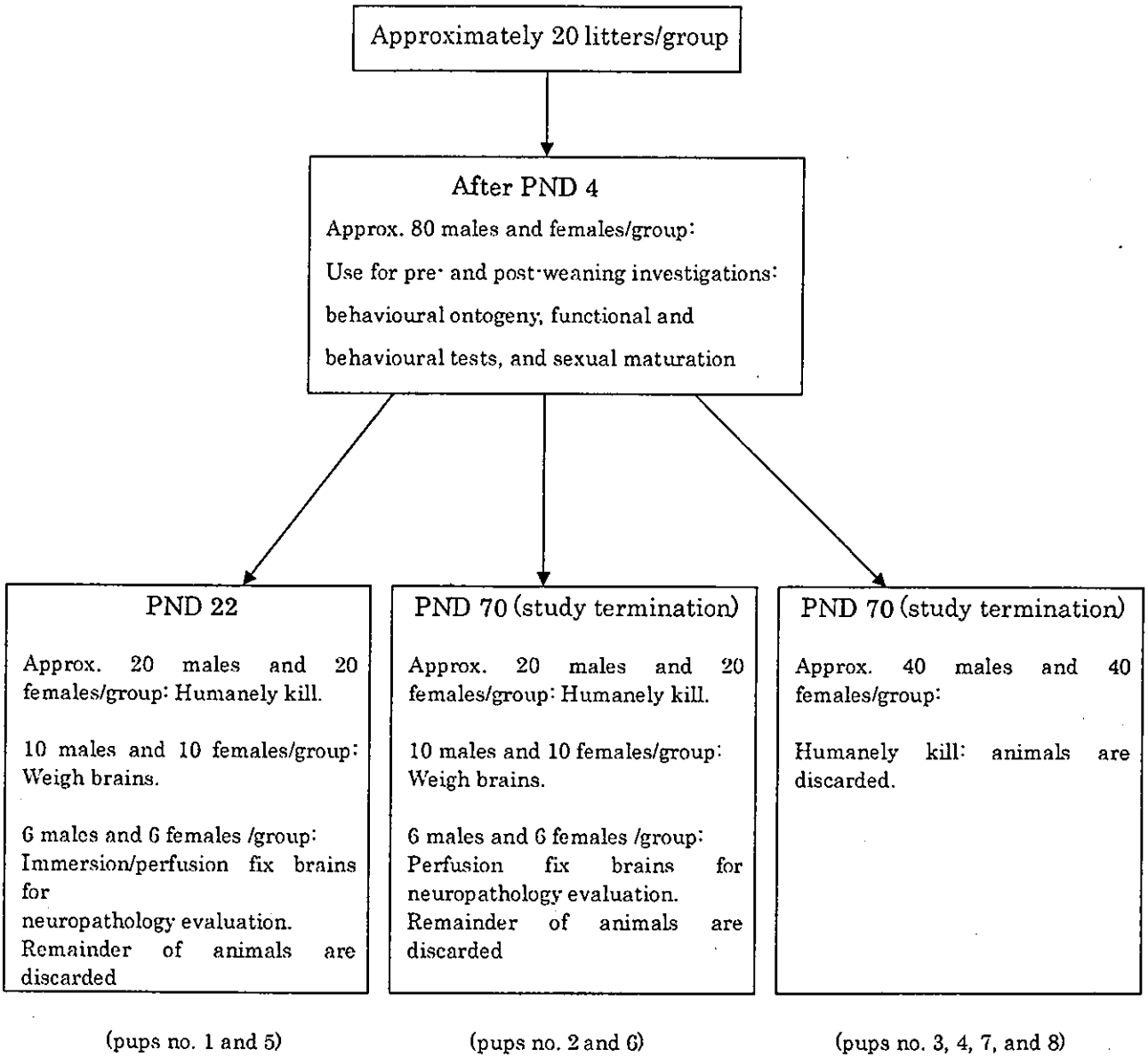
Organisation for Economic Co-operation and Development (OECD) (1995) *Draft Report of the OECD Ad Hoc Working Group on Reproduction and Developmental Toxicity.* Copenhagen, Denmark, 13–14 June 1995.

Organisation for Economic Co-operation and Development (OECD) (1996) *Final Report of the Consultation Meeting on Developmental Neurotoxicity.* Copenhagen, Denmark, 17–18 June 1996.

Organisation for Economic Co-operation and Development (OECD) (2003) *Report of the Expert Consultation Meeting in Developmental Neurotoxicity Testing.* Washington, US, 23–25 October 2000.

## 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: inter-species | intra-species | use of LOAEL | study period | nature of toxicity | TDI or VSD* (mg/kg/day) | allocation (%) | body weight (kg) | water consump. (L) | guideline value (mg/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 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

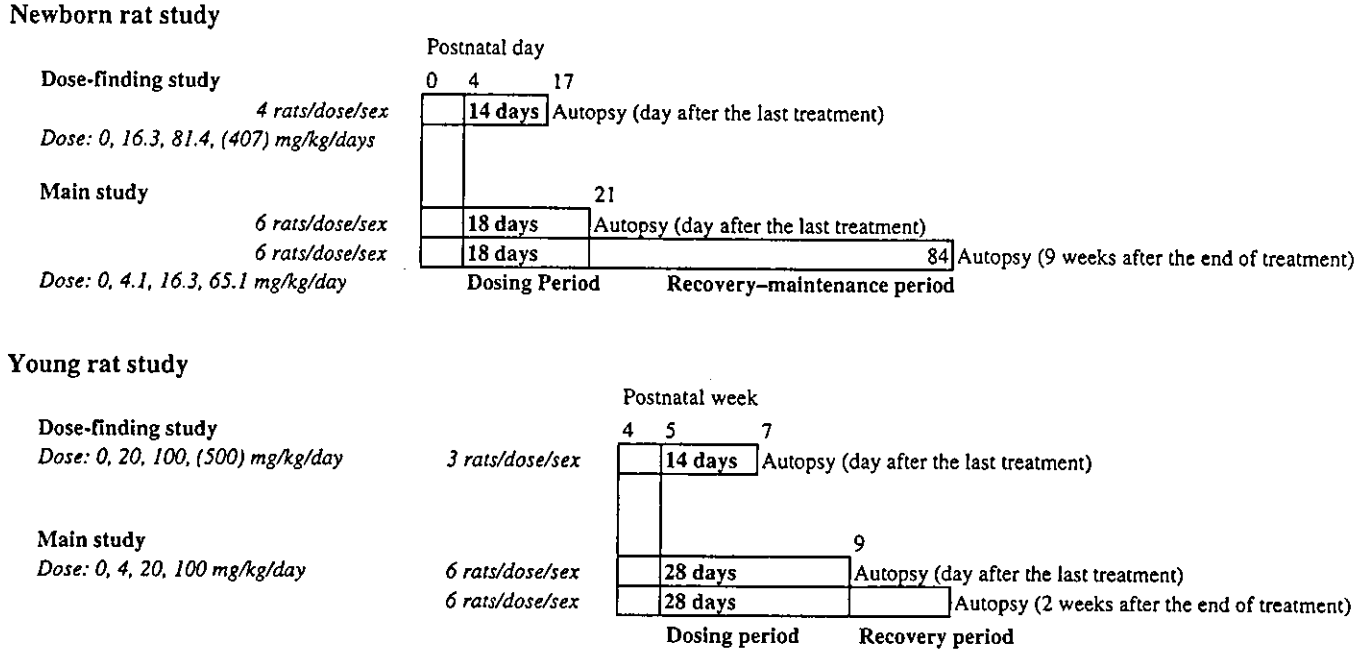


**Fig. 1** Time schedule of the newborn and young rat studies.

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