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Effect of Age on the Repeated-Dose Liver Micronucleus Assay Using 2,4-Dinitrotoluene

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Objective

The repeated-dose liver micronucleus (RDLMN) assay is being developed toward an IWGT-recommended protocol and potential OECD guideline adoption. During this process, the potential impact of animal age and the applicability to animals starting dosing at 8 weeks of age have been identified as key issues, particularly for integration into repeated-dose toxicity studies. To address these questions, the IWGT Liver MN subgroup selected compounds showing weak positive responses at 6-week-start dosing.

In the present study, we evaluated 2,4-dinitrotoluene (2,4-DNT) to compare RDLMN responses in rats aged 6 or 8 weeks at the start of administration. In addition, hepatocellular proliferation was assessed using Ki-67 and the mitotic index, and differences in MNHEP frequencies among liver lobes were examined to evaluate the suitability of different liver lobes for liver MN assay.

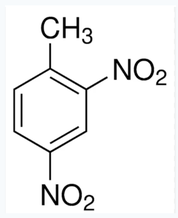
Materials and Methods

【Animals】

Male Crl:CD(SD) rats (5 animals/group) were purchased from The Jackson Laboratory Japan, Inc. The animals were 6 or 8 weeks old at the start of dosing.

【Test substance】

✓ 2,4-dinitrotoluene (2,4-DNT, CAS No. 121-14-2)
2,4-DNT was selected as a model compound because it induces relatively weak liver micronucleus responses; mortality has been reported at 200 mg/kg in 6-week-old rats [1].



✓ Positive control: Diethylnitrosamine (DEN, CAS No. 55-18-5)

【Dose levels and treatment】

2,4-DNT was administered orally to rats once daily for 28 consecutive days at doses of 0 (0.5% methylcellulose solution; vehicle), 25, 50, 100, and 150 mg/kg. DEN was administered at 12.5 mg/kg using the same dosing schedule.

【Liver micronucleus assay】

✓ Twenty-four hours after the final dosing, the liver was sampled and fixed in 10% phosphate-buffered formalin (formalin).

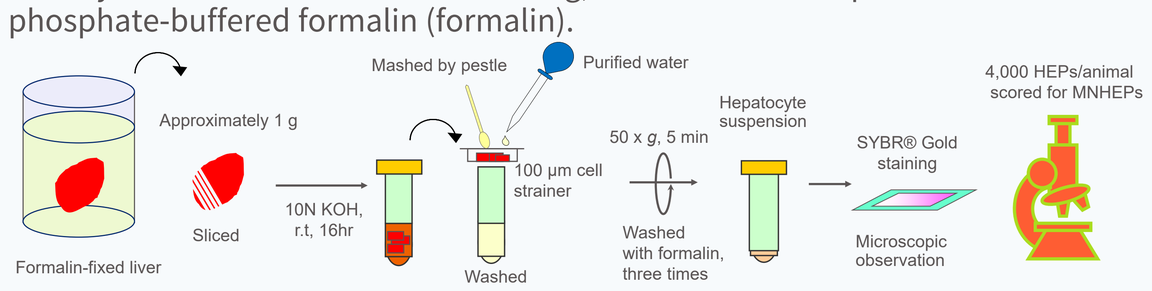


Figure 1 Slide preparation for liver MN assay using formalin-fixed method

✓ For liver lobe comparison, left lateral, left medial, right medial, right lateral, and caudate lobes from 6-week-old animals at 0 and 150 mg/kg were evaluated.

【Evaluation of cell proliferation】

✓ Liver sections were immunostained with a rabbit polyclonal anti-Ki-67 (Abcam, ab15580). Images were scanned using Aperio ScanScope, and 4000 HEPs/animal were scored to determine the frequency of Ki-67-positive hepatocytes.

✓ The mitotic index was determined during MNHEP evaluation.

【Statistical analysis】

✓ MNHEP frequencies were analyzed using the Kastenbaum and Bowman method.

✓ Other parameters were analyzed using Dunnett's test or Steel's test based on Bartlett's test.

Results

☐ Clinical signs: No treatment-related clinical signs were observed in either age group.

☐ Body weight changes: Reduced body weight gain was observed at 150 mg/kg.
6-week-old group: 22% lower than vehicle controls
8-week-old group: 16% lower than vehicle controls

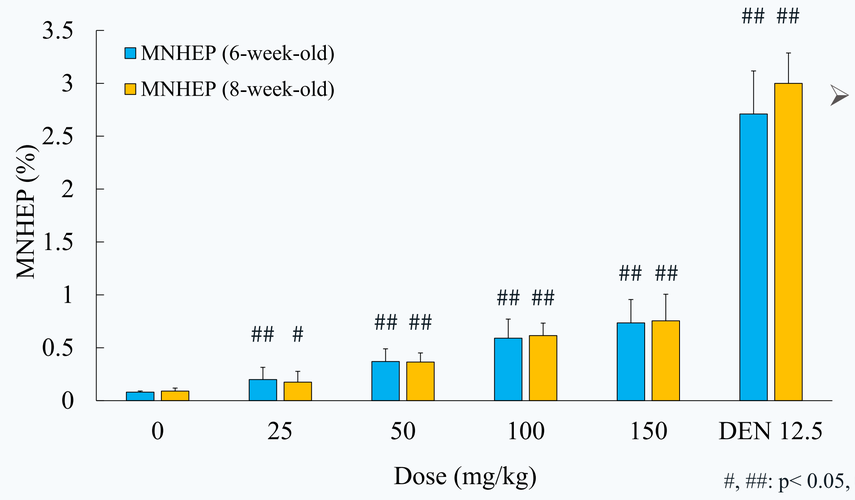
☐ Liver weights: Relative liver weights were increased in both age groups.

☐ Histopathological findings in the liver: Histopathological changes tended to be slightly more pronounced in the 6-week-old group than in the 8-week-old group.

	6-week-old group					8-week-old group				
Dose (mg/kg)	0	25	50	100	150	0	25	50	100	150
Hypertrophy, nucleus/cytoplasm, hepatocyte										
minimal	0	0	4	3	2	0	0	1	5	4
mild	0	0	0	2	3	0	0	0	0	1
Oval cell proliferation										
minimal	0	0	0	4	1	0	0	0	5	3
mild	0	0	0	1	4	0	0	0	0	2
Pigment deposition, Kupffer cells										
minimal	0	0	0	0	5	0	0	0	0	5

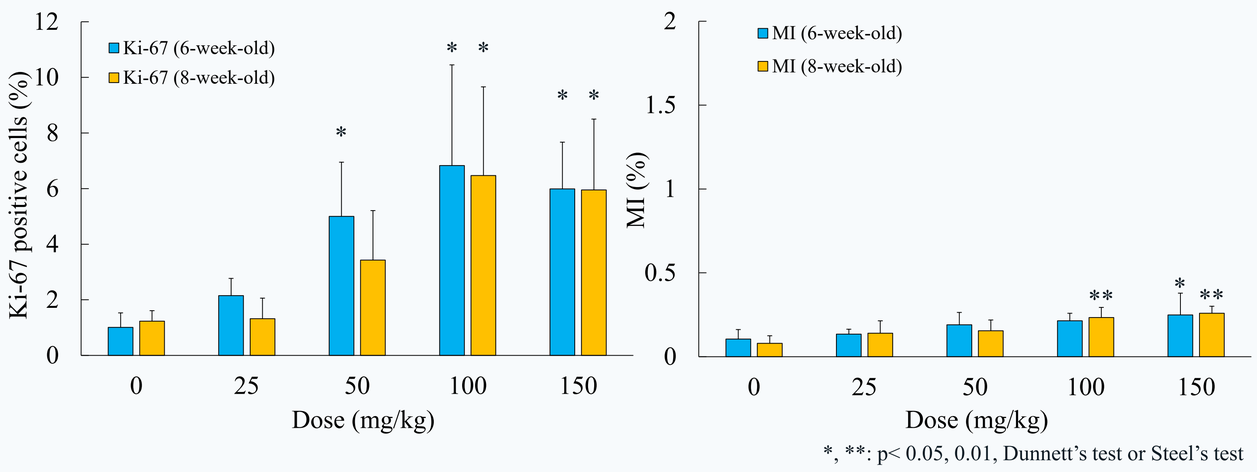
Table 1 Histopathological findings in the liver

➢ No treatment-related clinical signs were observed; however, reduced body weight gain (−22% or −16% vs. vehicle controls) and liver histopathological findings indicated that the selected high dose was appropriate.



#, ##: p< 0.05, 0.01, Kastenbaum and Bowman method

Figure 2 Results of the liver micronucleus assay of 2,4-DNT
– Comparison between 6-week-old and 8-week-old rats



*, **: p< 0.05, 0.01, Dunnett's test or Steel's test

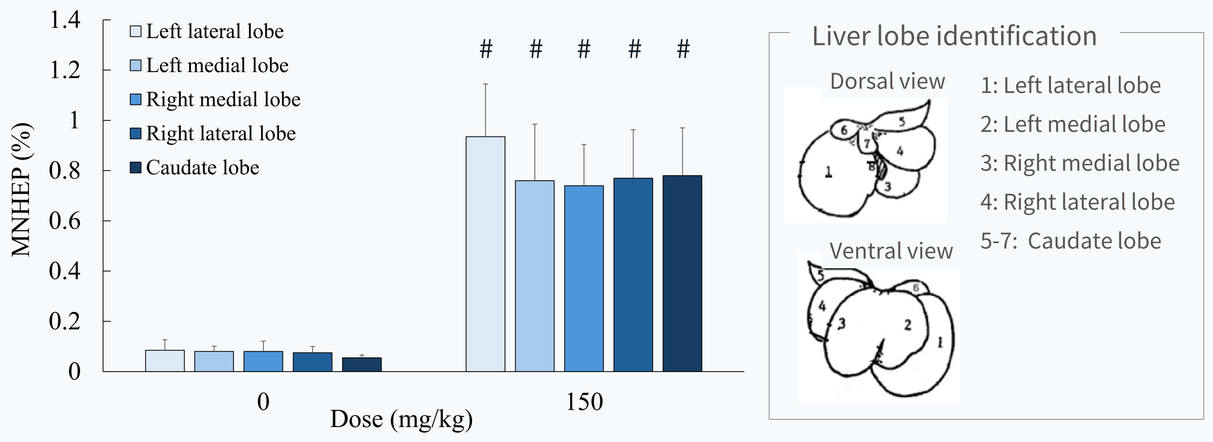
Figure 3 Evaluation of cell proliferation of 2,4-DNT
– Comparison between 6-week-old and 8-week-old rats

➢ A dose-dependent increase in Ki-67-positive hepatocytes was observed in both age groups, indicating increased hepatocellular proliferation.

➢ Histopathological changes tended to be slightly more pronounced in the 6-week-old group, consistent with the somewhat higher Ki-67-positive hepatocyte frequencies observed in younger animals.

➢ The mitotic index was increased; however, its low baseline value (~0.1% in vehicle controls) may limit sensitivity, particularly for antiproliferative compounds.

➢ Histopathological findings together with increased Ki-67-positive hepatocytes suggested regenerative hepatocellular proliferation. This proliferative response may have reduced the impact of age-related declines in basal hepatocellular proliferative activity, resulting in comparable liver MN responses in the 6- and 8-week-old groups.



#: p< 0.01, Kastenbaum and Bowman method, vs. corresponding vehicle control lobe

Figure 4 Results of the liver micronucleus assay of each liver lobe
– Comparison of MNHEP frequencies among liver lobes

➢ MNHEP frequencies were significantly increased in the 150 mg/kg group compared with the vehicle control group in all liver lobes.

➢ Liver MN induction by 2,4-DNT was comparable among the liver lobes.

Conclusion

1. Both 6- and 8-week-old rats showed sufficient sensitivity to detect relatively weak liver MN responses induced by 2,4-DNT.
2. Liver MN induction by 2,4-DNT was accompanied by regenerative hepatocellular proliferation.
3. Ki-67 exhibited a wider dynamic range than the mitotic index and may be a useful marker for interpreting liver MN assay results.
4. Comparable MNHEP frequencies across liver lobes suggest flexibility in liver lobe selection for liver MN assay.

Reference
[1] Maeda A et al. (2015) Mutat Res 780-781: 41-45

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