



# Effectiveness of the liver micronucleus assay using juvenile mice

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**ABSTRACT.** This study investigated the effectiveness of the liver micronucleus (MN) assay using juvenile mice. Therefore, we analyzed various hepatic cytochrome P450 (CYP)-mediated activities of ethoxyresorufin *O*-deethylation, pentoxyresorufin *O*-dealkylation, tolbutamide hydroxylation, bufuralol 1'-hydroxylation, aniline hydroxylation and midazolam 4-hydroxylation by CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A, respectively, in non-treated male ICR mice aged between 3 and 8 weeks. The enzyme efficiency levels in 3- and 4-week-old mice were approximately similar to or higher than those in 8-week-old mice, except for CYP1A and CYP2E in 3- and 4-week-old mice, respectively. Since these results suggest that juvenile mice have sufficient activities for most CYP enzymes, we also conducted a liver MN assay using diethylnitrosamine (DEN), a rodent hepatocarcinogen, on male ICR mice aged between 3 and 6 weeks. A peripheral blood (PB) MN assay was performed simultaneously in 4-week-old mice. Assays incorporating DEN produced positive results in 3- and 4-week-old mice and showed a dose-dependent increase in the micronucleated hepatocyte frequencies at 4 weeks. Both the liver MN assay in 5- and 6-week-old mice and the PB MN assay had negative results when using DEN. These results suggest that 3- and 4-week-old mice have micronuclei-inducing potential in the liver to detect genotoxic compounds using the liver MN assay.

**KEY WORDS:** CYP, genotoxicity assay, juvenile mouse, liver micronucleus assay

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*In vivo* rodent micronucleus (MN) assays have been widely performed using bone marrow (BM) and peripheral blood (PB) to assess the genotoxicity of chemicals; however, some genotoxic compounds that require metabolic activation in the liver give negative results [19]. Some active metabolites of such chemicals have a very short lifespan and do not accumulate in the bone marrow at concentrations high enough to induce the formation of micronuclei [29].

The MN assay using the liver, a major target for carcinogenesis and a key organ for drug metabolism [9], is a useful method for detecting hepatocarcinogens and genotoxic chemicals that are mainly activated by hepatic metabolic enzymes, e.g., cytochrome P450 (CYP). Since cell proliferation for the production of micronuclei in the adult animal liver is time-consuming, liver MN assays have been developed in various aspects. These improvements include treatment with mitogens [5], performing partial hepatectomy (PH) [12, 13, 35, 36] and using juvenile rats [29–33], young adult rats [10, 20, 21, 34] or mice [9] with repeated treatments. Careful consideration should be given when using the co-treatment method with mitogens, since there may be interactions with the test chemicals. In the PH method, some metabolic enzyme activities of the liver decrease after the procedure [26]. Although the repeated-dose liver MN (RDLMN) assay can be integrated into standard toxicity studies, it requires a lengthy and continuous administration, e.g., 14- or 28-day treatments, and a significant amount of test compounds [37]. In the juvenile rat method, the 4-week-old rats do not have similar activity levels of the hepatic CYP2C subfamily compared with adult rats [29]. However, with the exception of CYP2C, most CYP activity levels progressively increase after birth and reach a level similar to adult rats at approximately 30 days in age [15], and the hepatocyte proliferation at 4 weeks can still be observed [24]. Thus, the juvenile rat method can be easily conducted in a short period of time with a single or double administration without additional mitogens or surgical operations and can be useful as a genotoxicity assay.

Several studies have shown that the hepatocyte proliferation in mice continues until the postnatal day (PND) 30 [2, 28]. In the mouse liver, most gene expressions of CYP mRNA increase to a high and stable level between PND 20 and 30 [11, 25], and the

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activity of testosterone hydroxylase and aromatase increases to a level similar to adult mice by PND 15 [6]. Furthermore, Roy *et al.* reported that 1,4-dioxane, a rodent hepatocarcinogen which gave negative results in the mouse PB MN assay, and vinblastin sulfate, an aneugen, induce micronuclei formation in hepatocytes after treatment in 4-week-old mice [27]. Therefore, similar to the juvenile rat method, the liver MN assay using juvenile mice may be useful to assess the genotoxicity of chemicals and can be conducted with a smaller amount of reagents than rats. However, few studies have reported the micronuclei-inducing potential in juvenile mouse liver after the administration of genotoxic compounds.

In this study, in order to investigate the effectiveness of the juvenile mice liver MN assay, we studied the age effects on hepatic CYP activities using non-treated mice. Furthermore, we investigated the liver MN assay using young mice after the administration of diethylnitrosamine (DEN), a well-known genotoxic hepatocarcinogen in rodents that produces negative results in the mouse BM and PB MN assay. We also conducted simultaneous liver and peripheral blood (PB) MN assays using DEN to evaluate dose dependency.

## MATERIALS AND METHODS

### Animals

A total of 108 male Crl:CD1 (ICR) mice were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The animals were quarantined and acclimated for at least 5 days. The animals were housed under a 12-hr light-dark cycle in an air-conditioned room between 20–26°C and humidity between 30–70%. They received food and water *ad libitum*. The experiments in this study were conducted in accordance with the care and use of laboratory animals guideline which was approved by the Institutional Animal Care and Use Committee of Shionogi Pharmaceutical Research Center and the Committee of Animal Experiments of Tokyo University of Agriculture and Technology.

### Chemicals and dosages

Resorufin, ethoxyresorufin and pentoxyresorufin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Tolbutamide, hydroxyl- tolbutamide, midazolam, 4-hydroxymidazolam, bufuralol and 1'-hydroxybufuralol were purchased from Daiichi Chemical (Tokyo, Japan). Aniline and *p*-aminophenol were obtained from Wako Pure Chemical (Osaka, Japan). All the other chemicals were of analytical or HPLC grade.

Diethylnitrosamine (DEN) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Prior to administration, DEN was dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) and administered by oral gavage. The dose for DEN was selected based on data that showed positive results in the juvenile rat liver MN assay [29–33]. Negative control animals received physiological saline (DEN solvent).

### Experimental design

In experiment I, the hepatic enzyme activities of the CYP isozymes were evaluated in non-treated male mice aged between 3 and 8 weeks. We determined the enzyme activities from three pooled liver samples. For 3- and 4-week-old mice, livers from six were pooled. For 6- and 8-week-old mice, livers from five were pooled. The animals were anesthetized with isoflurane, and the liver was isolated immediately after perfusion through the portal vein using ice-cold buffer solution (1.15% KCl, 0.2 mM EDTA-2Na, 0.1 mM dithiothreitol, 0.01 mM phenyl methyl sulphonyl fluoride and 20% glycerol) until the color of the liver turned white (or pale white). The liver was subsequently stored at –80°C until the preparation of microsomes.

In experiment II, we evaluated the effect of age and dose on the liver MN assay using a double-dosing/single-sampling method [33] without *in situ* perfusion [20]. We used young mice aged 3 to 6 weeks to examine age-related changes of the frequencies in the micronucleated hepatocytes (MNHEPs), since HEP proliferation in mice was not observed after the PND 30 [2, 28] and young mice would have sufficient activities for most CYP enzymes from the results of the experiment I. Four or five male mice per group were treated with physiological saline or DEN (50 mg/kg/day for mice aged 3, 5 and 6 weeks and 12.5, 25 and 50 mg/kg/day for mice aged 4 weeks) twice in a 24 hr interval at 10 ml/kg body weight. Four days after the second administration of the test chemicals, the mice were exsanguinated under isoflurane, and the liver was subsequently isolated. The PB MN assay was conducted simultaneously in the same animals 4 weeks in age, and blood samples were collected from the caudal vein approximately 24 hr after the second dosing.

### Preparation of hepatic microsomes

Hepatic microsomes were prepared as previously described [1, 38]. Approximately 5.5 g of liver tissue was homogenized in 10 ml buffer solution while cooled on ice. The homogenate was centrifuged at 10,000 ×g for 20 min at 4°C, and the supernatant was ultracentrifuged at 105,000 ×g for 90 min at 4°C. The pellet was resuspended in ice-cold buffer solution, and the obtained microsomal suspension was stored at –80°C until use. Microsomal protein concentrations were determined by the Bradford assay [4] using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, U.S.A.) and bovine serum albumin. CYP contents were determined as previously described by Omura and Sato (1964) [23].

### Enzyme assays

The hepatic enzyme activities of CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A were measured by the degree of ethoxyresorufin *O*-deethylation, pentoxyresorufin *O*-dealkylation, tolbutamide hydroxylation, bufuralol 1'-hydroxylation, aniline

hydroxylation and midazolam 4-hydroxylation, respectively. The assays were performed with the following concentration of substrates: ethoxyresorufin, 0.83–4.15  $\mu\text{M}$ ; pentoxyresorufin, 2.07–16.6  $\mu\text{M}$ ; tolbutamide, 1–10 mM; bufuralol, 4.20–67.2  $\mu\text{M}$ ; aniline, 0.5–10 mM; and midazolam, 7.67–246  $\mu\text{M}$  (6–7 concentration points/determination). The ethoxyresorufin *O*-deethylation and pentoxyresorufin *O*-dealkylation activities were quantified using the fluorometric measurement of resorufin, a primary metabolite for both substrates [1, 7]. The tolbutamide hydroxylation activity was quantified using measurements of hydroxytolbutamide by high-performance liquid chromatography (HPLC) with UV detection [1, 18]. The bufuralol 1'-hydroxylation activity was quantified using measurements of 1'-hydroxybufuralol by HPLC with fluorometric detection [1, 17]. The aniline hydroxylation activity was quantified using measurements of *p*-aminophenol by HPLC with UV detection [22]. Midazolam 4-hydroxylation activity was quantified using measurements of 4-hydroxymidazolam by HPLC with UV detection [1, 39].

### Enzyme analysis

The formation of each metabolite was consistent with single-enzyme Michaelis-Menten kinetics. Therefore, the following equation was fitted to the observed data using the nonlinear least-squares regression to calculate maximum velocity ( $V_{\text{max}}$ ) and the Michaelis constant ( $K_{\text{m}}$ ):

$$V = \frac{V_{\text{max}} \times S}{K_{\text{m}} + S}$$

The intrinsic clearance ( $CL_{\text{int}}$ ) of each reaction was obtained by the following equation:

$$CL_{\text{int}} = V_{\text{max}}/K_{\text{m}}$$

### Liver MN assay

The liver MN assay developed by Narumi *et al.* [20] was used with some modifications. The liver (excluding the gallbladder) was washed with cold Hank's balanced salt solution (HBSS; Sigma-Aldrich Inc.) and minced into small pieces with a pair of scissors. The liver pieces were incubated with a collagenase (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) solution (100 units/ml in HBSS) at 37°C for 1 hr and shaken at approximately 50  $\times g$  followed by a stronger and manual shake for approximately 50 times every 30 min. After incubation, the hepatocytes (HEPs) were isolated by pipetting and filtering the suspension through a cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ, U.S.A.). The obtained HEPs were fixed with 10% neutral buffered formalin (Wako Pure Chemical Industries, Ltd.) and centrifuged at 50  $\times g$  for 1 min. Finally, the HEPs were resuspended in 10% neutral buffered formalin and stored at room temperature until analysis. Immediately before observation, 10  $\mu\text{l}$  of the HEP suspension was mixed with 10  $\mu\text{l}$  of acridine orange (AO, 200  $\mu\text{g}/\text{ml}$ ) and 4',6-diamino-2-phenylindole dihydrochloride (DAPI, 10  $\mu\text{g}/\text{ml}$ ) staining solution. The stained mixture was deposited onto a glass slide and covered with a cover glass. The slide preparation was observed by fluorescence microscopy at 400  $\times$  magnification with UV excitation. The number of MNHEPs per 2,000 HEPs and metaphase (M-phase) cells per 2,000 HEPs was counted for each animal according to previous reports [30–33]. MNHEPs were identified by their round or distinct micronuclei that were stained similarly to the nucleus with a diameter that was a fourth or less than that of a nucleus. M-phase cells were defined as those in a division stage between prophase and telophase that had poorly defined nuclear envelopes, identifiable chromosomes or two unevenly shaped nuclei that strongly fluoresced.

### Peripheral blood MN assay

Ten to twenty microliters of blood samples were mixed with 20  $\mu\text{l}$  of 10% neutral buffered formalin and stored at room temperature until analysis [16]. Immediately before observation, 10  $\mu\text{l}$  of the suspension was mixed with 10  $\mu\text{l}$  of AO solution (200  $\mu\text{g}/\text{ml}$ ), deposited onto a glass slide and covered with a cover glass. The slide preparation was observed by fluorescence microscopy at 1,000  $\times$  magnification with blue-light excitation. The number of micronucleated reticulocytes (MNRETs) per 2,000 reticulocytes (RETs) and the number of RETs per 1,000 erythrocytes were recorded for each animal.

### Statistical analysis

For statistical analysis, the SAS software (SAS System for Windows, Release 9.4, SAS Institute Inc., Cary, NC, U.S.A.) was used, and the significance level was set at 5%. After confirming equal variances among the age groups by Bartlett's test, data of hepatic CYP activities were analyzed by one-way ANOVA. Dunnett's multiple comparison test was used as post-hoc test. Differences in the incidence of MNHEPs or MNRETs between the DEN and the negative (solvent) control group were analyzed statistically by Fisher's exact test. Dose dependency based on the incidence of MNHEPs or MNRETs was analyzed statistically by Cochran-Armitage trend test. Differences in the RETs-ratio between the test and the negative control groups were analyzed statistically by Dunnett's multiple comparison test. Differences in the final body weight and the liver weight after administration of the test chemicals between the 6 weeks-aged group and the other-aged groups were analyzed statistically by Dunnett's multiple comparison test.

## RESULTS

### Enzyme activities

Table 1 shows the  $V_{\text{max}}$  values of the reactions catalyzed by CYP1A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A in hepatic microsomes obtained from mice aged 3 to 8 weeks. When compared with mice aged at 8 weeks, those at 3 weeks showed

**Table 1.** Results of maximum velocity (Vmax) values of CYP isozymes in non-treated male mice aged 3 to 8 weeks

	CYP activity	Vmax (nmol/min/mg protein)			
		3-week-old mice	4-week-old mice	6-week-old mice	8-week-old mice
Ethoxyresorufin <i>O</i> -deethylation	CYP1A	0.154 ± 0.034 <sup>a)</sup>	0.215 ± 0.022	0.155 ± 0.014 <sup>a)</sup>	0.316 ± 0.036
Pentoxoresorufin <i>O</i> -dealkylation	CYP2B	0.0291 ± 0.0024	0.0440 ± 0.0031 <sup>a)</sup>	0.0261 ± 0.0017	0.0273 ± 0.0024
Tolbutamide hydroxylation	CYP2C	0.763 ± 0.153	0.479 ± 0.118	0.520 ± 0.091	0.597 ± 0.071
Bufuralol 1'-hydroxylation	CYP2D	0.582 ± 0.043	0.815 ± 0.053	0.704 ± 0.134	0.724 ± 0.076
Aniline hydroxylation	CYP2E	0.837 ± 0.218	2.25 ± 0.30 <sup>a)</sup>	1.30 ± 0.13	1.09 ± 0.16
Midazolam 4-hydroxylation	CYP3A	0.370 ± 0.036	0.952 ± 0.032 <sup>a)</sup>	0.113 ± 0.034 <sup>a)</sup>	0.375 ± 0.016

Data represent the mean ± SD of three pooled liver samples. Livers were pooled from five for 3- and 4-week-old mice and six for 6- and 8-week-old mice. a)  $P < 0.05$ , significant difference from the 8-week-old mice (Dunnett's multiple comparison test).

**Table 2.** Results of intrinsic clearance (CLint) of CYP isozymes in non-treated male mice aged 3 to 8 weeks

	CYP activity	CLint (ml/min/mg protein)			
		3-week-old mice	4-week-old mice	6-week-old mice	8-week-old mice
Ethoxyresorufin <i>O</i> -deethylation	CYP1A	0.222 ± 0.058	0.546 ± 0.154	0.322 ± 0.110	0.421 ± 0.083
Pentoxoresorufin <i>O</i> -dealkylation	CYP2B	0.102 ± 0.016	0.154 ± 0.014 <sup>a)</sup>	0.0605 ± 0.0007	0.0668 ± 0.0058
Tolbutamide hydroxylation	CYP2C	0.112 ± 0.043	0.120 ± 0.016	0.105 ± 0.077	0.162 ± 0.028
Bufuralol 1'-hydroxylation	CYP2D	0.0717 ± 0.0067	0.122 ± 0.006	0.154 ± 0.033	0.0868 ± 0.0048
Aniline hydroxylation	CYP2E	1.22 ± 0.33	0.477 ± 0.131 <sup>a)</sup>	1.61 ± 0.36	2.37 ± 0.25
Midazolam 4-hydroxylation	CYP3A	0.00816 ± 0.00087	0.0127 ± 0.0015	0.00277 ± 0.00164 <sup>a)</sup>	0.00834 ± 0.00096

Data represent the mean ± SD of three pooled liver samples. Livers were pooled from five for 3- and 4-week-old mice and six for 6- and 8-week-old mice. a)  $P < 0.05$ , significant difference from the 8-week-old mice (Dunnett's multiple comparison test).

**Table 3.** Results of Michaelis constant (Km) values of CYP isozymes in non-treated male mice aged 3 to 8 weeks

	CYP activity	Km ( $\mu$ M)			
		3-week-old mice	4-week-old mice	6-week-old mice	8-week-old mice
Ethoxyresorufin <i>O</i> -deethylation	CYP1A	0.757 ± 0.383	0.414 ± 0.111	0.521 ± 0.178	0.774 ± 0.212
Pentoxoresorufin <i>O</i> -dealkylation	CYP2B	0.291 ± 0.071	0.285 ± 0.007	0.432 ± 0.026	0.412 ± 0.063
Tolbutamide hydroxylation	CYP2C	7,360 ± 2,590	4,000 ± 1,010	7,350 ± 5,230	3,760 ± 780
Bufuralol 1'-hydroxylation	CYP2D	8.19 ± 1.24	6.67 ± 0.32	4.79 ± 1.75	8.38 ± 1.26
Aniline hydroxylation	CYP2E	748 ± 396	5,010 ± 1,730 <sup>a)</sup>	846 ± 284	466 ± 123
Midazolam 4-hydroxylation	CYP3A	45.5 ± 4.7	75.8 ± 7.3	59.7 ± 53.1	45.4 ± 4.7

Data represent the mean ± SD of three pooled liver samples. Livers were pooled from five for 3- and 4-week-old mice and six for 6- and 8-week-old mice. a)  $P < 0.05$ , significant difference from the 8-week-old mice (Dunnett's multiple comparison test).

significantly lower Vmax values for ethoxyresorufin *O*-deethylation (approximately half). However, the values of the other reactions were not significantly different. In contrast, 4-week-old mice showed significantly higher Vmax values for pentoxoresorufin *O*-dealkylation (approximately 1.5-fold), aniline hydroxylation (approximately 2-fold) and midazolam 4-hydroxylation (approximately 2.5-fold), whereas the values of the other reactions were not significantly different. In 6-week-old mice, significantly lower values were obtained from ethoxyresorufin *O*-deethylation and midazolam 4-hydroxylation.

The CLint of each reaction was also compared between 8-week-old mice and mice of other ages in Table 2. Three-week-old mice did not show any significant differences in any of the reactions examined in this study. In contrast, 4-week-old mice showed significantly higher and lower values of pentoxoresorufin *O*-dealkylation (approximately 2-fold) and aniline hydroxylation (approximately one-fifth), respectively, and 6-week-old mice showed significantly lower values of midazolam 4-hydroxylation (approximately one-third).

As shown in Table 3, the Km values for each of the reactions were similar among the ages, except for aniline hydroxylation in 4-week-old mice where significantly higher values were obtained.

There were no significant differences in the CYP contents of hepatic microsomes between mice at 8 weeks old and other ages (Table 4).

**Table 4.** Results of CYP contents of hepatic microsomes in non-treated male mice aged 3 to 8 weeks

CYP content (nmol/mg protein)	
3-week-old mice	0.495 ± 0.058
4-week-old mice	0.740 ± 0.061
6-week-old mice	0.784 ± 0.159
8-week-old mice	0.795 ± 0.111

Data represent the mean ± SD of three pooled liver samples. Livers were pooled from five for 3- and 4-week-old mice and six for 6- and 8-week-old mice.

**Table 5.** Results of the final body weight and the liver weight after administration of physiological saline or diethylnitrosamine to mice aged 3 to 6 weeks

DEN (mg/kg/day)	No. of animals	Final body weight (g)	Liver weight (g)
		Mean ± SD	Mean ± SD
3-week-old mice			
0 (physiological saline)	5	26.2 ± 2.2 <sup>b)</sup>	1.74 ± 0.17
50	5	20.2 ± 1.4 <sup>b)</sup>	1.19 ± 0.16 <sup>a)</sup>
4-week-old mice			
0 (physiological saline)	4	29.0 ± 1.9 <sup>a)</sup>	1.76 ± 0.18
50	4	24.5 ± 0.7 <sup>b)</sup>	1.24 ± 0.09
5-week-old mice			
0 (physiological saline)	4	32.1 ± 2.6	2.02 ± 0.25
50	4	27.5 ± 3.7	1.62 ± 0.47
6-week-old mice			
0 (physiological saline)	4	34.3 ± 2.2	2.03 ± 0.30
50	4	30.8 ± 0.7	1.49 ± 0.09

DEN: diethylnitrosamine. a)  $P < 0.05$ , significant difference from the 6-week-old mice (Dunnett's multiple comparison test). b)  $P < 0.01$ , significant difference from the 6-week-old mice (Dunnett's multiple comparison test).

**Table 6.** Results of the liver micronucleus assay after administration of physiological saline or diethylnitrosamine to mice aged 3 to 6 weeks

DEN (mg/kg/day)	No. of animals	MNHEPs (%) / 2,000 HEPs	M-phase cells (%) / 2,000 HEPs
		Mean ± SD	Mean ± SD
3-week-old mice			
0 (physiological saline)	5	0.44 ± 0.21	0.40 ± 0.07
50	5	2.27 ± 0.22 <sup>a)</sup>	0.15 ± 0.11
4-week-old mice			
0 (physiological saline)	4	0.08 ± 0.06	0.10 ± 0.07
50	4	1.33 ± 0.41 <sup>a)</sup>	0.01 ± 0.03
5-week-old mice			
0 (physiological saline)	4	0.14 ± 0.09	0.00 ± 0.00
50	4	0.13 ± 0.06	0.03 ± 0.03
6-week-old mice			
0 (physiological saline)	4	0.05 ± 0.04	0.03 ± 0.03
50	4	0.13 ± 0.03	0.01 ± 0.03

DEN: diethylnitrosamine, MNHEPs: micronucleated hepatocytes, HEPs: hepatocytes, a)  $P < 0.01$ , significant difference from the solvent (physiological saline) control (Fisher's exact test).

#### Liver MN and PB MN assay

The mean final body weights and liver weights after administration of physiological saline or DEN were compared between 6-week-old mice and mice of other ages in Table 5. The mean final body weights in the physiological saline or DEN groups at 3 and 4 weeks old were significantly lower than those in the group at 6 weeks old. The mean liver weight in the DEN group at 3 weeks old was significantly lower than that in the group at 6 weeks old.

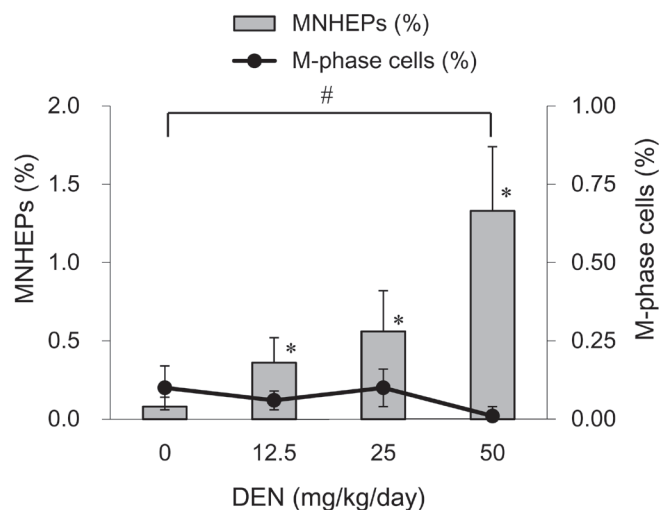
Table 6 shows the results of the liver MN assay after the administration of physiological saline or 50 mg/kg/day DEN to mice aged 3 to 6 weeks old. The mean frequencies of MNHEPs after a 50 mg/kg DEN administration in 3- and 4-week-old mice increased significantly compared with the negative control (2.27 and 1.33%, respectively). In contrast, these values in 5- and 6-week-old mice remarkably decreased (0.13% and 0.13%, respectively). There were no significant differences compared with the negative control.

As shown in Fig. 1, the mean MNHEP frequencies after treatment with DEN (12.5, 25 and 50 mg/kg/day) in 4-week-old mice increased dose dependently by approximately 4- to 16-fold compared with the negative control. In contrast, there were no significant differences between the mean MNRET and RET frequencies after DEN dosing compared to the negative control (Fig. 2).

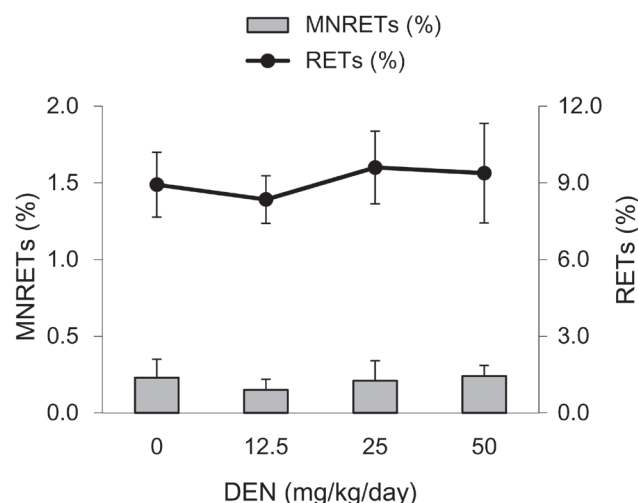
## DISCUSSION

In order to investigate whether juvenile mice have sufficient hepatic CYP activities compared with young adult mice (in which the latter are generally used for *in vivo* rodent BM or PB MN assays), age-related changes of these activities were evaluated in mice aged between 3 and 8 weeks. The hepatic enzyme activities of CYP1A (ethoxyresorufin *O*-deethylation), CYP2B (pentoxyresorufin *O*-dealkylation), CYP2C (tolbutamide hydroxylation), CYP2D (bufuralol 1'-hydroxylation), CYP2E (aniline hydroxylation) and CYP3A (midazolam 4-hydroxylation) in mice aged at 3 and 4 weeks were approximately equal to or higher than mice aged at 8 weeks; however, these observations did not apply to CYP1A and CYP2E in 3- and 4-week-old mice, respectively. In a previous study, enzyme activities of CYP1A and CYP3A (testosterone 6 $\beta$ -hydroxylation), CYP2C and CYP2B (testosterone 16 $\alpha$ -hydroxylation), CYP2B (testosterone 16 $\beta$ -hydroxylation) and CYP2B (aromatase activity) progressively increased with age and reached levels similar to adulthood by PND 15 with the exception of CYP2C (testosterone 2 $\alpha$ -hydroxylation) in CD-1 mouse liver [6]. Moreover, it was reported most CYP gene expressions in mouse liver were progressively increased up to PND 20–30 and reach the same levels as those of PND 45 or 60 [11, 25]. Although the mRNAs were expressed in the perinatal period and gradually decreased after birth in CYP2D26, CYP3A16, CYP3A41a/b and CYP3A44, many gene expressions in CYP2B, CYP2C, CYP2D and CYP3A were increased steadily between PND 20 and 30 and then reached plateau after PND 30 [11, 25]. From these results, it is considered the hepatic enzyme activities of CYP2B, CYP2C, CYP2D and CYP3A would reach enough levels to detect genotoxicity of chemicals in juvenile mice at 3 and 4 weeks old along with young adult mice. Due to the low activity of CYP2C in PND 4–30 rats [3, 15], the liver MN assay using juvenile rats may not detect genotoxic compounds, since it requires CYP2C activation [29]. However, our results suggest that the assay using juvenile mice can detect them.

The  $V_{max}$  values of ethoxyresorufin *O*-deethylation in mice aged at 3 and 6 weeks were about half the level of those in mice aged at 8 weeks. However, these values are higher than those in previous reports for PND 30 rats [3] and young adult rats [8], suggesting that can detect the genotoxicity of chemicals activated by CYP1A, such as 2-acetylaminofluorene [10, 31]. Accordingly,



**Fig. 1.** Frequencies of micronucleated hepatocytes (MNHEPs, evaluated from 2,000 hepatocytes) and metaphase (M-phase) cells (evaluated from 2,000 hepatocytes) after administration of physiological saline and DEN (12.5, 25 and 50 mg/kg/day) to 4-week-old male mice. Data are the mean of four animals with standard deviation. \* $P < 0.01$ , significantly different from solvent control (Fisher's exact test). # $P < 0.01$ , significantly different from solvent control (Cochran-Armitage trend test).



**Fig. 2.** Frequencies of micronucleated reticulocytes (MNRETs, evaluated from 2,000 reticulocytes) and reticulocytes (RETs, evaluated from 1,000 erythrocytes) after administration of physiological saline and DEN (12.5, 25 and 50 mg/kg/day) to male mice aged at 4 weeks. Data are the mean of four animals with standard deviation.

it seems that mice aged at 3 weeks or older have enough CYP1A activities to generate genotoxic metabolites in the liver; however, further studies of the juvenile mouse liver MN assay with other genotoxic chemicals that require metabolic activation by CYP1A should be conducted.

Although the  $K_m$  value of midazolam 4-hydroxylation in 6-week-old mice was almost the same level to that of 8-week-old mice, the  $V_{max}$  and  $CL_{int}$  values of that in 6-week-old mice were about one-third the level of those in 8-week-old mice. However, the  $V_{max}$  and  $K_m$  values in mice at 6 weeks old are approximately equal to those of rats at 9 weeks old in a previous report [1]. Thus, hepatic CYP3A activity in mice aged at 6 weeks would be sufficient level compared with those of young adult rats commonly used for *in vivo* rodent BM or PB MN assays.

While 3-week-old mice had almost the same level of CYP2E activity as 8-week-old mice, 4-week-old mice showed lower  $CL_{int}$  and higher  $V_{max}$  and  $K_m$  values of aniline hydroxylation compared with mice aged at 8 weeks. While Hart *et al.* [11] reported the mRNA expression of CYP2E1 quickly increases after PND 0 and reaches a plateau by PND 5 in mouse liver, Peng *et al.* [25] demonstrated the expression in mouse liver was continually increased to a peak at PND 20, followed by approximately half decrease between PND 20 and 30 and then relatively constant thereafter. Although the decrease of CYP2E1 mRNA might be related to the phenomena observed only in 4-week-old mice, the reason for the low affinity to aniline at 4 weeks is unknown. However, since positive results were obtained in the liver MN assay in mice aged at 3 and 4 weeks, it is likely that these mice have sufficient CYP2E activity to induce micronuclei formation in hepatocytes in response to DEN administration, which is mainly activated by CYP2E [14].

The MNHEP frequencies after DEN administration to 4-week-old mice increased dose-dependently, which is comparable with the data of liver MN assay using juvenile rats [29, 33]. In contrast, the PB MN assay with DEN was negative in 4-week-old mice, and there were no significant differences between the MNRET and RET frequencies after DEN dosing compared to the negative control. DEN is carcinogenic in all animal species tested, and one of the major target organs is liver [19]. It is considered that the genotoxic metabolite of DEN activated by hepatic CYP2E causes the hepatocarcinogenesis [14]. While DEN induced positive results in *in vitro* genotoxicity assay, such as Ames test and chromosomal aberration test, in the presence of an exogenous metabolic system, no micronucleus induction was observed after DEN administration in BM or PB MN assay using adult male CD-1 mice [19] and juvenile rats [30]. This negative result in the PB MN assay may be due to the short lifespan of active DEN metabolites in the liver which makes it difficult to accumulate in the bone marrow at concentrations high enough to induce micronuclei formation [29]. Thus, the juvenile mouse liver MN assay could detect such indirect genotoxic chemicals that give negative results in rodent BM or PB MN assay. In addition, the simultaneous liver and PB MN assay system could provide more useful information, such as organ specificity, by comparing micronuclei incidences in two different tissues [30–33].

Despite the activity level of hepatic CYP2E of 6-week-old mice having similar levels to that of 8-week-old mice, a dramatic decrease in MNHEP frequencies with DEN was observed in 5- and 6-week-old mice. This may have resulted from the marked decrease in cell division which is necessary to form micronuclei after chromosome damage in the liver. Apte *et al.* [2] reported

an increase in cell proliferation by the presence of proliferating cell nuclear antigen (PCNA)-positive hepatocytes that were observed in the S phase from PND 5 to 20. They also observed a significant decrease in PCNA-positive cells after PND 30 in ICR mouse livers. Furthermore, it has been reported that PCNA-positive hepatic cells in C57BL/6 mice increased from PND 10 to PND 25 compared with PND 0, and the number of PCNA-positive cells decreased markedly after PND 30 [28]. Suzuki *et al.* [29] demonstrated that the MNHEP frequencies decreased age-dependently after a single 50 mg/kg DEN administration, with the highest values in F344 rats aged at 3 weeks and low values at 7 and 9 weeks due to an age-related decrease in HEP proliferation. In this study, we also examined the frequencies of M-phase cells (mitotic index) used in previous reports of rat liver MN assay [10, 20, 30–33] as a parameter of cell proliferation in the mouse liver. However, the frequencies were too low to evaluate an age effect. In spite of the low mitotic index, DEN induced MNHEPs in 3- and 4-week-old mice at similar or higher levels compared with data of the previous reports in the juvenile rat method [30–33], suggesting that mice aged 3 and 4 weeks would have sufficient HEP division ability to generate MNHEPs. In addition, the final body weight and liver weight increased with age in our study. An increase in liver and body weight was observed between PND 0 and PND 30 mice, and the increase in liver weight was due to increased cell proliferation [2, 28]. Therefore, the age-related increase of liver weight in young mice would be caused by HEP proliferation. The mitotic index of HEPs is usually observed at very low levels in the juvenile rat [30–33] or in the RDLMN method [10, 20]. Otherwise, Hagio *et al.* [9] reported that HEP proliferation could be evaluated by detecting Phospho-Histone H3 antibody-positive cells, a marker of cell proliferation, in the RDLMN assay using adult mice with DEN treatments. Therefore, mitotic index would be not a good indicator for HEP proliferation, and another indicator might be useful for evaluation of cell proliferation in rodent liver. However, the method for measuring HEP proliferation is not standardized in the rodent liver MN assay [37].

In conclusion, this study demonstrated that juvenile mice (3- and 4-week-old mice) have sufficient enzyme activity levels for most CYP isozymes and have the micronuclei-inducing potential for detecting the genotoxicity of DEN which is mainly activated by CYP2E in the liver. While further studies are required for the liver MN assay using juvenile mice with other hepatocarcinogens or genotoxic compounds that require metabolic activation in the liver, the juvenile mouse liver MN assay along with the juvenile rat method could be a useful genotoxicity assay. Moreover, the combination of liver and PB MN assay in juvenile mice can reduce animal numbers and amount of reagents.

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