any supportive data for the carcinogenic potential of this chemical as revealed in the long-term and promotion bioassays.

MX induced statistically significant positive responses in both initiation and promotion cell transformation assays. These findings agree with positive outcomes using a C3H 10T1/2 cell transformation assay system (21) and also give supportive information to the carcinogenicity of MX (2). However, tumours were not observed after *in vivo* inoculation of a large number of transformed cells harvested from the initiation assay in which MX was used as the initiator and TPA as the promoter. This may be owing to the fact that the BALB/c 3T3 cells of the transformation assay were derived from a BALB/c mouse strain as used for the tumourigenicity assay, or may be related to the rather short *in vivo* expression period. We did not perform a similar experiment using immune-deficient nude mice. However, within 2–4 months Boone and Jacobs reported the induction of tumours in BALB/c mice by inoculation of transformed cells (22). Although we carried out the study for 2–3 weeks, the period might have been too short to develop nodules. Cells isolated from transformed foci in the initiation assay did not induce any nodules after inoculation to BALB/c mice, the strain of mouse from which the transformation assay cells were derived. Taken together, we could not adjudge the malignancy of transformed cells induced by MX when used as an initiator.

The possibility of an *in vivo* promoting effect of MX was revealed by the positive result in the promotion assay using BALB/c 3T3 cells. Moreover, this result was supported by the demonstration that MX inhibited GJIC, which is a characteristic of many tumour promoters evaluated using the metabolic cooperation assay (8). The major role of GJIC is considered to be the maintenance of homeostasis in multicellular organisms, and it is believed that second messenger transfer through GJIC is important for cell growth control (23,24). Tumour-promoting chemicals such as TPA and analogues, DDT and aldrin inhibit GJIC (25–27), and this *in vitro* test for tumour promoters is recommended as a useful tool for detecting non-genotoxic carcinogens (28). This activity of MX in the current GJIC assay is consistent with a recent report on GJIC inhibition in BALB/c 3T3 cells (29).

MX appears to have weak genotoxicity in mammalian systems *in vivo*, and it is probable that the tumour promoting activity of MX is important for explaining its carcinogenic activity. Although many regulatory bodies assess chemical safety based on the dogma that genotoxic carcinogens do not have any threshold, we propose that risk assessment of MX takes into account the chemical's likely threshold as a tumour promoter.

References

1. Meier,J.R., Knohl,R.B., Coleman,W.E., Ringhand,H.P., Munch,J.W., Kaylor,W.H., Streicher,R.P. and Kopfler,F.C. (1987) Studies on the potent bacterial mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone: aqueous stability, XAD recovery and analytical determination in drinking water and in chlorinated humic acid solutions. *Mutat. Res.*, **204**, 363–373.
2. Komulainen,H., Kosuma,V.M., Vaittinen,S.L., Vartiainen,T., Kaliste-Korhonen,E., Lotjonen,S., Tuominen,R.K. and Tuomisto,J. (1997) Carcinogenicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone in the rat. *J. Natl Cancer Inst.*, **89**, 848–856.
3. Nishikawa,A., Kinae,N., Furukawa,F., Mitsui,M., Enami,T., Hasegawa,T. and Takahashi,M. (1994) Enhancing effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone (MX) on cell proliferation and lipid peroxidation in the rat gastric mucosa. *Cancer Lett.*, **85**, 151–157.
4. Meier,J.R., Blazak,W.F. and Knohl,R.B. (1987) Mutagenic and clastogenic properties of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone: a potent bacterial mutagen in drinking water. *Environ. Mol. Mutagen.*, **10**, 411–424.
5. Tikkanen,L. and Kronberg,L. (1990) Genotoxic effects of various chlorinated butenoic acids identified in chlorinated drinking water. *Mutat. Res.*, **240**, 109–116.
6. Brunborg,G., Holme,J.A., Soderlund,E.J., Hongslo,J.K., Vartiainen,T., Lotjonen,S. and Becher,G.H. (1991) Genotoxic effects of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) in mammalian cells *in vitro* and in rats *in vivo*. *Mutat. Res.*, **260**, 55–64.
7. Jansson,K. and Hyttinen,J.M. (1994) Induction of gene mutation in mammalian cells by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), a chlorine disinfection by-product in drinking water. *Mutat. Res.*, **322**, 129–132.
8. Matsumura,H., Watanabe,M., Matsumoto,K. and Ohta,T. (1994) 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) induces gene mutations and inhibits metabolic cooperation in cultured Chinese hamster cells. *J. Toxicol. Environ. Health*, **43**, 65–72.
9. Miyamae,Y., Iwasaki,K., Kinae,N., Tsuda,S., Murakami,M., Tanaka,M. and Sasaki,Y.F. (1997) Detection of DNA lesions induced by chemical mutagens using the single-cell gel electrophoresis (comet) assay, 2. Relationship between DNA migration and alkaline condition. *Mutat. Res.*, **393**, 107–113.
10. Nunn,J.W., Davies,J.E. and Chipman,J.K. (1997) Production of unscheduled DNA synthesis in rodent hepatocytes *in vitro*, but not *in vivo*, by 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX). *Mutat. Res.*, **373**, 67–73.
11. Sasaki,Y.F., Nishidate,E., Izumiyama,F., Watanabe-Akanuma,M., Kinae,N., Matsusaka,N. and Tsuda,S. (1997) Detection of *in vivo* genotoxicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) by the alkaline single cell gel electrophoresis (Comet) assay in multiple mouse organs. *Mutat. Res.*, **393**, 47–53.
12. Holme,J.A., Haddeland,U., Haug,K. and Brunborg,G. (1999) DNA damage induced by the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) in mammalian cells *in vitro* and in mice. *Mutat. Res.*, **441**, 145–153.
13. Kakunaga,T. and Yamasaki,H. (eds) (1985) Transformation assay of established cell lines: mechanisms and application. IARC Scientific Publication No. 67, IARC, Lyon.
14. Kakunaga,T. and Crow,J.D. (1980) Cell variants showing differential susceptibility to ultraviolet light-induced transformation. *Science*, **209**, 505–507.
15. Tsuchiya,T. and Umeda,M. (1995) Improvement in the efficiency of the *in vitro* transformation assay method using BALB/3T3 A31-1-1 cells. *Carcinogenesis*, **16**, 1887–1894.
16. Murray,A.W. and Fitzgerald,D.J. (1979) Tumour promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. *Biochem. Biophys. Res. Commun.*, **91**, 395–401.
17. Yotti,L.P., Chang,C.C. and Trosko,J.E. (1979) Elimination of metabolic cooperation in Chinese hamster cells by a tumour promoter. *Science*, **206**, 1089–1091.
18. Stedman,D.B. and Welsch,F. (1985) Effects of 6-thioguanine on communication competence and factors affecting reversibility of phorbol ester inhibition in V79 cells measured by the metabolic cooperation assay and by dye coupling. *Carcinogenesis*, **6**, 1599–1605.
19. Padmapriya,A.A., Just,G. and Lewis,N.G. (1985) Synthesis of 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone, a potent mutagen. *Can. J. Chem.*, **63**, 828–832.
20. Yamasaki,H., Krutovskikh,V., Mesnil,M., Columbano,A., Tsuda,H. and Ito,N. (1993) Gap junctional intercellular communication and cell proliferation during rat liver carcinogenesis. *Environ. Health Perspect.*, **101**, 191–197.
21. Laaksonen,M., Maki-Paakkanen,J., Kronberg,L. and Komulainen,H. (2003) Effects of chlorohydroxyfuranones on 3-methylcholanthrene-induced neoplastic transformation in the two-stage transformation assay in C3H 10T1/2 cells. *Arch. Toxicol.*, **77**, 594–600.
22. Boone,C.W. and Jacobs,J.B. (1976) Sarcomas routinely produced from putatively nontumorigenic Balb/3T3 and C3H/10T1/2 cells by subcutaneous inoculation attached to plastic platelets. *J. Supramol. Struct.*, **5**, 131–137.
23. Loewenstein,W.R. (1979) Junctional intercellular communication and the control of growth. *Biochim. Biophys. Acta*, **560**, 1–65.
24. Yamasaki,H. (1990) Gap junctional intercellular communication and carcinogenesis. *Carcinogenesis*, **11**, 1051–1058.

25. Fitzgerald,D.J. and Murray,A.W. (1983) A new intercellular communication assay: its use in studies on the mechanism of tumour promotion. *Cell Biol. Int. Rep.*, **6**, 235–242.
26. Bohrman,J.S., Burg,J.R., Elmore,E., Gulati,D.K., Barfnecht,T.R., Niemeier,R.W., Dames,B.L., Toraason,M. and Langenbach,R. (1988) Interlaboratory studies with the Chinese hamster V79 cell metabolic cooperation assay to detect tumor-promoting agents. *Environ. Mol. Mutagen.*, **12**, 33–51.
27. Noda,K. (1984) Effects of various chemicals including tumor promoters on metabolic cooperation. *Yokohama Med. J.*, **35**, 407–420.
28. Sakai,A., Iwase,Y., Nakamura,Y., Sasaki,K., Tanaka,N. and Umeda,M. (2002) Use of a cell transformation assay with established cell lines, and a metabolic cooperation assay with V79 cells for the detection of tumor promoters: a review. *Altern. Lab. Anim.*, **30**, 33–59.
29. Hakulinen,P., Maki-Paakkanen,J., Naarala,J., Kronberg,L. and Komulainen,H. (2004) Potent inhibition of gap junctional intercellular communication by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in BALB/c 3T3 cells. *Toxicol. Lett.*, **151**, 439–449.

Received on January 5, 2005; revised on June 8, 2005; accepted on July 15, 2005

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Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)—Mammalian Mutagenicity Study Group (MMS)

Hiroshi Suzuki [a,*], Naohiro Ikeda [b], Kazuo Kobayashi [c], Yukari Terashima [c], Yasushi Shimada [d], Takayoshi Suzuki [e], Toshiyuki Hagiwara [f], Shigeki Hatakeyama [g], Koko Nagaoka [h], Junichi Yoshida [i], Yukiko Saito [j], Jin Tanaka [k], Makoto Hayashi [e]

[a] *Ina Research Inc., 2148-188 Nishiminowa, Ina-shi, Nagano 399-4501, Japan*
[b] *Safety and Environmental Research Center, Kao Corporation, 2606 Akabane, Ichikaimachi, Haga, Tochigi 321-3497, Japan*
[c] *Toxicology Research Laboratory, R&D Kissei Pharmaceutical Co. Ltd., 2320-1 Maki, Hotaka, Minamiazumi, Nagano 399-8305, Japan*
[d] *Hokko Chemical Industry Co. Ltd., 2165 Toda, Atsugi-shi, Kanagawa 243-0023, Japan*
[e] *Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[f] *Laboratory Animal Science and Toxicology Laboratories, Sankyo Co. Ltd., 717 Horikoshi, Fukuroi, Shizuoka 437-0065, Japan*
[g] *Nisshin Kyorin Pharmaceutical Co. Ltd., 5-3-1 Tsurugaoka, Oi-cho, Iruma-gun, Saitama 356-8511, Japan*
[h] *Fukushima Research Laboratories, Toa Eiyo Ltd., 1 Tanaka, Yuno, Iizuka, Fukushima 960-0280, Japan*
[i] *Central Research Laboratories, Kaken Pharmaceutical Co. Ltd., 301 Gensuke, Fujieda-shi, Shizuoka 426-8646, Japan*
[j] *Mitsubishi Chemical Safety Institute Ltd., 14 Sunayama, Hasaki-machi, Kashima-gun, Ibaraki 314-0255, Japan*
[k] *Biosafety Research Center, Foods, Drugs and Pesticides, 582-2 Shioshinden, Fukude-cho, Iwata-gun, Shizuoka 437-1213, Japan*

Received 25 August 2004; received in revised form 18 February 2005; accepted 15 March 2005

Abstract

We conducted simultaneous liver and peripheral blood micronucleus assays in young rats with seven rodent hepatocarcinogens—4,4′-methylenedianiline (MDA), quinoline, *o*-toluidine, 4-chloro-*o*-phenylenediamine (CPDA), dimethylnitrosamine (DMN), *p*-dimethylaminoazobenzene (DAB), and di(2-ethylhexyl)phthalate (DEHP)—and two mutagenic chemicals—kojic acid and methylmethanesulfonate (MMS).

* Corresponding author. Tel.: +81 265 73 8611; fax: +81 265 73 8612.
E-mail address: h-suzuki@ina-research.co.jp (H. Suzuki).

1383-5718/$ – see front matter
doi:10.1016/j.mrgentox.2005.03.012

Quinoline, DMN, and DAB were positive in the liver assay, while *o*-toluidine, kojic acid, DAB, and MMS were positive in the peripheral blood assay. *o*-Toluidine, kojic acid, and DAB are reportedly negative in mouse bone marrow micronucleus assays, indicating a species difference.

Our results revealed a correlation between micronucleus induction in hepatocytes and hepatocarcinogenicity. This technique can be useful for the detection of micronucleus-inducing chemicals that require metabolic activation, and it enables simultaneous comparison of the micronucleus-inducing potential of chemicals in the liver and peripheral blood in the same individual.

Keywords: Young rat; Liver micronucleus; Peripheral blood micronucleus; Hepatocarcinogen

1. Introduction

In vivo rodent bone marrow (BM) micronucleus assay results correlate highly with carcinogenicity in many organs, but the test is rather insensitive to indirect and liver carcinogens [1]. The micronucleus-inducing potential of such chemicals can be detected in in vivo liver micronucleus assays [2–4], which can be conducted by the partial hepatectomy (PH) method [2,5,6], co-treatment with mitogens [7,8] or an in vivo/in vitro assay system [9]. These all have serious disadvantages. In the PH method, P-450, styrenemonooxygenase, epoxide hydrolase, and glutathione-*S*-epoxide transferase activity is decreased [10], and the method is time-consuming because it involves surgery. In the co-treatment method with mitogens, the mitogens can interact with the test chemicals [11]. The in vivo/in vitro assay system requires much effort, time, and expense.

Searching for better approach, we evaluated liver micronucleus assay that uses 4-week-old rats [11]. We evaluated the assay using the hepatocarcinogen diethylnitrosamine (DEN) [12]. In 4-week-old rats, not only liver growth but also P450 activity are at their maximum and glucuronic acid, sulfate, glutathione, and glycine conjugation levels are the same as in mature animals [13], as are the levels of hexobarbital hydroxylation, *N*-demethylation of ethylmorphine, *O*-demethylation of *p*-nitroanisole and hydroxylation of aniline [14]. Since the usefulness of this method has not been clearly demonstrated, we organized a collaborative study to evaluate it with nine model chemicals. We conducted the peripheral blood micronucleus assay [15,16] simultaneously to evaluate another organ in the same animal. Our results demonstrated the relationship in young rats between the hepatocarcinogenicity and hepatocyte micronucleus-inducing potential of the test chemicals.

2. Materials and methods

2.1. Collaboration

Eleven research laboratories collaborated in this study (Table 1).

2.2. Animals

Male Fischer F344 or SD rats, 3 weeks of age, were purchased from Charles River Japan Inc., and used at 4 weeks of age. The animals were housed under a 12-h light–dark cycle and allowed free access to commercial pellets and tap water.

2.3. Chemicals

4,4′-Methylenedianiline (MDA, CAS No. 101-77-9), kojic acid (CAS No. 501-30-4), quinoline (CAS No. 91-22-5), *o*-toluidine (CAS No. 95-53-4), 4-chloro-*o*-phenylenediamine (CPDA, CAS No. 95-83-0), and dimethylnitrosamine (DMN, CAS No. 62-75-9) were purchased from Wako Pure Chemical Industries Ltd.; *p*-dimethylaminoazobenzene (DAB, CAS No. 60-11-7), di(2-ethylhexyl)phthalate (DEHP, CAS No. 117-81-7), and methylmethanesulfonate (MMS, CAS No. 66-27-3) from Aldrich. Diethylnitrosamine (DEN, CAS No. 55-18-5) was purchased from Wako Pure Chemical Industries Ltd. or Tokyo Kasei Co. Ltd., and cyclophosphamide (CP, CAS No. 50-18-0) was purchased from ICN Biochemicals or Aldrich.

MDA, *o*-toluidine, CPDA, and DAB were suspended in olive oil, quinoline and DEHP in corn oil. Kojic acid was suspended in 1% sodium carboxymethylcellulose. DMN was dissolved in distilled water, MMS in physiological saline. DEN and CP, the positive control substances, were dissolved in distilled water, and the same lot chemical was used in all laboratories.

Table 1
Study participants

	Laboratory	Investigators
1	Biosafety Research Center, Foods, Drugs and Pesticides	Jin Tanaka
2	Hokko Chemical Industry Co. Ltd.	Yasushi Shimada
3	Ina Research Inc.	Hiroshi Suzuki[a], Kana Komatsu Akiko Koeda, Tadashi Imamura
4	Kaken Pharmaceutical Co. Ltd.	Junichi Yoshida
5	Kao Corporation	Naohiro Ikeda
6	Kissei Pharmaceutical Co. Ltd.	Kazuo Kobayashi, Yukari Terashima, Kaori Yasue
7	Mitsubishi Chemical Safety Institute Ltd.	Yukiko Saito
8	National Institute of Health Sciences	Takayoshi Suzuki, Makoto Hayashi
9	Nisshin Kyorin Pharmaceutical Co. Ltd.	Shigeki Hatakeyama
10	Sankyo Co. Ltd.	Toshiyuki Hagiwara, Ayumi Okazaki
11	Toa Eiyo Ltd.	Koko Nagaoka

[a] Chief study organizer.

2.4. Doses

We used 1/2 and 1/4 of the LD_{50} value of each chemical as the high and low dose. When the LD_{50} values were unclear, we estimated them by small-scale experiments according to the method of Lorke [17]. Negative control animals received the respective vehicle. Positive control animals received DEN at 40 mg/kg (liver micronucleus assay) or CP at 10 mg/kg (peripheral blood micronucleus assay). Each group consisted of four or five animals. Dosing was conducted once intraperitoneally or orally. With the exception of MMS, each chemical was evaluated by two laboratories.

2.5. Liver micronucleus assay

Rats were anesthetized with ethylether 3, 4 or 5 days after a single administration of test chemical or 5 days after administration of the negative or positive control chemicals. Hepatocytes were isolated by the collagenase perfusion method, rinsed with 10% neutral formalin two or three times, centrifuged at $50 \times g$ for 1 min, suspended in 10% neutral formalin, and stored under refrigeration. For staining, 10–20 μL of the suspension was mixed with an equal volume of acridine orange (AO)–4′6-diamidino-2-phenylindole dihydrochloride (DAPI) [12]. Approximately 10–20 μL of stained suspension was dropped onto a clean glass slide and covered with a cover slip (24 mm × 40 mm).

Microscopic preparations were evaluated with the aid of a fluorescence microscope (×400 or greater) with UV excitation. The number of micronucleated hepatocytes (MNHEPs) among 2000 hepatocytes (two fields) was recorded for each animal. MNHEPs were defined as hepatocytes with round or distinct micronuclei that stained like the nucleus, with the ≤1/4 diameter of the nucleus [7,18]. The number of mitotic cells per 2000 hepatocytes was determined.

2.6. Peripheral blood micronucleus assay

A small amount of blood was collected from a tail vessel on Day 2 after treatment, at which time most chemicals induce the maximum response [19]. It was stained by either of the following methods: (1) 5–10 μL was dropped on to AO-coated slides, covered with cover glasses, and stored in a deep freezer until analysis [15], or (2) 10 μL suspension was mixed with about 30 μL of 10% neutral formalin and stored at room temperature, the samples were mixed with an equal volume of AO solution (500 μg/mL) in the ratio of 1:1 and smeared on a glass slide immediately before analysis. Specimens were evaluated with the aid of a fluorescent microscope (×600 or greater) with B excitation. The number of micronucleated reticulocytes (MNRETs) among 2000 reticulocytes (RETs) and the number RETs among 1000 erythrocytes were recorded for each animal.

2.7. Statistical analysis

We determined the statistical significance of the incidence of micronucleated hepatocytes or reticulocytes using Kastenbaum and Bowman's method [20] and that of reticulocytes with the Student *t*-test.

3. Results

3.1. Liver micronucleus assay

Table 2 shows the results of the liver micronucleus assay. Quinoline, DMN, and DAB were positive in both

Table 2
Results of the liver micronucleus assay

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
MDA				
Lab 1				
0	4	5	0.11 ± 0.09	0.38 ± 0.35
200	4	3	0.14 ± 0.11	0.23 ± 0.10
	4	4	0.19 ± 0.13	1.35 ± 0.94
	4	5	0.16 ± 0.09	0.73 ± 0.49
300	4	3	0.10 ± 0.04	0.19 ± 0.18
	3	4	0.08 ± 0.10	0.43 ± 0.40
	4	5	0.10 ± 0.09	0.50 ± 0.19
400	2	3	0.38 ± 0.04[a]	0.23 ± 0.11
	2	4	0.20 ± 0.14	0.28 ± 0.32
	3	5	0.18 ± 0.08	0.20 ± 0.13
DEN*	4	5	1.21 ± 0.08[a]	0.25 ± 0.12
Lab 2				
0	4	5	0.00 ± 0.00	0.06 ± 0.09
150	4	3	0.00 ± 0.00	0.18 ± 0.12
	4	4	0.05 ± 0.04	0.20 ± 0.12
	4	5	0.03 ± 0.03	0.00 ± 0.00
300	3	3	0.10 ± 0.09	0.13 ± 0.13
	3	4	0.02 ± 0.03	0.07 ± 0.03
	4	5	0.08 ± 0.06	0.08 ± 0.03
DEN*	4	5	0.44 ± 0.20[b]	0.18 ± 0.09
Kojic acid				
Lab 1				
0	4	5	0.06 ± 0.03	0.66 ± 0.26
1000	4	3	0.06 ± 0.06	0.98 ± 0.43
	4	4	0.06 ± 0.05	0.85 ± 0.25
	4	5	0.08 ± 0.05	1.56 ± 0.14
2000	4	3	0.04 ± 0.05	0.44 ± 0.13
	4	4	0.08 ± 0.05	0.49 ± 0.35
	4	5	0.09 ± 0.08	1.04 ± 0.29
DEN*	4	5	0.66 ± 0.13[a]	0.65 ± 0.31
Lab 2				
0	5	5	0.07 ± 0.06	0.61 ± 0.22
1000	5	3	0.04 ± 0.02	0.50 ± 0.23
	5	4	0.10 ± 0.06	0.63 ± 0.17
	5	5	0.11 ± 0.07	0.93 ± 0.06
2000	5	3	0.08 ± 0.08	0.57 ± 0.21
	5	4	0.07 ± 0.03	0.57 ± 0.08
	5	5	0.05 ± 0.04	0.60 ± 0.12
DEN*	5	5	1.04 ± 0.16[a]	0.95 ± 0.08
Quinoline				
Lab 1				
0	4	5	0.09 ± 0.06	0.48 ± 0.33
75	4	3	0.16 ± 0.08	0.18 ± 0.10
	4	4	0.39 ± 0.20[a]	0.15 ± 0.07
	4	5	0.20 ± 0.09[b]	0.25 ± 0.27

Table 2 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
150	4	3	0.58 ± 0.46[a]	0.34 ± 0.21
	4	4	0.33 ± 0.09[a]	0.39 ± 0.15
	4	5	0.35 ± 0.22[a]	0.18 ± 0.16
DEN*	4	5	0.21 ± 0.14[b]	0.48 ± 0.33
Lab 2				
0	5	5	0.03 ± 0.03	0.44 ± 0.10
75	5	3	0.36 ± 0.10[a]	0.35 ± 0.12
	5	4	0.22 ± 0.06[a]	0.33 ± 0.10
	5	5	0.12 ± 0.06	0.36 ± 0.10
150	5	3	1.22 ± 0.09[a]	0.33 ± 0.06
	5	4	0.93 ± 0.22[a]	0.42 ± 0.15
	5	5	0.61 ± 0.07[a]	0.23 ± 0.06
DEN*	5	5	0.84 ± 0.12[a]	0.76 ± 0.10
o-Toluidine				
Lab 1				
0	4	5	0.05 ± 0.06	0.41 ± 0.09
300	4	3	0.10 ± 0.07	0.16 ± 0.20
	4	4	0.10 ± 0.09	0.09 ± 0.05
	4	5	0.11 ± 0.10	0.40 ± 0.37
600	4	3	0.05 ± 0.06	0.21 ± 0.17
	4	4	0.06 ± 0.05	0.08 ± 0.06
	4	5	0.01 ± 0.03	0.06 ± 0.08
DEN*	4	5	0.85 ± 0.17[a]	0.30 ± 0.11
Lab 2				
0	4	5	0.04 ± 0.05	0.59 ± 0.30
300	4	3	0.04 ± 0.07	0.28 ± 0.06
	4	4	0.05 ± 0.07	0.45 ± 0.17
	4	5	0.08 ± 0.12	0.66 ± 0.36
600	4	3	0.04 ± 0.08	0.27 ± 0.18
	4	4	0.04 ± 0.05	0.31 ± 0.14
	4	5	0.01 ± 0.03	0.64 ± 0.26
DEN*	4	5	0.68 ± 0.15[a]	0.46 ± 0.09
CPDA				
Lab 1				
0	4	5	0.11 ± 0.02	0.34 ± 0.13
150	4	3	0.11 ± 0.13	0.10 ± 0.07
	4	4	0.20 ± 0.09	0.18 ± 0.09
	4	5	0.15 ± 0.16	0.46 ± 0.42
300	4	3	0.21 ± 0.09	0.01 ± 0.03
	4	4	0.21 ± 0.13	0.04 ± 0.03
	4	5	0.23 ± 0.13	0.09 ± 0.12
DEN*	4	5	0.88 ± 0.34[a]	0.25 ± 0.18
Lab 2				
0	4	5	0.09 ± 0.05	0.65 ± 0.09
150	4	3	0.05 ± 0.00	0.48 ± 0.09
	4	4	0.08 ± 0.09	0.36 ± 0.09
	4	5	0.06 ± 0.05	0.76 ± 0.27

Table 2 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
300	4	3	0.06 ± 0.08	0.25 ± 0.09
	4	4	0.04 ± 0.05	0.28 ± 0.12
	4	5	0.10 ± 0.07	0.31 ± 0.20
DEN*	4	5	0.68 ± 0.17[a]	0.34 ± 0.13
DMN				
Lab 1				
0	4	5	0.04 ± 0.03	0.66 ± 0.30
5	4	3	0.36 ± 0.36[a]	1.03 ± 0.12
	4	4	0.35 ± 0.25[a]	0.76 ± 0.44
	4	5	0.33 ± 0.24[a]	0.41 ± 0.27
10	4	3	0.31 ± 0.23[a]	0.45 ± 0.27
	4	4	0.51 ± 0.33[a]	0.34 ± 0.10
	4	5	0.36 ± 0.19[a]	0.86 ± 0.17
DEN*	4	5	1.04 ± 0.33[a]	0.41 ± 0.18
Lab 2				
0	4	5	0.05 ± 0.00	0.78 ± 0.43
5	4	3	0.15 ± 0.07	0.38 ± 0.23
	4	4	0.34 ± 0.24[a]	0.55 ± 0.20
	4	5	0.36 ± 0.26[a]	0.73 ± 0.27
10	4	3	0.26 ± 0.09[a]	0.55 ± 0.36
	4	4	0.51 ± 0.20[a]	0.61 ± 0.22
	4	5	0.46 ± 0.09[a]	0.70 ± 0.27
DEN*	4	5	0.86 ± 0.30[a]	0.64 ± 0.33
DAB				
Lab 1				
0	4	5	0.03 ± 0.03	0.10 ± 0.07
71	4	3	0.19 ± 0.08[a]	0.18 ± 0.15
	4	4	0.11 ± 0.08[b]	0.23 ± 0.13
	4	5	0.08 ± 0.10	0.44 ± 0.10
142	4	3	0.35 ± 0.08[a]	0.21 ± 0.10
	4	4	0.16 ± 0.03[a]	0.11 ± 0.03
	4	5	0.14 ± 0.03[b]	0.13 ± 0.12
DEN*	4	5	0.63 ± 0.27[a]	0.63 ± 0.18
Lab 2				
0	4	5	0.19 ± 0.11	0.19 ± 0.18
120	4	3	0.36 ± 0.10[b]	0.59 ± 0.17
	4	4	0.48 ± 0.09[a]	0.29 ± 0.12
	4	5	0.61 ± 0.02[a]	0.39 ± 0.12
240	4	3	0.25 ± 0.06	0.28 ± 0.21
	4	4	0.41 ± 0.07[b]	0.36 ± 0.16
	4	5	0.38 ± 0.13[b]	0.44 ± 0.19
DEN*	3	5	0.95 ± 0.33[a]	0.15 ± 0.05
DEHP				
Lab 1				
0	4	5	0.05 ± 0.04	0.95 ± 0.19
1000	4	3	0.05 ± 0.04	0.33 ± 0.46
	4	4	0.05 ± 0.04	0.20 ± 0.08
	4	5	0.06 ± 0.09	0.43 ± 0.10

Table 2 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
2000	4	3	0.04 ± 0.05	0.28 ± 0.49
	4	4	0.05 ± 0.04	0.23 ± 0.10
	4	5	0.05 ± 0.06	0.63 ± 0.34
DEN*	4	5	1.66 ± 0.24[a]	1.13 ± 0.13
Lab 2				
0	4	5	0.08 ± 0.06	0.73 ± 0.36
1000	4	3	0.04 ± 0.05	0.43 ± 0.06
	4	4	0.09 ± 0.06	0.65 ± 0.47
	4	5	0.06 ± 0.05	0.26 ± 0.06
2000	4	3	0.09 ± 0.08	0.23 ± 0.16
	4	4	0.09 ± 0.09	0.15 ± 0.06
	4	5	0.09 ± 0.09	0.28 ± 0.17
DEN*	4	5	0.98 ± 0.52[a]	0.41 ± 0.16
MMS				
Lab 1				
0	4	5	0.05 ± 0.06	0.56 ± 0.34
40	4	3	0.08 ± 0.06	0.89 ± 0.30
	4	4	0.01 ± 0.03	0.66 ± 0.21
	4	5	0.04 ± 0.05	0.66 ± 0.40
80	4	3	0.11 ± 0.05	0.60 ± 0.24
	4	4	0.11 ± 0.08	0.58 ± 0.40
	4	5	0.08 ± 0.05	0.80 ± 0.41
DEN*	4	5	0.88 ± 0.12[a]	0.69 ± 0.42

MDA, 4,4′-methylenedianiline; DEN*, diethylnitrosamine (as a positive control, 40 mg/kg); CPDA, 4-chloro-*o*-phenylenediamine; DMN, dimethylnitrosamine; DAB, *p*-dimethylaminoazobenzene; DEHP, di (2-ethylhexyl) phthalate; MMS, methylmethanesulfonate.

[a] Significantly different from the solvent control (Kastenbaum and Bowman test; $P < 0.01$).

[b] Significantly different from the solvent control (Kastenbaum and Bowman test; $P < 0.05$).

laboratories. Deaths occurred at 400 mg/kg of MDA in sampling groups as follows: two animals on Day 3, two on Day 4, and one on Day 5. Thus, the positive response in samples harvested on Day 3 was based on only two animals. At 300 mg/kg of MDA, one animal died on Day 4. MDA was negative at 300 mg/kg in each sampling day. The other five chemicals were negative.

The appearance of mitotic cells was confirmed in all laboratories with all chemicals.

3.2. Peripheral blood micronucleus assay

Table 3 shows the results of the peripheral blood micronucleus assay. Kojic acid, *o*-toluidine, and DAB were positive in both laboratories, MMS in the one that tested it. Quinoline was positive in one of the two laboratories. CPDA was significantly cytotoxic, decreasing the % RET in both laboratories.

4. Discussion

We conducted the liver and peripheral blood micronucleus assays concurrently in young rats with nine mutagenic and/or carcinogenic chemicals. Table 4 compares the data generated in this collaboration with published bone marrow and hepatocarcinogenicity data.

The mean incidence of MNHEPs (%) for 70 rats in the solvent control groups was 0.07 ± 0.06%. This low incidence suggests the robustness of the assay.

Quinoline, DMN and DAB were positive in the liver micronucleus assay. The MNHEP (%) induced by 150 mg/kg quinoline tended to decrease with sampling time in both labs. This may have been due to inhibition of hepatocyte proliferation, as evidenced by the decrease in mitotic cells. The same may be applicable to DAB at 142 mg/kg. Although a statistically significant increase in MNHEP (%) was induced by 400 mg/kg

Table 3
Results of the peripheral blood micronucleus assay

Chemical and dose (mg/kg)	No. of animals	MNRET (%) mean ± S.D.	RET (%) mean ± S.D.
MDA			
Lab 1			
0	4	0.06 ± 0.06	14.0 ± 1.3
200	4	0.09 ± 0.03	11.6 ± 1.2[d]
400	3	0.15 ± 0.05	14.4 ± 3.3
DEN*	4	0.05 ± 0.04	13.0 ± 1.7
CP**	4	0.73 ± 0.10[a]	9.0 ± 1.0[c]
Lab 2			
0	4	0.04 ± 0.03	
150	4	0.13 ± 0.03	
300	4	0.09 ± 0.08	NT
DEN*	4	0.01 ± 0.03	
CP**	4	0.93 ± 0.42[b]	
Kojic acid			
Lab 1			
0	4	0.19 ± 0.12	8.3 ± 1.2
1000	4	0.15 ± 0.04	6.2 ± 0.4[d]
2000	4	0.70 ± 0.24[a]	7.1 ± 0.6
DEN*	4	0.16 ± 0.08	7.6 ± 1.6
CP**	4	1.43 ± 0.24[a]	6.6 ± 0.6[d]
Lab 2			
0	5	0.07 ± 0.05	11.6 ± 0.3
1000	5	0.16 ± 0.04[a]	11.2 ± 0.6
2000	5	0.38 ± 0.04[a]	11.7 ± 0.8
CP**	5	0.93 ± 0.12[a]	10.4 ± 0.9
Quinoline			
Lab 1			
0	4	0.13 ± 0.03	4.2 ± 0.6
75	4	0.11 ± 0.06	5.0 ± 0.5
150	4	0.10 ± 0.00	4.0 ± 0.9
DEN*	4	0.10 ± 0.07	4.7 ± 0.2
CP**	4	1.14 ± 0.43[a]	3.6 ± 0.5
Lab 2			
0	5	0.07 ± 0.03	12.2 ± 0.4
75	5	0.08 ± 0.00	11.9 ± 0.3
150	5	0.17 ± 0.03[a]	11.3 ± 0.9
CP**	5	0.95 ± 0.06[a]	10.3 ± 1.0
o-Toluidine			
Lab 1			
0	4	0.08 ± 0.06	13.6 ± 0.7
300	4	0.25 ± 0.11[b]	17.1 ± 2.1[d]
600	4	0.36 ± 0.09[a]	18.7 ± 3.6
DEN*	4	0.08 ± 0.06	16.8 ± 3.5
CP**	4	0.86 ± 0.25[a]	13.7 ± 2.2
Lab 2			
0	4	0.21 ± 0.08	11.0 ± 1.5
300	4	0.19 ± 0.03	13.5 ± 2.7

Table 3 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	MNRET (%) mean ± S.D.	RET (%) mean ± S.D.
600	4	0.46 ± 0.11[a]	13.6 ± 3.7
CP**	4	0.93 ± 0.21[a]	9.2 ± 0.7
CPDA			
Lab 1			
0	4	0.05 ± 0.06	7.5 ± 2.4
150	4	0.10 ± 0.07	5.8 ± 1.4
300	4	0.08 ± 0.06	3.6 ± 0.4[d]
DEN*	4	0.05 ± 0.04	6.8 ± 1.0
CP**	4	0.90 ± 0.35[a]	6.4 ± 0.8
Lab 2			
0	4	0.05 ± 0.06	12.0 ± 2.2
150	4	0.08 ± 0.03	12.1 ± 3.1
300	4	0.13 ± 0.06	7.8 ± 0.5[d]
DEN*	4	0.03 ± 0.03	12.5 ± 1.3
CP**	4	0.76 ± 0.14[a]	12.3 ± 3.2
DMN			
Lab 1			
0	4	0.08 ± 0.06	17.7 ± 1.9
5	4	0.04 ± 0.05	17.4 ± 3.1
10	4	0.15 ± 0.08	16.9 ± 2.5
DEN*	4	0.03 ± 0.03	13.5 ± 2.3
CP**	4	1.01 ± 0.49[a]	12.6 ± 1.2[c]
Lab 2			
0	4	0.11 ± 0.09	16.1 ± 3.7
5	4	0.19 ± 0.14	15.6 ± 2.1
10	4	0.18 ± 0.09	15.4 ± 2.5
DEN*	4	0.25 ± 0.07	17.4 ± 1.7
CP**	4	0.89 ± 0.19[a]	15.4 ± 3.2
DAB			
Lab 1			
0	4	0.03 ± 0.05	
71	4	0.05 ± 0.06	NT
142	4	0.43 ± 0.23[a]	
CP**	4	0.64 ± 0.13[a]	
Lab 2			
0	4	0.05 ± 0.04	13.6 ± 2.1
120	4	0.03 ± 0.03	14.0 ± 2.8
240	4	0.25 ± 0.13[a]	21.9 ± 6.4[d]
CP**	4	0.55 ± 0.16[a]	11.8 ± 1.4
DEHP			
Lab 1			
0	4	0.14 ± 0.09	29.0 ± 2.5
1000	4	0.18 ± 0.16	21.7 ± 1.3[c]
2000	4	0.18 ± 0.06	22.3 ± 1.1[c]
DEN*	4	0.06 ± 0.05	14.7 ± 2.2[c]
CP**	4	1.23 ± 0.34[a]	13.1 ± 2.6[c]

Table 3 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	MNRET (%) mean ± S.D.	RET (%) mean ± S.D.
Lab 2			
0	4	0.16 ± 0.05	23.1 ± 2.4
1000	4	0.25 ± 0.14	27.1 ± 7.4
2000	4	0.16 ± 0.05	23.1 ± 3.8
DEN*	4	0.19 ± 0.08	21.9 ± 3.3
CP**	4	1.06 ± 0.13[a]	21.9 ± 2.2
MMS			
Lab 1			
0	4	0.14 ± 0.10	8.5 ± 1.0
40	4	2.04 ± 0.79[a]	12.5 ± 4.8
80	4	0.96 ± 0.57[a]	3.8 ± 0.2[c]
DEN*	4	0.18 ± 0.05	12.4 ± 1.5[c]
CP**	4	1.54 ± 1.03[a]	7.8 ± 1.8

MDA, 4,4′-methylenedianiline; DEN*, diethylnitrosamine (the first positive control, 40 mg/kg); CP**, cyclophosphamide (the second positive control, 10 mg/kg); CPDA, 4-chloro-*o*-phenylenediamine; DMN, dimethylnitrosamine; DAB, *p*-dimethylaminoazobenzene; DEHP, di (2-ethylhexyl) phthalate; MMS, methylmethanesulfonate. NT: not tested.

[a] Significantly different from the solvent control (Kastenbaum and Bowman test; $P < 0.01$).

[b] Significantly different from the solvent control (Kastenbaum and Bowman test; $P < 0.05$).

[c] Significantly different from the solvent control (Student *t*-test; $P < 0.01$).

[d] Significantly different from the solvent control (Student *t*-test; $P < 0.05$).

MDA in samples harvested 3 days after dosing, the data were from only two animals. In conjunction with Lab 2 results, MDA was considered to be negative in this assay. Quinoline, DMN and DAB, are chemicals were also positive in the presence of metabolic activation in in vitro genotoxicity assays [21–23]. Quinoline and DMN induce hepatocellular carcinoma in mice and rats [24–26], while DAB induces hepatocellular carcinoma in rats, but not in mice [27]. Four chemicals that were negative in this study have been reported to be carcinogenic. MDA and CPDA induce hepatocellular carcinoma in male and female mice [28–30] and neoplastic nodules in rats [29–31]. *o*-Toluidine induces hepatocellular carcinomas and hemangiosarcomas in mice and multiple organs tumors in rats [32]. DEHP induces hepatocellular carcinoma in mice and rats [33], but this chemical, unlike quinoline, DMN, and DAB, is a peroxisome proliferator, not a genotoxic carcinogen [34]. So the negative results in this assay are understandable. MMS induces carcinomas in the nasal cavity, central nervous system, and injection sites [35], but did not induce micronuclei in this study. *O*-Alkylation

Table 4
Micronucleus assay results for nine chemicals in this study compared with results from published bone marrow and hepatocarcinogenicity assays

Chemical	MN		BM		Hepatocarcinogenicity	
	L	PB	Mouse	Rat	Mouse	Rat
MDA	–	–	– [1]	ND	+ [28,29]	+ [28,29]
Kojic acid	–	+	– [41,50]	ND	– [42]	ND
Quinoline	+	–, +	+ (–) [44,45]	ND	+ [25]	+ [24]
o-Toluidine	–	+	– [1]	ND	+ [32]	– [32]
CPDA	–	–	+ [16]	+ (–) [16]	+ [30,31]	+ [30,31]
DMN	+	–	– (+) [46,47]	ND	+ [26]	+ [26]
MMS	–	+	+ [16]	+ [16]	ND	– [35]
DAB	+	+	– [1]	ND	ND	+ [27]
DEHP	–	–	– [1]	ND	+ [33]	+ [33]

MN, micronucleus assay; L, liver; PB, peripheral blood; BM, bone marrow micronucleus assay; parentheses show peripheral blood micronucleus assay. ND, no data found.

is more efficient than *N*-alkylation in the formation of micronuclei [36–38], and considering that MMS causes primarily *N*-7-methylguanine formation [39], the negative results were expected. Regarding P450 levels in young rats, 1A2, 2A1, 2B1, 2B2, 2E1, 3A1, and 3A2 levels increase with age, and reaching a maximum at approximately 30 days. Thereafter, the levels are suppressed by growth hormones, and 2C7, 2C11, 2C12, and 2C22 levels increase [40]. Therefore, P450 changes may have affected the results of the assay. Quinoline, DMN, and DAB are clearly genotoxic in in vitro only following metabolic activation [21–23], which makes them suitable for this assay.

In summary, quinoline, DMN, and DAB, which are rat hepatocarcinogens, induced liver micronuclei in this study. MDA, kojic acid [41,42], *o*-toluidine, and CPDA, which possess weak or uncertain hepatocarcinogenic potential in rats, did not, nor did DEHP, a non-genotoxic rat hepatocarcinogen. All chemicals, except MMS, were evaluated in two laboratories with similar results, as noted.

Although we did not statistically analyze the incidence of mitosis, we observed an increase or decrease for each chemical. The mitotic index reflected only three time points and did not give any information about the total number of mitoses. Thus, a correlation between MNHEP (%) and mitotic index was not always evident. These results may reflect increased mitotic activity or cytotoxic action of test chemicals on dividing hepatocytes [43].

The mean incidence of MNRETs (%) for 70 rats in the solvent control groups was $0.10 \pm 0.08\%$. This low incidence suggests the robustness of the assay like the liver assay.

Quinoline at 150 mg/kg was positive in the peripheral blood micronucleus assay only in Lab 2. Inconsistent results for quinoline have been reported before: the compound was positive in mouse bone marrow micronucleus assays [44] and negative in transgenic mouse peripheral blood micronucleus assays [45]. Thus, quinoline induces micronuclei in the liver but may not in hepatopoietic tissue. DMN was negative in peripheral blood micronucleus assay in rats, but has been reported to be positive in transgenic mice [46]. It is also negative in the mouse bone marrow micronucleus assay [47]. These results may reflect the fact that *N*-nitroso chemicals are difficult to evaluate in bone marrow micronucleus assays [1]. The rate of *N*-hydroxylation of DAB is higher in rats than in mice [48]. *N*-hydroxylation of amino azo dyes generates mutagenic metabolites [49], which may yield different results. Although kojic acid and *o*-toluidine were positive in this study, they are negative in mouse bone marrow micronucleus assays [50,51]. The MNRET (%) for CPDA in Lab 2 tended to increase in a dose-dependent manner, though it did not reach a statistically significant level. Because of the MNRET (%) were not dose-dependent in Lab 1, CPDA was considered to be negative. Although, CPDA was negative in this assay, it is positive in mouse bone marrow assays [16]. Thus, species differences are evident for kojic acid, *o*-toluidine and CPDA. The results of MMS, a direct alkylating agent, were consistent with those of mouse/rat bone marrow micronucleus assays [16].

In the present study, we evaluated known hepatocarcinogenic chemicals for micronucleus-inducing effects in 4-week-old rats. For some chemicals, our peripheral blood results differed from those reported by others, perhaps because younger rats are more sensitive to mutagens [52]. Accordingly, the simultaneous liver and peripheral blood assay system may bring out different result to previously reported one. In this assay, rodent hepatocarcinogens have been mainly used. Further evaluation using other organ carcinogens should be performed to assess this system in future.

As shown with quinoline, DMN, and DAB, the liver MN assay detected chemicals that required metabolic activation. Thus, it could be used to confirm positive responses in in vitro genotoxicity assays. These assays could expand the information obtained, for example, in the in vivo/in vitro UDS (unscheduled DNA synthesis) assay or the in vivo single cell gel electrophoresis (Comet) assay.

In conclusion, this assay system enabled us to simultaneously detect hepatocyte and peripheral blood micronucleus induction in the same animal. We also obtained information on differences in clastogen sensitivity between rats and mice. More chemicals should be studied to elucidate the validity and the sensitivity of this assay system.

Acknowledgment

This article was communicated by the Mammalian Mutagenicity Study group (MMS) of the Environmental Mutagen Society of Japan (JEMS).

References

[1] T. Morita, N. Asano, T. Awogi, Y. Sasaki, S. Sato, H. Shimada, S. Sutou, T. Suzuki, A. Wakata, T. Sofuni, M. Hayashi, Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (Groups 1, 2A, and 2B). The summary report of the 6th collaborative study by CSGMT/JEMS·MMS, Mutat. Res. 389 (1997) 3–122.

[2] F.A. Angelosanto, Tissues other than bone marrow that can be used for cytogenetic analyses, Environ. Mol. Mutagen. 25 (1995) 338–343.

[3] I. Cliet, E. Fournier, C. Melcion, A. Cordier, In vivo micronucleus test using mouse hepatocytes, Mutat. Res. 216 (1989) 321–326.

[4] V.S. Zhurkov, L.P. Sycheva, O. Salamatova, I.F. Vyskubenko, E.G. Feldt, N.I. Sherenesheva, Selective induction of micronuclei in the rat/mouse colon and liver by 1,2-dimethylhydrazine: a seven-tissue comparative study, Mutat. Res. 368 (1996) 115–120.

[5] A.D. Tates, I. Neuteboom, M. Hofker, L. den Engelse, A micronucleus technique for detecting clastogenic effects of mutagens/carcinogens (DEN, DMN) in hepatocytes of rat liver in vivo, Mutat. Res. 74 (1980) 11–20.

[6] A.D. Tates, L. den Engelse, The role of short-lived lesions in the induction of micronuclei in rat liver by ethylnitrosourea and methyl methanesulfonate: the importance of experimental design, Mutat. Res. 210 (1989) 271–279.

[7] I. Braithwaite, J. Ashby, A non-invasive micronucleus assay in the rat liver, Mutat. Res. 203 (1988) 23–32.

[8] J. Ashby, P.A. Lefevre, The rat-liver carcinogen *N*-nitrosomorpholine initiates unscheduled DNA synthesis and induces micronuclei in the rat liver in vivo, Mutat. Res. 225 (1989) 143–147.

[9] S. Sawada, T. Yamanaka, K. Yamatsu, C. Furihata, T. Matsushima, Chromosome aberrations, micronuclei and sister-chromatid exchanges (SCEs) in rat liver induced in vivo by hepatocarcinogens including heterocyclic amines, Mutat. Res. 251 (1991) 59–69.

[10] A.M. Rossi, M. Romano, L. Zaccaro, R. Pulci, M. Salmona, DNA synthesis, mitotic index, drug-metabolising systems and cytogenetic analysis in regenerating rat liver, Mutat. Res. 182 (1987) 75–82.

[11] J.W. Parton, M.L. Garriott, An evaluation of micronucleus induction in bone marrow and in hepatocytes isolated from collagenase perfused liver or from formalin-fixed liver using four-week-old rats treated with known clastogens, Environ. Mol. Mutagen. 29 (1997) 379–385.

[12] H. Suzuki, T. Shirotori, M. Hayashi, A liver micronucleus assay using young rats exposed to diethylnitrosamine: methodological establishment and evaluation, Cytogenet. Genome Res. 104 (2004) 299–303.

[13] G.I. Sipes, A.J. Gandolfi, Biotransformation of toxicants, in: M.O. Amdur, J. Doull, C.D. Klassen (Eds.), Casarett and Doull's Toxicology, The Basic science of poisons, McGraw-Hill, New York, 1993, pp. 88–126.

[14] R.A. Furner, T.E. Gram, R.E. Stitzel, The influence of age, sex and drug treatment on microsomal drug metabolism in four rat strains, Biochem. Pharmacol. 18 (1969) 1635–1641.

[15] M. Hayashi, T. Morita, Y. Kodama, T. Sofuni, M. Ishidate Jr., The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange coated slides, Mutat. Res. 245 (1990) 245–249.

[16] A. Wakata, Y. Miyamae, S. Sato, T. Suzuki, T. Morita, N. Asano, T. Awogi, K. Kondo, M. Hayashi, Evaluation of the rat micronucleus test with bone marrow and peripheral blood: summary of the 9th collaborative study by CSGMT/JEMS·MMS, Environ. Mol. Mutagen. 32 (1998) 84–100.

[17] D. Lorke, A new approach to practical acute toxicity testing, Arch. Toxicol. 54 (1983) 275–287.

[18] P.I. Countryman, J.A. Heddle, The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes, Mutat. Res. 41 (1976) 321–331.

[19] The collaborative study group for the micronucleus test, micronucleus test with mouse peripheral blood erythrocytes by acridine orange supravital staining: the summary report of the 5th collaborative study by the CSGMT/JEMS·MMS, Mutat. Res. 278 (1992) 83–98.

[20] M.A. Kastenbaum, K.O. Bowman, Tables for determining the statistical significance of mutation frequencies, Mutat. Res. 9 (1970) 527–549.

[21] M. Ishidate Jr. (Ed.), Data Book of Chromosomal Aberration Test In Vitro, Revised ed., Elsevier, Amsterdam, 1988.

[22] A.T. Natarajan, A.D. Tates, P.P.W. Van Buul, M. Meijers, N. De Vogel, Cytogenetic effects of mutagens/carcinogens after activation in a microsomal system in vitro. Part I: Induction of chromosome aberrations and sister chromatid exchanges by diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) in CHO cells in the presence of rat-liver microsomes, Mutat. Res. 37 (1976) 83–90.

[23] E. Zeiger, B. Anderson, S. Haworth, T. Lawlor, K. Mortelmans, W. Speck, *Salmonella* mutagenicity tests. III: Results from the testing of 255 chemicals, Environ. Mol. Mutagen. 9 (Suppl. 9) (1987) 1–110.

[24] K. Hirano, Y. Shinohara, H. Tsuda, S. Fukushima, M. Takahashi, N. Ito, Carcinogenic activity of quinoline on rat liver, Cancer Res. 36 (1976) 329–335.

[25] Y. Shinohara, T. Ogiso, M. Hananouchi, K. Nakanishi, T. Yoshimura, N. Ito, Effect of various factors on the induction of liver tumors in animals by quinoline, Gann 68 (1977) 785–796.

[26] IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, vol. 17, *N*-Nitrosodimethylamine, International Agency for Research on Cancer, Lyon, France, 1978.

[27] IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, vol. 8, *para*-Dimethylaminoazobenzene, International Agency for Research on Cancer, Lyon, France, 1975.

[28] IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, vol. 39, 4,4′-Methylenedianiline and its Dihydrochloride, International Agency for Research on Cancer, Lyon, France, 1986.

[29] E.K. Weisburger, A.S. Murthy, H.S. Lilja, J.C. Lamb IV, Neoplastic response of F344 rats and B6C3F1 mice to the polymer and dyestuff intermediates 4,4′-methylenebis(*N*,*N*-dimethyl)-

benzenamine,4,4′-oxydianiline,and 4,4′-methylenedianiline, J. Natl. Cancer Inst. 72 (6) (1984) 1457–1463.
[30] IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 27, 4-Chloro-*ortho*-phenylenediamine and 4-Chloro-*meta*-phenylenediamine, International Agency for Research on Cancer, Lyon, 1982.
[31] E.K. Weisburger, A.S. Murthy, R.W. Fleischman, M. Hagopian, Carcinogenicity of 4-chloro-*o*-phenylenediamine, 4-chloro-*m*-phenylenediamine, and 2-chloro-*p*-phenylenediamine in Fischer 344 rats and B6C3F$_1$ mice, Carcinogenesis 1 (1980) 495–499.
[32] IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 77, *ortho*-Toluidine, International Agency for Research on Cancer, Lyon, 2000.
[33] IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 29, Some Industrial Chemicals and Dyestuffs, International Agency for Research on Cancer, Lyon, 1982.
[34] T. Suga, Hepatocarcinogenesis by peroxisome proliferators, J. Toxicol. Sci. 29 (1) (2004) 1–12.
[35] IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 71, Methyl-methanesulfonate, International Agency for Research on Cancer, Lyon, 1987.
[36] A.D. Tates, I. Neuteboom, A.H.M. Rotteveel, N. de Vogel, G.J. Menkveld, L. den Engelse, Persistence of preclastogenic damage in hepatocytes of rats exposed to ethylnitrosourea, diethylnitrosamine, dimethylnitrosamine and methyl methanesulfonate. Correlation with DNA *O*-alkylation, Carcinogenesis 7 (7) (1986) 1053–1058.
[37] L. Den Engelse, R.J. De Brij, E. Scherer, B.G. Floot, Persistence and accumulation of (potential) single-strand breaks in liver DNA of rats treated with ethyl methanesulphonate, Cancer Lett. 11 (1980) 199–208.
[38] T. Suzuki, M. Hayashi, X. Wang, K. Yamamoto, T. Ono, B.C. Myhr, T. Sofuni, A comparison of the genotoxicity of ethylnitrosourea and ethyl methanesulfonate in *lacZ* transgenic mice (Muta^{TM} Mouse), Mutat. Res. 395 (1997) 75–82.
[39] D.T. Beranek, Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents, Mutat. Res. 231 (1990) 11–30.
[40] R. Kato, Y. Yamazoe, Sex-specific cytochrome P450 as a cause of sex- and species-related differences in drug toxicity, Toxicol. Lett. 64–65 (1992) 661–667.
[41] G.J. Nohynek, D. Kirkland, D. Marzin, H. Toutain, C. Leclere-Ribaud, H. Jinnai, An assessment of the genotoxicity and human health risk of topical use of kojic acid [5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one], Food Chem. Toxicol. 42 (2004) 93–105.
[42] N. Fujimoto, H. Watanabe, T. Nakatani, G. Roy, A. Ito, Induction of thyroid tumors in (C57BL/6N × C3H/N)F$_1$ mice by oral administration of kojic acid, Food Chem. Toxicol. 36 (1998) 697–703.
[43] K. Müller, P. Kasper, L. Müller, An assessment of the in vitro hepatocyte micronucleus assay, Mutat. Res. 292 (1993) 213–224.
[44] M.A. Hamoud, T. Ong, M. Petersen, J. Nath, Effects of quinoline and 8-hydroxyquinoline on mouse bone marrow erythrocytes as measured by the micronucleus assay, Teratogen. Carcinogen. Mutagen. 9 (1989) 111–118.
[45] T. Suzuki, Y. Miyata, K. Saeki, Y. Kawazoe, M. Hayashi, T. Sofuni, In vivo mutagenesis by the hepatocarcinogen quinoline in the *lacZ* transgenic mouse: evidence for its in vivo genotoxicity, Mutat. Res. 412 (2) (1998) 161–166.
[46] T. Suzuki, T. Itoh, M. Hayashi, Y. Nishikawa, F. Furukawa, M. Takahashi, T. Sofuni, Organ variation in the mutagenicity of dimethylnitrosamine in Big Blue mice, Environ. Mol. Mutagen. 28 (4) (1996) 348–353.
[47] D. Jenssen, C. Ramel, Factors affecting the induction of micronuclei at low doses of X-rays, MMS and dimethylnitrosamine in mouse erythroblasts, Mutat. Res. 58 (1) (1978) 51–65.
[48] T. Kimura, M. Kodama, C. Nagata, A correlation of the rate of *N*-hydroxylation of aminoazo dyes with their carcinogenic activity in the rat, Carcinogenesis 3 (12) (1982) 1393–1396.
[49] S. Ohnishi, M. Murata, M. Degawa, S. Kawanishi, Oxidative DNA damage induced by an *N*-hydroxy metabolite of carcinogenic 4-dimethylaminoazobenzene, Jpn. J. Cancer Res. 92 (2001) 23–29.
[50] M. Nonaka, H. Omura, T. Sofuni, M. Hayashi, Kojic acid did not induce micronuclei in mouse bone marrow hematopoietic cells, MMS Commun. 4 (2) (1996) 109–112.
[51] Y. Nakai, K. Hirabayashi, Y. Takahashi, D. Miura, Y. Kasahara, K. Morita, Y. Izawa, The genetic toxicology of *o*-toluidine with special reference to its non-clastogenicity in vivo, MMS Commun. 2 (1994) 99–108.
[52] S. Hamada, K. Nakajima, T. Serikawa, M. Hayashi, The effect of aging on the results of the rat micronucleus assay, Mutagenesis 18 (3) (2003) 273–275.

 doi:10.1111/j.1741-450.2006.0098.x

ORIGINAL ARTICLE

Susceptibility of newborn rats to 3-ethylphenol and 4-ethylphenol compared with that of young rats

Mika Takahashi[1], Mutsuko Hirata-Koizumi[1], Nobuo Nishimura[2], Yoshihiko Ito[3], Masao Sunaga[4], Sakiko Fujii[4], Eiichi Kamata[1], Ryuichi Hasegawa[1], and Makoto Ema[1]

[1]National Institute of Health Sciences, Tokyo, [2]Gotemba Laboratory, Bozo Research Center, Gotemba, [3]Research Institute for Animal Science in Biochemistry and Toxicology, Sagamihara and [4]Safety Research Institute for Chemical Compounds, Sapporo, Japan

ABSTRACT **Newborn rat studies were conducted with oral administration of 3-ethylphenol (3EP) and 4-ethylphenol (4EP) on postnatal days (PND) 4–21 to allow comparison of no observed adverse effect level (NOAEL) and unequivocally toxic level (UETL) with those from 28-day studies of young rats starting at 5–6 weeks of age. In the newborn rat studies, slightly lowered body weight was observed after 3EP treatment, and deaths, hypoactivity, Straub tail, deep respiration and delayed righting reflex were clearly observed after 4EP treatment. In the young rat studies, salivation, staggering gait, changes in the liver including high values of liver weight and alanine aminotransferase or total cholesterol and the lesions in the forestomach were clearly observed after 3EP and 4EP treatments. NOAELs of 3EP and 4EP in the newborn rat studies appeared to be almost 3 times lower than those in the young rat studies. As a clear toxicity of 3EP was not observed in newborn rats, UETLs were not established for 3EP. Regarding 4EP, UETL of young rats was 4–5 times higher than that of newborn rats. In conclusion, newborn rats were 3–5 times more susceptible to 3EP and 4EP than young rats.**

Key Words: 3-ethylphenol, 4-ethylphenol, newborn rats, repeated-dose toxicity, young rats

INTRODUCTION

The possible toxic effect of chemical substances on the development of 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. In the EPA children's environmental health yearbook, US EPA (1998) has already stated comprehensively that children have their special vulnerability to certain toxic substances such as drugs and environmental chemicals. The special vulnerability in children to toxic substances may results from a combination of toxicokinetic, toxicodynamic and exposure factors, and kinetic factors are of importance mainly in the early postnatal period, largely as the result of immature elimination systems, i.e. metabolizing enzymes and/or renal function (Schwenk *et al.* 2002). There is much less information about differences between children and adults with regard to toxicodynamics (Schwenk *et al.* 2002). Regarding exposure factors, children play close to the ground and are constantly licking their fingers or mouthing toys or objects. As a result, mouthing becomes a potentially significant exposure route (US EPA 2002).

The potential toxic effects of chemicals cannot be anticipated from data on adults, and a data set on exposed children is essential for assessment of children's health. In this context, we have determined the toxicity of chemicals in newborn rats after direct dosing and compared it with that in young rats. We have already reported the differences in the susceptibility to toxicities of chemicals between newborn and young rats for 4-nitrophenol and 2,4-dinitrophenol (Koizumi *et al.* 2001), for 3-aminophenol (Koizumi *et al.* 2002), for 3-methylphenol (Koizumi *et al.* 2003), for tetrabromobisphenol A (Fukuda *et al.* 2004), for 2,4,6-trinitrophenol (Takahashi *et al.* 2004), for 1,3-dibromopropane and 1,1,2,2-tetrabromoethane (Hirata-Koizumi *et al.* 2005). With regard to the no observed adverse effect level (NOAEL), these reports showed that the toxic response in newborn rats was at most 3–4 times (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol and 3-methylphenol) higher than that in young rats. On the other hand, the toxic response in newborn rats was 5 times (1,3-dibromopropane) and 8 times (1,1,2,2-tetrabromoethane) lower than that in young rats. The toxicological profiles of 4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, 1,3-dibromopropane and 1,1,2,2-tetrabromoethane were similar between newborn rats and young rats. The nephrotoxicity of tetrabromobisphenol A was specific for newborn rats. We also reported that the toxicity profiles induced by 2,4,6-trinitrophenol were markedly different between newborn and young rats.

3-Ethylphenol (3EP) is a photographic chemical intermediate and an intermediate for the cyan coupler of photographic paper (Horikawa *et al.* 1998). 4-Ethylphenol (4EP) is a chemical compound widely used as a source material of reactive polymers, antioxidants, drugs, agricultural chemicals and dyes (Chemical Products' Handbook 2004). These chemicals are listed in the 2004 OECD list of high production volume (HPV) chemicals (OECD 2004a). The HPV chemicals list contains those chemicals that are produced at levels greater than 1000 tons per year in at least one member country/region of OECD. Regarding the toxicity information on these two chemicals, only a few studies are available. Thompson *et al.* (1995) showed that 4EP was metabolized to a reactive quinone methide intermediate by rat liver enzymes and that this oxidation mechanism played a significant role in the cytotoxic effect of 4EP. This intermediate was subsequently trapped with glutathione to produce two diastereomeric conjugates. Recently, 28-day repeated dose oral toxicity studies of 3EP and 4EP in young rats were conducted as part of the Japanese Existing Chemical Safety Program and published in the annual toxicity

Correspondence: Makoto Ema, DVM, PhD, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.
Email: ema@nihs.go.jp

Received July 22, 2005; revised and accepted October 4, 2005.

testing report (MHLW 2001a,b), in which no observed effect level was evaluated.

In the present paper, we re-evaluated the toxicity of 3EP (MHLW 2001a) and 4EP (MHLW 2001b) in young rats in terms of NOAEL and unequivocally toxic level (UETL). We considered that the findings in the main test of repeated dose study and the dose-finding study were useful for characterizing the toxicity of chemicals. NOAEL is the highest tested dose in a study that did not produce any observable adverse effects and is expressed in terms of the weight of a test substance given daily per unit weight of a test animal. UETL has been used only for our comparative toxicity analysis as a clear toxic dose. It is generally not readily definable because it depends on the type of toxicity (Hirata-Koizumi *et al.* 2005). We determined the toxicity of 3EP and 4EP in newborn rats, compared and discussed NOAELs and UETLs of 3EP and 4EP for young and newborn rats.

MATERIALS AND METHODS

Chemicals

3EP (3-ethylphenol, CAS no. 620-17-7, purity 96.2%) was obtained from Taoka Chemical Co., Ltd. (Osaka, Japan) and 4EP (4-ethylphenol, CAS no. 123-07-9, purity 98.4% for the newborn rat study and 98.3% for the young rat study) was obtained from Maruzen Petrochemical Co., Ltd. (Tokyo, Japan) and they were dissolved in olive oil.

Animals

In the newborn rat study, pregnant SPF Crj:CD(SD)IGS rats (gestation day 14–15) were purchased from Atsugi Breeding Center, Charles River Japan (Yokohama, Japan) and allowed to deliver spontaneously. The day on which parturition was completed was designated as postnatal day (PND) 0. Pups (newborn rats) were separated from dams on PND 3 and were suckled by foster mothers. In the young rat study, four-week old males and females of the same strain were purchased from the same farm as in the newborn rat study.

The animals were maintained in an environmentally controlled room set at 20–26°C with a relative humidity of 45–65% 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 (CRF-1, Oriental Yeast, Tokyo, Japan or Laboratory MR Stock, Nosan Corporation, Yokohama, Japan) and water. The animals were euthanized by exsanguination under anesthesia using ether.

Study design

Time schedule for 3EP and 4EP studies is shown in Figure 1.

18-day repeated dose study in newborn rats

Dose-finding study. Twenty-four male and 24 female newborns for 3EP or 20 male and 20 female newborns for 4EP were randomly selected and assigned to four dose groups, including a control group. Six foster mothers for 3EP and five for 4EP were used. One foster mother suckled four male and four female pups. Newborn rats (6/sex/dose for 3EP, 5/sex/dose for 4EP) were given 3EP at 0, 30, 100 or 300 mg/kg/day or 4EP at 0, 100, 300 or 1000 mg/kg/day by gavage once a day on PND 4–21 (for 18 days) and killed on PND 22 after overnight starvation. General condition, body weights, hematology, blood biochemistry, necropsy and organ weights were examined. The similar study design was applied to the main study.

Main study. Forty-eight males and 48 females for each chemical for two autopsy groups (the end of the dosing period and the recovery-maintenance period) were randomly selected and assigned to four dose groups, including a control group. Twelve foster mothers were used for each chemical. One foster mother suckled four male and four female newborn rats up to weaning on PND 21. After weaning, newborn rats of the recovery-maintenance group were individually maintained for 9 weeks. Newborn rats (6/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 30, 100 or 300 mg/kg/day on PND 4–21 (for 18 days) and killed on PND 22 after overnight starvation. The dosage levels were determined based on the results of the dose-finding study. Recovery-maintenance groups (6/sex/dose for each chemical) given the same dosage were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks of age, almost the same age as young rats at the end of the recovery period.

General condition was observed at least once a day for newborn rats during the dosing period (separated from each foster mother) and during the recovery-maintenance period. Body weight was measured before dosing, more than two times per week during the dosing period and at seven-day intervals thereafter. Food consumption was measured about 2 times per week only during the recovery-maintenance period. Some developmental landmarks were assessed (OECD 2004b), such as piliation, incisor eruption, eye opening, testes descent and vaginal opening. All newborn rats were examined for abnormalities of reflex ontogeny; e.g. pupillary

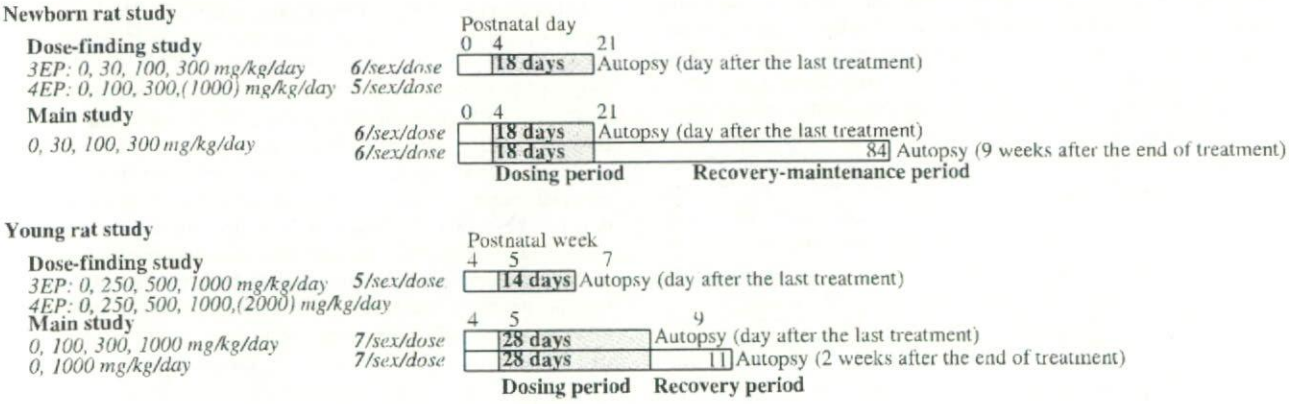

Fig. 1 Time schedule of newborn and young rat studies of 3-ethylphenol (3EP) and 4-ethylphenol (4EP).

reflex, Preyer's reflex, corneal reflex, righting reflex and air righting reflex on PND 20 or 21.

In urinalysis, color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, urine sediment, specific gravity, osmotic pressure and volume of urine were examined in the late recovery-maintenance period. Newborn rats were killed on PND 22 or 85. On the day of the sacrifice, blood was collected from the abdominal aorta. Hematological parameters, such as the red blood cell count, hemoglobin concentration, hematocrit value, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte ratio, 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, alanine aminotransferase (ALT), γ-glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, cholinesterase, phospholipids, calcium, inorganic phosphorus, sodium, potassium and chloride levels in the serum, were also determined. After a gross examination, the brain, pituitary gland, heart, thymus, liver, kidneys, spleen, adrenals, thyroids, lungs, testes/ovaries and epididymides/uterus were weighed. The organs were fixed with 10% buffered formation-phosphate and paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination. The studies using newborn rats were conducted at Gotemba Laboratory, Bozo Research Center Inc. (Gotemba, Japan) for 3EP and at Research Institute for Animal Science in Biochemistry and Toxicology (Sagamihara, Japan) for 4EP under Good Laboratory Practice (GLP) conditions (MHW 1988), and accordance with 'Guidelines for Animal Care and Use' of these laboratories.

28-day repeated dose study in young rats

Dose-finding study. Five-week-old rats (5/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 250, 500, 1000 or 2000 (only for 4EP) mg/kg/day for 14 days and killed the day following the last administration after overnight starvation. General condition, body weights, food consumption, hematology, blood biochemistry, necropsy and organ weights were examined.

Main study. Five-week-old rats (7/sex/dose for each chemical) were given 3EP or 4EP by gavage once a day at 0, 100, 300 or 1000 mg/kg/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 1000 mg/kg/day) (7/sex/dose for each chemical) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. The rats were examined for general condition, body weights, food consumption, urinalysis, hematology, blood biochemistry, necropsy findings, organ weights and histopathological findings. The study using young rats was conducted at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan) for 3EP and 4EP under GLP conditions (MHW 1988), and accordance with 'Guidelines for Animal Care and Use' of these laboratories.

Statistical analysis

Continuous data were analyzed with Bartlett's test for homogeneity of variance. If the data were homogeneous, one-way analysis of variance and Dunnett's test were conducted for group comparisons between the control and individual chemical-treated groups. If not homogenous or in case of quantitative urinalysis data, analysis was performed using the Kruskal–Wallis test. In consequence, if a significant difference was detected, the Dunnett type test or Mann–Whitney's *U*-test was conducted. In the newborn rat study, categorical data for general appearance and reflex ontogeny were analyzed by Fisher's exact probability test or Mann–Whitney's *U*-test. A probability less than 5% was considered statistically significant.

RESULTS

18-day study of 3EP in newborn rats

In the dose-finding study, body weights were considerably lowered in males (max. 9% decrease) and females (max. 6% decrease) at 300 mg/kg/day during the dosing period when compared to controls. However, the decreases were not statistically significant due to variations of the data.

Only slight changes were found in the main study as shown in the Table 1 and Figure 2. At 300 mg/kg/day, body weights were significantly lower than controls in males on PND 11–17 (max. 6% decrease) and females on PND 11–21 (max. 7% decrease). Significantly high value of relative liver weight was observed in males at 300 mg/kg/day and in females at 100 and 300 mg/kg/day at the end of the dosing period; however, it was not considered toxicologically significant because of the absence of changes in parameters of blood biochemistry and histopathological findings related to liver damage. There were no effects on the developmental landmarks at any dose. There were no effects of 3EP treatment at the end of the recovery-maintenance period.

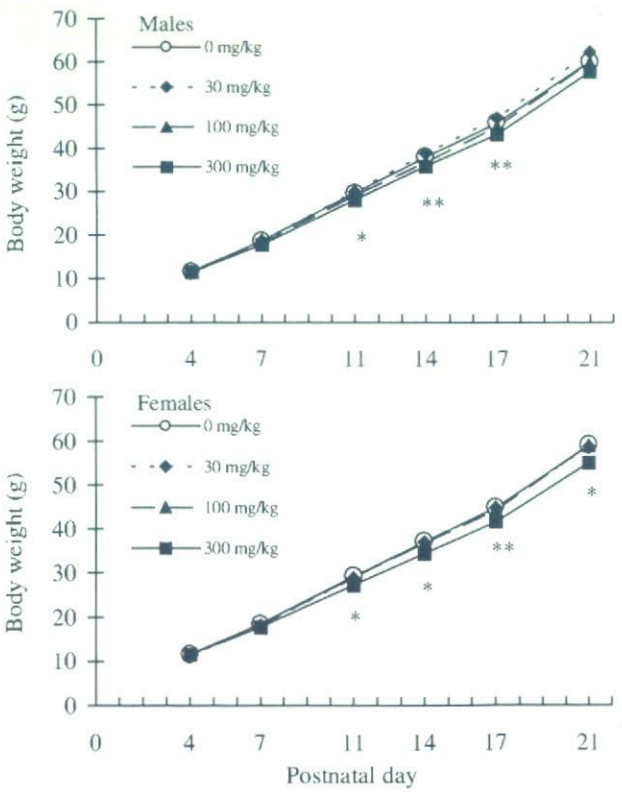

Fig. 2 Body weight curves in 18-day study of 3-ethylphenol (3EP) in newborn rats.

Table 1 Main findings of 3-ethylphenol (3EP) at the end of the dosing in the newborn and the young rat main studies

	Newborn rat study (mg/kg/day)				Young rat study (mg/kg/day)			
	0	30	100	300	0	100	300	1000
Male								
No. animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	2
No. animals examined	6	6	6	6	6‡	7	7	7
ALT (IU/L)	36 ± 7	36 ± 4	41 ± 9	35 ± 5	24 ± 2	25 ± 3	27 ± 4	40 ± 2**
Total cholesterol (mg/dL)	85 ± 8	86 ± 17	83 ± 11	99 ± 18	55 ± 8	53 ± 9	59 ± 15	61 ± 7
Relative liver weight (g/100 g BW)	3.00 ± 0.16	3.14 ± 0.10	3.18 ± 0.11	3.42 ± 0.21**	3.11 ± 0.19	2.98 ± 0.14	3.36 ± 0.24	3.62 ± 0.25**
Relative kidney weight (g/100 g BW)	1.10 ± 0.09	1.08 ± 0.03	1.10 ± 0.06	1.05 ± 0.06	0.81 ± 0.02	0.80 ± 0.05	0.80 ± 0.11	0.91 ± 0.06**
Forestomach, hyperplasia	0	0	0	0	0	0	0	7
Female								
No. animals examined	12	12	12	12	14	7	7	14
Clinical toxic signs†	0	0	0	0	0	0	0	5
No. animals examined	6	6	6	6	7	7	7	7
ALT (IU/L)	34 ± 3	30 ± 4	32 ± 4	30 ± 6	22 ± 4	22 ± 3	22 ± 2	28 ± 6*
Total cholesterol (mg/dL)	89 ± 10	90 ± 21	96 ± 18	94 ± 10	56 ± 15	57 ± 7	61 ± 7	76 ± 15**
Relative liver weight (g/100 g BW)	2.93 ± 0.10	3.03 ± 0.12	3.14 ± 0.10*	3.39 ± 0.17**	3.10 ± 0.14	3.09 ± 0.16	3.28 ± 0.18	3.68 ± 0.25**
Relative kidney weight (g/100 g BW)	1.07 ± 0.07	1.15 ± 0.08	1.13 ± 0.06	1.15 ± 0.05	0.82 ± 0.05	0.83 ± 0.03	0.85 ± 0.07	0.86 ± 0.04
Forestomach, hyperplasia	0	0	0	0	0	0	0	7

Values are given as the mean ± SD. *$P < 0.05$ and **$P < 0.01$ indicate significantly different from control group. BW, body weight.

†Staggering gait, prone/lateral position, tremor or soiled perigenital fur; ‡data from one animal were excluded because its hard palate was accidentally broken on day 23 of dosing.

28-day study of 3EP in young rats

In the dose-finding study, one female showed staggering gait and a lateral position for three hours after the first dosing at 1000 mg/kg/day. At this dose, significantly high values of relative liver weight and ALT in males and relative liver weight and total cholesterol in females were observed. At 500 mg/kg/day, significantly high values of ALT in males and relative liver weight in females were observed.

In the main study (Table 1 and Fig. 3), adverse effects as below were found at 1000 mg/kg/day. Clinical signs, such as staggering gait, a prone/lateral position and soiled perigenital fur, were observed in 2/14 males and 5/14 females. Staggering gait and a prone and/or lateral position occasionally occurred 10 min after dosing and lasted one hour. Soiled perigenital fur was also observed in 1/14 males and 3/14 females at this dose. Body weight of males was significantly lowered on days 2 and 7 of dosing. In urinalysis, significantly high volumes of urine and water consumption and significantly low protein were observed in males and females at the end of the dosing period. In blood biochemistry, significantly high values of ALT in males and females and total cholesterol in females were observed. In the necropsy findings, thinning of the limiting ledge in the forestomach in 5/7 males and 2/7 females were observed at the end of the dosing period. Significantly high values of relative liver weight in males and females and relative kidney weight in males were observed at the end of the dosing period. Hyperplasia of the squamous cell in the forestomach was observed in all 7 males and all 7 females at the end of the dosing period. There were no effects of 3EP treatment at the end of the recovery period.

18-day study of 4EP in newborn rats

In the dose-finding study, deaths occurred at 300 mg/kg/day in one female each on days 6 and 8 of dosing, and at 1000 mg/kg/day in all rats by day 3 of dosing. In these dead rats, hypoactivity was observed and additionally, deep respiration, pale skin and/or dehydration were observed. In the surviving rats, hypoactivity during the dosing period was found in 3/5 males and 1/3 females at 300 mg/kg/day.

The main findings in the main study are shown in Table 2 and Figure 4. Clinical signs, such as hypoactivity, hypothermia, tremor, Straub tail, deep respiration and emaciation, were observed in 10/12 males and all 12 females at 300 mg/kg/day. Hypoactivity in males and females and hypothermia, tremor, Straub tail, deep respiration and emaciation in females were significantly more frequent at this dose and these clinical signs disappeared by day 9 of dosing for males and day 13 of dosing for females. At 300 mg/kg/day, 2/12 females were found dead on days 10 and 12 of dosing. One of them showed dark red lung and congestive edema of the lung and the other showed distention of the gastrointestinal tract and atrophy of the thymic cortex at necropsy. The delay in the righting reflex was observed in 4/12 males at 300 mg/kg/day, in 1/12 females at 100 mg/kg/day and in 1/10 females at 300 mg/kg/day. At 300 mg/kg/day, body weights of males and females were significantly lower on PND 7–21. Significantly high relative weight of the liver was observed in males and females at 300 mg/kg/day at the end of the dosing period. There were no changes in the parameters of blood biochemistry or histopathological findings related to liver damage. There were no effects of 4EP treatment at the end of the recovery-maintenance period.

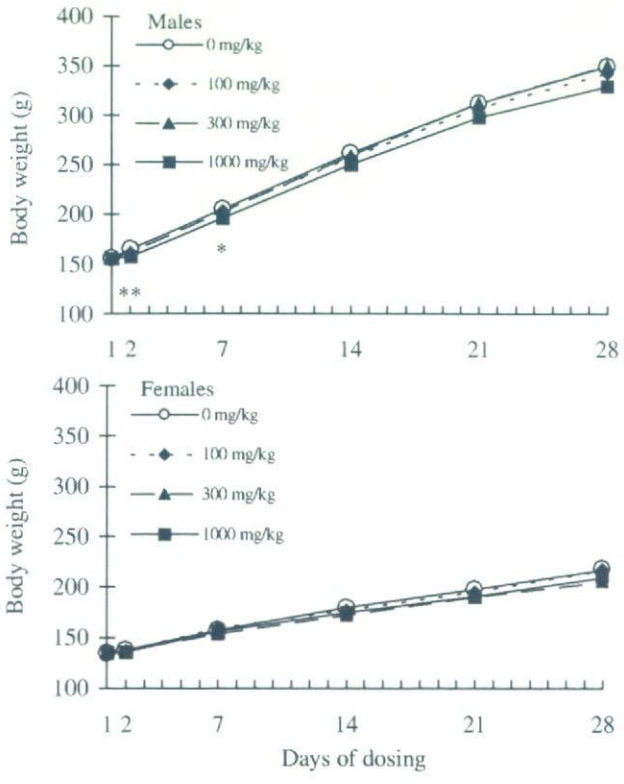

Fig. 3 Body weight curves in 28-day study of 3-ethylphenol (3EP) in young rats.

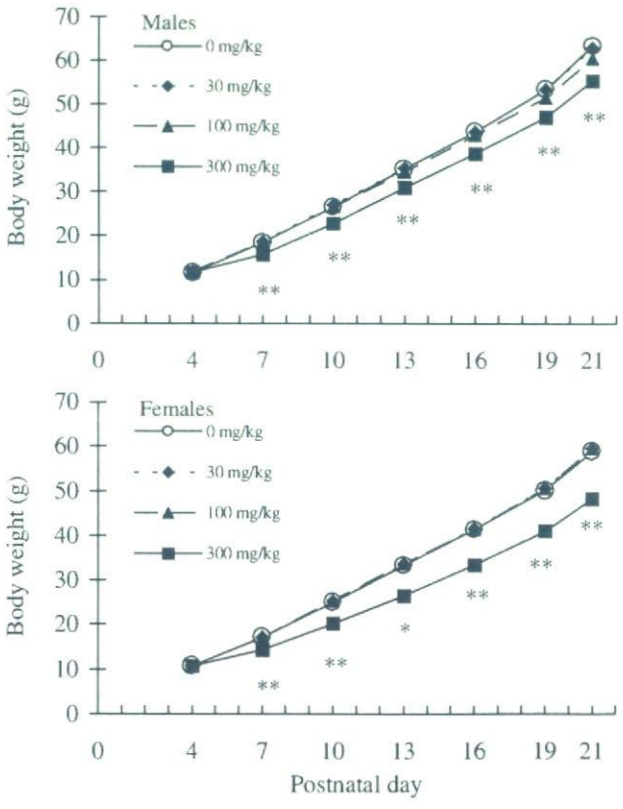

Fig. 4 Body weight curves in 18-day study of 4-ethylphenol (4EP) in newborn rats.