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## Regular article

# Evaluation of the *in vivo* Mutagenicity of Nickel Subsulfide in the Lung of F344 *gpt* delta Transgenic Rats Exposed by Intratracheal Instillation: A Collaborative Study for the *gpt* delta Transgenic Rat Mutation Assay

Tomoyuki Kamigaito[1,6], Tadashi Noguchi[1], Kazunori Narumi[2], Rie Takashima[2],
Shuichi Hamada[2], Hisakazu Sanada[3], Masayuki Hasuko[4], Hiroyuki Hayashi[5],
Kenichi Masumura[4] and Takehiko Nohmi[4]

[1]Japan Bioassay Research Center, Japan Industrial Safety and Health Association, Kanagawa, Japan
[2]Safety Assessment Department, Mitsubishi Chemical Medience Corporation, Ibaraki, Japan
[3]Central Research Laboratories, Kaken Pharmaceutical Co., Ltd., Shizuoka, Japan
[4]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan
[5]Toxicology Laboratory, Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd., Yokohama, Japan

This study was conducted to evaluate the effectiveness of a transgenic rat mutation assay using F344 *gpt* delta rats. We investigated the mutagenic potential in the lung of nickel subsulfide ($Ni_3S_2$), an insoluble fine-crystalline-metallic compound and a carcinogen in the rodent and human lung. $Ni_3S_2$ carcinogenicity has been proposed to act via both genotoxic and non-genotoxic mechanisms. $Ni_3S_2$ was intratracheally instilled into male *gpt* delta rats at doses of 0.5 and 1 mg/animal once a week for four weeks; these doses of $Ni_3S_2$ are high enough to induce inflammation in the lung. Following a period of 28 and 90 days after the first administration, the *gpt* mutant frequencies (MFs) in lung were determined in four independent laboratories, and Spi⁻ selection for larger deletion mutations was done in one laboratory. The *gpt* MFs of the rats treated with $Ni_3S_2$ were not increased: all four laboratories obtained similar results with no statistical differences. The Spi⁻ MFs were also not increased by exposure to $Ni_3S_2$. These results indicate that intratracheally instilled $Ni_3S_2$ is non-mutagenic in the lung of *gpt* delta transgenic rats; however, whether $Ni_3S_2$ is non-mutagenic in the lung or it induces mutations which are not detectable by transgenic rodent mutation assays requires further investigation.

Key words: F344 *gpt* delta transgenic rat, nickel subsulfide, *gpt* assay, Spi⁻ assay

## Introduction

Transgenic animals, such as Big Blue® rats and mice (1,2), Muta™ mice (3) and *gpt* delta rats and mice (4,5), are powerful tools for the detection of *in vivo* mutagenicity. The transgenic rodent mutation assay using *gpt* delta transgenic mice and rats was developed by Nohmi *et al.* (4,5). This assay system is composed of two detection methods, the 6-thioguanine (6-TG) assay for point mutations and Spi⁻ selection for deletion (6,7). This system is convenient for the identification of gene mutation by DNA sequencing due to small gene size (456 bp). In addition, positive selections are easier to evaluate mutant frequencies (MFs) than conventional color selections. Spontaneous MFs are comparatively lower than that of other transgenic rodent mutation assay system (7). Among the numerous substances which have been evaluated using this system are a variety of chemical compounds (8–10), radiation (11,12), and micro/nanoparticles (13), validating the usefulness of this transgenic rodent mutation assay system (7).

Recently, a protocol or guideline for the use of transgenic rodent gene mutation assays was discussed and proposed by the International Workshop on Genotoxicity Testing (IWGT) (14–16). It recommends a repeat-dose regimen with daily treatments for a period of 28 days and sampling time for 3 days following the final treatment. This protocol is being developed into an OECD Test Guideline (17). IWGT also suggests alternative treatment regimens; for example, weekly dose administration may be appropriate for some evaluations and a longer sampling time may be more appropriate if slowly proliferating tissues are of interest (16). We adopted the fundamental protocol of a collaborative

[6]Correspondence to: Tomoyuki Kamigaito, Japan Bioassay Research Center, Japan Industrial Safety and Health Association, 2445 Hirasawa, Hadano, Kanagawa 257-0015, Japan. Tel: +81-463-82-3911, Fax: +81-463-82-3860, E-mail: t-kamigaito@jisha.or.jp

study, which doses were a negative control group and a minimum of two dose revels (17).

The purpose of our study was to evaluate *in vivo* mutagenicity of nickel subsulfide ($Ni_3S_2$) by transgenic gene mutation assays in the lung as the target organ using F344 *gpt* delta rat. $Ni_3S_2$ is an insoluble fine-crystalline-metallic compound and a representative rodent and human lung carcinogenic metal.

Epidemiological studies of nickel refinery workers have demonstrated increased mortality from lung and nasal cancer, and it is apparent that the cancer risks have been associated with exposure to insoluble nickel compounds such as $Ni_3S_2$ and nickel oxide (18,19). $Ni_3S_2$ clearly has carcinogenic activity in the lungs of male and female F344/N rats exposed by inhalation, but it is not carcinogenic in male or female B6C3F1 mice exposed by inhalation (20). Injection of $Ni_3S_2$ has been shown to cause numerous carcinogenic lesions in rodents depending on the site of injection: lesions include sarcomas, rhabdomyosarcomas, fibrosarcomas, fibrous histiocytomas, mesotheliomas, renal-cell neoplasms, retinoblastomas, melanomas, and gliomas (19,21–27).

A variety of nickel compounds, including $Ni_3S_2$, are able to interact with molecular oxygen and generate reactive oxygen species (ROS) (27); however, like most carcinogenic metal ions, nickel compounds have weak or no mutagenic activity in bacteria (27–33), except when present at high toxic concentrations (34). In mammalian cells, carcinogenic nickel compounds are also generally poor mutagens (27,29,30,32,33,35). These compounds are, however, clastogenic in mammalian cells, inducing DNA strand breaks and accompanying chromosomal aberrations, sister-chromatid exchanges and the formation of micronuclei (29,30,32,36–39), and these clastogenicities are thought to be the mechanism by which $Ni_3S_2$ transforms mammalian cells *in vitro* (27,35,37,39).

Kawanishi *et al.* showed that $Ni_3S_2$ induced the formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) DNA adducts and DNA strand breaks, both *in vitro* and after intratracheal instillation into the lungs of Wistar rats (40). In these studies, intratracheal instillation of $Ni_3S_2$ also caused inflammation in the lungs of the rats. In contrast, Mayer *et al.* reported that $Ni_3S_2$ has no mutagenic activity in *lacZ* transgenic mice and *lacI* transgenic rats treated by single inhalation exposure for 2 h at a dose close to the maximum tolerated dose (MTD) of $Ni_3S_2$, even though it was mutagenic in *lacI* transgenic cells *in vitro* (41).

We assessed the *in vivo* mutagenicity of $Ni_3S_2$ administered into the lung by intratracheal instillation using *gpt* delta transgenic rats. The administration was limited to once a week for 4 weeks and lung sampling was at 7 days after fourth treatment due to the reduction of body weight over a few days by treatment under

anesthesia, instead of 28 consecutive daily treatments and sampling time in IWGT recommendation. To manifest maximum mutation frequency, the rats were sacrificed 28 or 90 days after the first administration. The period of 90 days between the initial administration of $Ni_3S_2$ and sacrifice (a little less than 10 weeks after the final administration of $Ni_3S_2$) was chosen to allow the lung to recover from $Ni_3S_2$ induced inflammation.

## Materials and Methods

Four laboratories (Table 1) participated in a collaborative study to evaluate the transgenic rodent gene mutation assay using *gpt* delta rats recommended by IWGT. This study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC). The animals were cared for in accordance with the Guideline for the care and use of laboratory animals in JBRC.

**Chemicals:** Nickel subsulfide ($Ni_3S_2$) (CAS.No. 12035-72-2) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The purity of the $Ni_3S_2$ particles (150-mesh) was 99.7%. The particle characterization of the $Ni_3S_2$ particle was determined using scanning electron microscopy (SU-8000, Hitachi Ltd., Tokyo Japan). The mean size was $2.9 \pm 1.74$ μm in diameter and more than 90% of the $Ni_3S_2$ particles had diameters less than 5 μm (Fig. 1). Perfluorocarbon (PF-5060) (CAS.No. 96508-42-1) was obtained from 3M (St. Paul, MN, USA).

**Animals and treatment:** Five-week-old male *gpt* delta rats [F344/NSlc-Tg(*gpt* delta)] were obtained from Japan SLC (Shizuoka, Japan). The inbred F344/NSlc-Tg (*gpt* delta) rat was established by backcrossing outbred *gpt* delta SD males with inbred F344 females. This strain has been backcrossed more than 15 times and is genetically homogeneous (42). The *gpt* delta transgenic rat contains approximately 5 to 10 copies of the lambda EG10 transgene in chromosome 4 and is maintained as a heterozygote (6). The animals were quarantined for one week and acclimated for 6 weeks to allow growth to a

**Table 1.** Participants in the collaborative study

| Lab | Investigators |
|---|---|
| A | T. Kamigaito, T. Noguchi: Japan Bioassay Research Center |
| B | K. Narumi, R. Takashima, S. Hamada: Mitsubishi Chemical Medience Corporation |
| C | H. Sanada: Central Research Laboratories, Kaken Pharmaceutical Co., Ltd. |
| D | K. Masumura, M. Hasuko, T. Nohmi: National Institute of Health Sciences |

The *gpt* MFs in all treated groups were studied by Labs A–D.
The Spi⁻ MFs in all treated groups were studied by Lab D.
*Animal housing, treatments to animals and tissue collection for $Ni_3S_2$- and ENU-treated groups were conducted by Japan Bioassay Research Center and Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd. respectively.

body weight of about 250 g. The animals were housed individually in stainless steel wire mash cages under barrier system room controlled environmental conditions (temperature of $24 \pm 3°C$, and relative humidity of $55 \pm 15\%$). Fluorescent lighting was controlled automatically



Fig. 1. Characteristics of nickel subsulfide particles in test substance. Upper panel: Scanning electron micrograph of $Ni_3S_2$ particles. Bar shows $10 \mu m$. Lower panel: Frequency distribution (bar) and cumulative distribution (dotted line) in $Ni_3S_2$ particl.

to provide a 12-h light-dark cycle. All animals were given basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water *ad libitum*. Twelve-week-old rats were divided by stratified randomization into 5-body weight matched groups.

Perfluorocarbon is a volatile organic compound (bp 56°C) which does not affect respiratory function in rodents and is an efficient vehicle for $Ni_3S_2$ particle delivery to pulmonary alveoli by intratracheal instillation (43). $Ni_3S_2$ particles homogenously suspended in perfluorocarbon were administrated into the endotrachea of the rats by intratracheal instillation, using a microspray cannula (Series 1A-1B Intratracheal Aerosolizer, PennCentury, Inc., PA, USA). $Ni_3S_2$ was administered at doses of 0 (vehicle control), 0.5, or 1.0 mg/animal in 0.3 mL perfluorocarbon once a week for four weeks. Each dose was administered to five rats under isoflurane anesthesia. Administration was limited to one time a week due to the harmful effects of the anesthesia. The dose of administered $Ni_3S_2$ was determined so as not to exceed the highest exposure dose produced only minimal interference with lung defense mechanisms based on particle clearance (40,44,45). Animals were sacrificed 28 days or 90 days after the first administration. The lung tissue was removed, weighed and a piece of the organ was fixed in 10% neutral buffered formalin and prepared and stained with hematoxylin and eosin. The remaining tissue was frozen in liquid nitrogen. Frozen lung tissue was broken, divided, packed with dry ice and sent to each of the collaborating laboratories for DNA extraction and mutation analysis (Table 1).

As a positive control, the livers of *N*-ethyl-*N*-nitrosourea (ENU) treated transgenic rats were analyzed. This treatment was conducted by Pharmaceutical
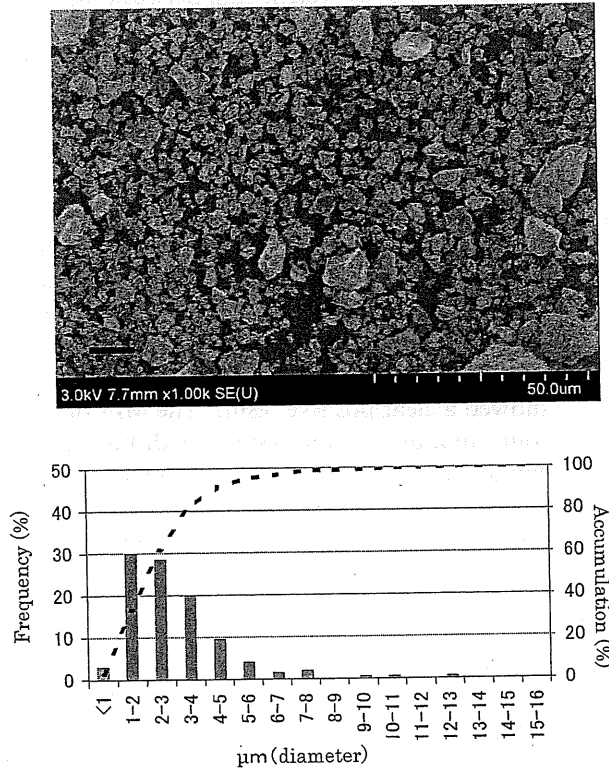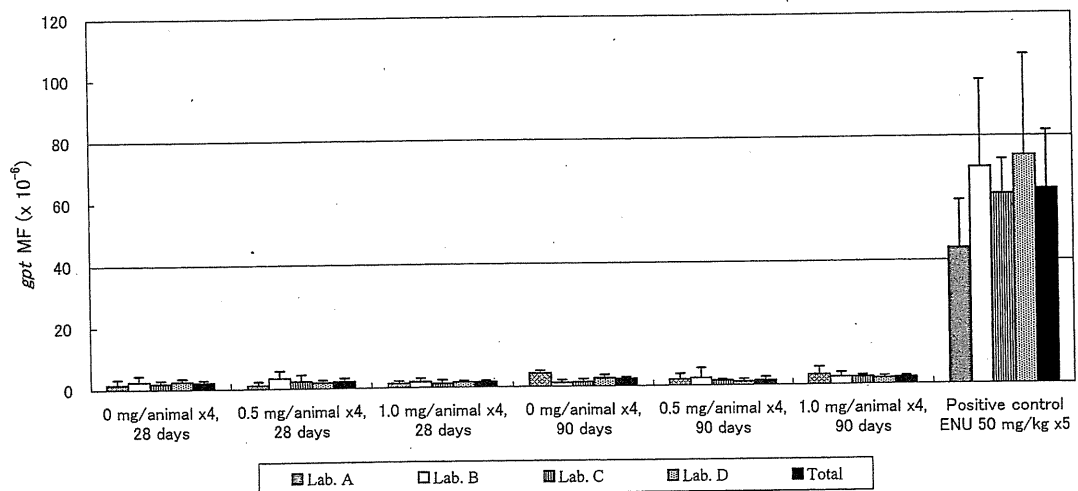


Fig. 2. *gpt* Mutant frequencies of each laboratoriy in the lung of male F344 *gpt* delta rats treated with $Ni_3S_2$ by intratracheal instillation and liver treated with ENU (positive control).

Research Center of Meiji Seika Pharma Co., Ltd. (Kanagawa, Japan). ENU was intraperitoneally administered to the gpt delta F344 male rats at a dose of 50 mg/kg once a day for five consecutive days. Twenty-six days after the final treatment the rats were killed and the livers were removed, frozen and distributed to the collaborating research laboratories (46).

**DNA isolation and mutation assay:** The gpt mutation assay was conducted at four collaborating laboratories and the Spi⁻ assay was at a single laboratory using previously reported methods (4,6). A RecoverEase™ DNA Isolation Kit (Agilent Technologies, Santa Clara, CA) was used to extract genomic DNA according to the manufacturer's instructions. The transgene was rescued from rat genomic DNA by in vitro lambda phage packaging using Transpack™ Packaging Extract (Agilent Technologies) according to the manufacturer's instructions.

For the gpt assay, the E.coli YG6020 strain which expresses Cre recombinase was infected with the rescued lambda phage. Total colonies and 6-TG resistant mutant colonies confirmed by replating were counted to calculate the frequencies of point mutations (gpt assay).

For the Spi⁻ assay, the E.coli XL1-Blue MRA strain and the XL1-Blue MRA(P2) strain were infected with the rescued lambda phage. Total recued plaques formed in E.coli XL1-Blue plates (representing total rescued lambda phage) and mutant plaques formed in the XL1-Blue MRA(P2) plates and confirmed by replating in E.coli WL95(P2) (representing mutant phages with deletion mutations) were counted to assess the frequencies of deletion mutations.

**Statistical analysis:** Bartlett's test was preliminarily used to determine whether the variances of data among control and treated groups were different or not. Body weight, organ weight and mutant frequency were analyzed by Dunnett's' multiple test because the variances of the data was not statistically different between each group. Incidences of histopathological lesions were analyzed by Fisher exact test. The level of significance was set at a $p$ value of less than 0.05 (two-sided analysis with).

## Results

Rats were exposed by intratracheal instillation to 0, 0.5 and 1.0 mg $Ni_3S_2$/animal once a week for four weeks. The total doses of each treated group were estimated to be 7 and 14 mg/kg, respectively. There was no difference in body weight gain between the $Ni_3S_2$-treated rats and the controls at 28 or 90 days after the first administration of $Ni_3S_2$. However, the absolute lung weight and the relative lung weight were increased in the 1.0 mg/animal group at 28 days compared to the untreated control group. No significant difference was observed at 90 days (Table 2).

**Mutant frequency (MF):** A summary of the gpt MFs determined in the four participating laboratories is shown in Table 3 and Fig. 2. There were no significant differences between the gpt MFs of the treated rats and the vehicle controls, at 28 days or 90 days. Under the same experimental conditions for mutation assay, ENU showed a clear positive result. The MFs obtained by the four laboratories were similar with no statistical differences between them as shown in appendix A–D (available at http:www.j-ems.org/journal/). The gpt MFs of the vehicle control groups in the four laboratories were $1.63 \pm 1.73$, $2.54 \pm 1.95$, $1.87 \pm 1.13$ and $2.60 \pm 0.98 \times 10^{-6}$ at 28 days and $4.40 \pm 0.88$, $1.28 \pm 0.94$, $1.44 \pm 1.00$ and $2.70 \pm 1.08 \times 10^{-6}$ at 90 days, respectively.

The gpt MF in the livers of gpt delta rats treated with ENU was $62.88 \pm 18.92 \times 10^{-6}$, dramatically increased compared to the gpt MFs in the lung of the vehicle control ($2.28 \pm 0.66$ or $2.50 \pm 0.46 \times 10^{-6}$), and the spontaneous gpt MF in the liver of F344 gpt delta rat previously reported ($4.4 \times 10^{-6}$) (7). Again, there was no statistical difference in the gpt MFs reported by the four different laboratories: $43.90 \pm 15.51$, $70.05 \pm 28.31$, $61.30 \pm 11.27$ and $73.74 \pm 32.81 \times 10^{-6}$.

The Spi⁻ MFs were determined in only one of the participating laboratories (Table 4). Similar to the gpt MF results, there were no significant differences between the Spi⁻ MFs of the treated rats and the vehicle controls.

Table 2. Body weight and lung weight of male F344 gpt delta rats treated with $Ni_3S_2$

| Group | Sampling time (days) | No. of animals | Body weight | | Lung weight | |
|---|---|---|---|---|---|---|
| | | | at first treatment (g) | at dissection (g) | Absolute (g) | Relative (%) |
| 0 mg/animal × 4 | 28 | 5 | 289 ± 8 | 286 ± 11 | 1.302 ± 0.052 | 0.46 ± 0.02 |
| 0.5 mg/animal × 4 | 28 | 5 | 291 ± 11 | 291 ± 12 | 1.327 ± 0.067 | 0.46 ± 0.03 |
| 1.0 mg/animal × 4 | 28 | 5 | 288 ± 11 | 287 ± 7 | 1.420 ± 0.076* | 0.49 ± 0.02* |
| 0 mg/animal × 4 | 90 | 5 | 283 ± 8 | 372 ± 5 | 1.247 ± 0.063 | 0.34 ± 0.02 |
| 0.5 mg/animal × 4 | 90 | 5 | 284 ± 11 | 372 ± 8 | 1.277 ± 0.081 | 0.34 ± 0.02 |
| 1.0 mg/aniaml × 4 | 90 | 5 | 288 ± 7 | 374 ± 6 | 1.309 ± 0.042 | 0.35 ± 0.01 |

*Significant difference compared with control group (Dunnett's test, $p < 0.05$).

**Table 3.** Summary of *gpt* mutant frequencies in the lung of *gpt* delta rats treated with Ni$_3$S$_2$ by intratracheal administration and liver treated with ENU (positive control)

| Treatment | Smpling time | Animal No. | Organ | Number of packagings | Total Population | Number of mutants | Mutant frequency ($\times 10^{-6}$) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Mean ± SD |
| 0 mg/animal × 4 | 28 days | 1001 | lung | 17 | 1,704,000 | 3 | 1.76 | |
| | | 1002 | lung | 14 | 2,907,000 | 8 | 2.75 | |
| | | 1003 | lung | 14 | 4,026,000 | 10 | 2.48 | |
| | | 1004 | lung | 14 | 3,693,000 | 11 | 2.98 | |
| | | 1005 | lung | 14 | 4,200,000 | 6 | 1.43 | 2.28 ± 0.66 |
| 0.5 mg/animal × 4 | 28 days | 1101 | lung | 14 | 2,669,500 | 6 | 2.25 | |
| | | 1102 | lung | 14 | 3,577,500 | 5 | 1.40 | |
| | | 1103 | lung | 13 | 2,625,000 | 11 | 4.19 | |
| | | 1104 | lung | 18 | 3,445,500 | 5 | 1.45 | |
| | | 1105 | lung | 10 | 2,851,500 | 6 | 2.10 | 2.28 ± 1.13 |
| 1 mg/animal × 4 | 28 days | 1201 | lung | 13 | 3,142,500 | 8 | 2.55 | |
| | | 1202 | lung | 15 | 3,687,000 | 4 | 1.08 | |
| | | 1203 | lung | 13 | 3,823,500 | 7 | 1.83 | |
| | | 1204 | lung | 15 | 4,333,500 | 6 | 1.38 | |
| | | 1205 | lung | 15 | 6,930,000 | 13 | 1.88 | 1.74 ± 0.55 |
| 0 mg/animal × 4 | 90 days | 1011 | lung | 11 | 4,149,000 | 11 | 2.65 | |
| | | 1012 | lung | 11 | 3,348,000 | 8 | 2.39 | |
| | | 1013 | lung | 11 | 5,779,500 | 17 | 2.94 | |
| | | 1014 | lung | 9 | 4,371,000 | 12 | 2.75 | |
| | | 1015 | lung | 13 | 5,701,500 | 10 | 1.75 | 2.50 ± 0.46 |
| 0.5 mg/animal × 4 | 90 days | 1111 | lung | 10 | 3,832,500 | 6 | 1.57 | |
| | | 1112 | lung | 11 | 2,611,500 | 3 | 1.15 | |
| | | 1113 | lung | 10 | 5,424,000 | 6 | 1.11 | |
| | | 1114 | lung | 13 | 2,565,000 | 9 | 3.51 | |
| | | 1115 | lung | 12 | 7,806,000 | 5 | 0.64 | 1.59 ± 1.12 |
| 1 mg/animal × 4 | 90 days | 1211 | lung | 7 | 3,405,000 | 8 | 2.35 | |
| | | 1212 | lung | 7 | 4,500,000 | 9 | 2.00 | |
| | | 1213 | lung | 8 | 3,385,500 | 10 | 2.95 | |
| | | 1214 | lung | 5 | 3,940,500 | 9 | 2.28 | |
| | | 1215 | lung | 7 | 4,006,500 | 7 | 1.75 | 2.27 ± 0.45 |
| ENU 50 mg/kg × 5 | 31 days | 51 | liver | 13 | 3,336,450 | 282 | 84.52 | |
| | | 52 | liver | 11 | 3,780,300 | 152 | 40.21 | |
| | | 53 | liver | 11 | 3,252,900 | 179 | 55.03 | |
| | | 54 | liver | 11 | 3,676,500 | 296 | 80.51 | |
| | | 55 | liver | 11 | 3,140,550 | 170 | 54.13 | 62.88 ± 18.92[†] |

[†]The *gpt* MF in the livers of *gpt* delta rats treated with ENU was markedly increased compared to the spontaneous *gpt* MF in F344 *gpt* delta rat livers of $4.4 \times 10^{-6}$ previously reported (7).

The Spi⁻ MF for vehicle controls ($6.49 \pm 3.93 \times 10^{-6}$) was higher than the spontaneous Spi⁻ MF in the lung reported for the *gpt* delta mouse ($2.8 \times 10^{-6}$) (7). The Spi⁻ MF in the livers of rats treated with ENU was $16.74 \pm 9.10 \times 10^{-6}$, in the present study, increased compared to the Spi⁻ MF in the lung of vehicle control and compared to the spontaneous Spi⁻ MF in F344 *gpt* delta rat liver of $2.8 \times 10^{-6}$ previously reported (7).

· **Histopathology:** For all the groups, histopathological changes due to the intratracheal instillation of vehicle and Ni$_3$S$_2$ were observed in the lung. Table 5 summarizes the histopathological findings of rats and their severity scores at each time point. At 28 days, a slight infiltration of inflammatory cells into the lung was observed in all animals including the negative control group. Eosinophils were found in the perivascular regions. Slight to moderate infiltration of alveolar macrophages was found in three animals of 1.0 mg/animal treatment group and this incidence was significantly greater than that of the control group. Furthermore, focal fibrosis was observed in two animals in the 0.5 mg/animal treatment group and two animals in the 1.0 mg/animal treatment group. Slight to moderate infiltration of alveolar macrophages was found in three animals of 1.0 mg/animal treatment group. At 90 days, focal fibrosis was observed in only two animals, one in the 0.5 mg/animal group and the other in the 1.0 mg/animal group, and mild infiltration of inflammatory cells was seen in only a few rats in the 0.5 mg/animal and control groups. Overall, inflammation in the lung at 90 days was milder than at 28 days, indicating recovery from the intratracheal instillation procedure.

**Table 4.** Summary of Spi⁻ mutant frequencies in the lung of *gpt* delta rats treated with $Ni_3S_2$ by intratracheal administration and liver treated with ENU (positive control)

| Treatment | Smpling time | Animal No. | Organ | Number of packagings | Total Population | Number of mutants | Mutant frequency ($\times 10^{-6}$) | Mean ± SD |
|---|---|---|---|---|---|---|---|---|
| 0 mg/animal × 4 | 28 days | 1001 | lung | 6 | 865,500 | 5 | 5.78 | |
| | | 1002 | lung | 6 | 1,665,000 | 4 | 2.40 | |
| | | 1003 | lung | 6 | 1,224,000 | 9 | 7.35 | |
| | | 1004 | lung | 6 | 951,000 | 4 | 4.21 | |
| | | 1005 | lung | 6 | 1,339,500 | 17 | 12.69 | 6.49 ± 3.93 |
| 0.5 mg/animal × 4 | 28 days | 1101 | lung | 4 | 631,500 | 3 | 4.75 | |
| | | 1102 | lung | 6 | 1,412,500 | 7 | 4.96 | |
| | | 1103 | lung | 6 | 656,000 | 3 | 4.57 | |
| | | 1104 | lung | 5 | 622,500 | 2 | 3.21 | |
| | | 1105 | lung | 5 | 1,426,500 | 8 | 5.61 | 4.62 ± 0.88 |
| 1 mg/animal × 4 | 28 days | 1201 | lung | 6 | 1,188,000 | 8 | 6.73 | |
| | | 1202 | lung | 6 | 1,371,000 | 8 | 5.84 | |
| | | 1203 | lung | 7 | 7 23,000 | 5 | 6.92 | |
| | | 1204 | lung | 7 | 456,000 | 6 | 13.16 | |
| | | 1205 | lung | 6 | 1,570,500 | 14 | 8.91 | 8.31 ± 2.93 |
| 0 mg/animal × 4 | 90 days | 1011 | lung | 3 | 697,500 | 3 | 4.30 | |
| | | 1012 | lung | 4 | 550,500 | 4 | 7.27 | |
| | | 1013 | lung | 4 | 1,380,000 | 2 | 1.45 | |
| | | 1014 | lung | 3 | 1,314,000 | 10 | 7.61 | |
| | | 1015 | lung | 3 | 1,620,000 | 8 | 4.94 | 5.11 ± 2.50 |
| 0.5 mg/animal × 4 | 90 days | 1111 | lung | 3 | 1,225,500 | 9 | 7.34 | |
| | | 1112 | lung | 4 | 1,212,000 | 9 | 7.43 | |
| | | 1113 | lung | 3 | 1,692,000 | 9 | 5.32 | |
| | | 1114 | lung | 4 | 708,000 | 4 | 5.65 | |
| | | 1115 | lung | 3 | 1,380,000 | 6 | 4.35 | 6.02 ± 1.34 |
| 1 mg/animal × 4 | 90 days | 1211 | lung | 6 | 1,110,000 | 3 | 2.70 | |
| | | 1212 | lung | 3 | 1,063,500 | 4 | 3.76 | |
| | | 1213 | lung | 3 | 1,012,500 | 5 | 4.94 | |
| | | 1214 | lung | 4 | 946,500 | 6 | 6.34 | |
| | | 1215 | lung | 5 | 2,233,500 | 7 | 3.13 | 4.18 ± 1.47 |
| ENU 50 mg/kg × 5 | 31 days | 51 | liver | 1 | 547,500 | 10 | 18.26 | |
| | | 52 | liver | 1 | 478,500 | 4 | 8.36 | |
| | | 53 | liver | 1 | 357,000 | 3 | 8.40 | |
| | | 54 | liver | 1 | 219,000 | 4 | 18.26 | |
| | | 55 | liver | 1 | 460,500 | 14 | 30.40 | 16.74 ± 9.10[†] |

[†]The Spi⁻ MF in the livers of *gpt* delta rats treated with ENU was markedly increased compared to the spontaneous Spi⁻ MF in F344 *gpt* delta rat livers of $2.8 \times 10^{-6}$ previously reported (7).

**Table 5.** Histopathological results in the lung of $Ni_3S_2$-treated male F344 *gpt* delta rats

| Group | Sampling time | No. of animals | Infiltration of inflammatory cells (including eosinophils, perivascular) | Fibrosis (focal) | Infiltration of alveolar macrophages |
|---|---|---|---|---|---|
| $Ni_3S_2$ 0 mg × 4 | 28 days | 5 | 5 (2+) | | |
| $Ni_3S_2$ 0.5 mg × 4 | 28 days | 5 | 5 (2+) | 2 (1+) | |
| $Ni_3S_2$ 1 mg × 4 | 28 days | 5 | 5 (2+) | 2 (1+) | 3* (1(1+), 2(2+)) |
| $Ni_3S_2$ 0 mg × 4 | 90 days | 5 | 4 (1+) | | |
| $Ni_3S_2$ 0.5 mg × 4 | 90 days | 5 | 3 (1+) | 1 (1+) | |
| $Ni_3S_2$ 1 mg × 4 | 90 days | 5 | 1 (1+) | | |

The number of the animals bearing the lesion in each exposed or control group were shown in the column. The parenthesized values indicate the number of the animals bearing the lesion with each of 4 different grades of severity, i.e., 1+ : slight, 2+ : moderate, 3+ : marked, 4+ : severe. Significant difference indicated by *$p \leq 0.05$ by Fisher exact test compared to each control.

## Discussion

The lung inflammation induced with the higher dose of $Ni_3S_2$ was characterized by the infiltration of alveolar macrophages and, furthermore, the fibrosis was observed in the both of $Ni_3S_2$-treated groups. These lung lesions indicated an inflammation and recovery from the inflammation. The histopathological results at 90-days suggested the recovery of inflammatory lesions. Similar results were obtained in lung toxicity study after 13-week inhalation exposure to $Ni_3S_2$ in F344 rats and mice (47). These results suggested that the treatment of conditions of the present study ensured that the rats were sufficiently challenged by $Ni_3S_2$ particles. And the period of sampling time was long enough to detect mutations because regeneration and/or recovery were observed in the lung tissues of the $Ni_3S_2$-treated groups at 28 or 90 days after the first treatment. In addition, a threshold of insoluble particles in lung clearances of F344 rats was estimated at 1–2 mg (45). The impaired lung clearance with overload of particles might induce tumor and fibrosis (48).

The gpt MFs in the lung were independently measured in four independent laboratories using a common standard method. The overall packaging efficiencies in each laboratory tended to be low, especially in one laboratory. One of the reasons was thought to poor DNA quality extracted. In addition, copy number of the transgene in transgenic animals may affect efficiency of recovery of the transgene. It is reasonable that gpt delta rat assays need more packaging than mouse assays, because copy number of the transgene par haploid is 5 ~ 10 in rat and about 80 in mouse (4,5). Another reason might be lower activity of packaging extract used.

The gpt MFs obtained by the four laboratories were similar, without any statistical differences between any of the laboratories. Administration of 0.5 or 1 mg $Ni_3S_2$/rat once a week for four weeks did not affect gpt MF in the lungs of F344 gpt delta rats: the gpt MFs were all similar to the previously reported spontaneous gpt MF in the lungs of gpt delta mice ($3.4 \times 10^{-6}$) (7). Further, no increase in the Spi MF was observed in the treated groups.

$Ni_3S_2$ is able to interact with molecular oxygen and generate reactive oxygen species (ROS) (27), and Kawanishi et al. (40) reported that $Ni_3S_2$ induced pathological inflammation and oxidative DNA damage in the lungs of rats treated with single intratracheal instillation of 0.5 or 1.0 mg/animal. $Ni_3S_2$ generation of DNA-damaging ROS coupled with induction of inflammation and its inhibition of DNA repair is thought to enhance its genotoxicity and tumorigenicity (27,40). This suggests that F344 gpt delta rats exposed by intratracheal instillation to high doses of $Ni_3S_2$ should acquire multiple mutations in the lung. In our study, however, although F344 gpt delta rats were administered $Ni_3S_2$ at doses high enough to induce inflammatory responses in the lung and the duration of the experiment was set as weekly dosing for four weeks with two sampling periods (28 or 90 days after the first treatment), $Ni_3S_2$ did not cause an increase in gpt or Spi⁻ MF in the lung. The lack of induction of mutation is in agreement with the resultsof in vitro mutagenesis assays (20,27–29,36–38). Moreover, administration of $Ni_3S_2$ by inhalation to the Muta™ Mouse and the Big Blue® rat did not induce mutations in the lacZ or the lacI genes (41). Taken together, these data suggest that in vivo mutagenicity of $Ni_3S_2$, such as induction of point mutations or small deletions, could not be observed by transgenic rodent mutation assays under those experimental conditions.

One possible reason why ROS-induced DNA damage does not lead to a detectable increase in mutations is that the damages are eliminated, either by the damage being repaired or by the cells undergoing apoptosis. Various studies have demonstrated that ROS induced by toxic metals are closely related to metal-induced apoptosis and carcinogenesis (49). Inhalation of high concentrations of amosite asbestos and bitumen fume by rats induces oxidative DNA damage (50–52). In the Big Blue® rat, a single intratracheal instillation of amosite asbestos 20–30 $\mu$m in length at a dose of 2 mg/animal resulted in a 2-fold increase in DNA mutations 16 weeks after treatment (53). In these animals, however, the amosite fibers were not eliminated from the lung and caused significant tissue damage with infiltration of neutrophils and macrophages. It is well known that long ($>10\,\mu$m) asbestos fibers induce ROS generation by phagocytes (54–56). Therefore, ROS generation stimulated by asbestos was augmented by neutrophils and macrophages, and the production of ROS continued throughout the experiment. In contrast to asbestos fibers, bitumen fumes, which did not cause prolonged inflammation or prolonged infiltration of phagocytes, did not induce an increase in MFs in the lungs of Big Blue® rats despite the formation of DNA adducts (50).

Two other genotoxic mechanisms may contribute to the carcinogenicity of $Ni_3S_2$ in the rat lung. Recently, it was demonstrated that $Ni_3S_2$ induced silencing of the gpt transgene present in G12 cells, a V79-derived transgenic Chinese hamster cell line containing the gpt transgene, without mutation of the transgene (57). The silencing of the gpt gene was shown to be due to DNA methylation. If DNA hypermethylation results in aberrant silencing of a tumor suppressor gene, it could participate in carcinogenesis (27,41,57). This type of epigenetic event would not be detected by the gpt or Spi⁻ assays we used to detect $Ni_3S_2$ induced mutations.

Another genotoxic mechanism is the generation of chromosomal breaks leading to mutations such as DNA strand breaks which lead to chromosomal aberrations. This type of damage will be rarely detected by the gpt or

Spi$^-$ assays we used to detect Ni$_3$S$_2$ induced mutations. For example, a single administration of Ni$_3$S$_2$ by nose only inhalation for 2 h at concentrations of 86 and 130 Ni$_3$S$_2$ mg/m$^3$ to Muta$^{TM}$ mice and Big Blue$^®$ rats (estimated doses of 6 and 10 mg/kg) did not cause increased mutations in nasal or lung mucosa despite clear induction of DNA strand breaks (41).

Another possibility is that the *gpt* or Spi$^-$ assays we used to detect Ni$_3$S$_2$ induced mutations were not sensitive enough to detect Ni$_3$S$_2$ generated mutations in the lung. Mutations are fixed into the genome of a cell primarily through DNA replication. Therefore, the population of stem cells in an organ is important for mutational events to occur, associating the sensitivity of an organ to a mutagen with the organ's population of stem cells and their cell division rate (16,58–60). Consequently, the primary target cells of Ni$_3$S$_2$ mutagenicity and carcinogenicity in the lung are epithelial type II cells, the lung stem cells. However, these cells make up only 12% of total alveolar cell population in 5-month-old male rats (61). In the liver, a tissue in which increases in *gpt* and Spi$^-$ MFs were readily detected, over 80% of the mass of the organ is made up of liver stem cell hepatocytes (62). Therefore, the low sensitivity to the Ni$_3$S$_2$ mutagenicity in the lung of transgenic rodents might be due to the low population of stem cells. In addition, the lung has a well developed antioxidant system and this would decrease the mutagenicity of ROS generating genotoxic compounds (63). Dimethylarsinic acid (DMA) induces DNA damage in the lung by formation of peroxyl radical species; however, DMA was ineffective in inducing mutations in the lungs of Muta$^{TM}$ Mice (64). The authors speculate that the mutagenicity of DMA in the lung might be too low to be detected in the Muta$^{TM}$ Mouse. Of course, as noted above, the lack of mutagenicity may have been due to removal of cells harboring DNA damage. On the other hand, when kaolin, manufactured insoluble micro/nanoparticles which induce lung carcinogenesis in rodents, were intratracheally instilled into *gpt* delta mice, they caused 2-fold increases in *gpt* and Spi$^-$ MFs in the lung (13). Other studies also reported the increase in *gpt* MFs in the lung of *gpt* delta mice treated with benzo[a]pyrene or 1,6-dinitropyrene by a single intratracheal instillation and diesel exhaust by inhalation for 24 weeks (65–67). Those suggest that the *gpt* and Spi$^-$ assays using *gpt* delta transgenic animals are sensitive enough to detect an increased MF if it exists.

Briefly, possible reasons why we could not detect mutations in the lungs of F344 *gpt* delta rats exposed to the carcinogen Ni$_3$S$_2$ are (1) Ni$_3$S$_2$ exerts its effects by a non-genotoxic mechanism; (2) the type of mutation induced by Ni$_3$S$_2$ could not be detected by the *gpt* or Spi$^-$ assays; (3) the type of mutation induced by Ni$_3$S$_2$ could be detected by the *gpt* and Spi$^-$ assays, but these assays are not sensitive enough to detect mutations under the experimental condition we used.

Investigation of genotoxic effects in a target organ after exposure to a compound *in vivo* is critical for understanding the mechanism of carcinogenicity and for risk assessment of carcinogens. Consequently, the transgenic rodent mutation assay has become a critically important method of investigating the effects which carcinogens have on the various organs of the body. Validation of an existing system or development of a new system for determining mutagenicity in the lung is critically important to determine the risk of workplace and environmental pollutants and to properly regulate the generation of hazardous materials.

In conclusion, the results of our study indicate that Ni$_3$S$_2$ does not induce the *gpt* or Spi$^-$ mutations in the lung of *gpt* delta rats. Therefore, this assay evaluates Ni$_3$S$_2$ as non-mutagenic in the lung of F344 *gpt* delta transgenic rats. The results obtained by four different laboratories were consistent. If the protocol is effective in measuring the mutations which occur in the target organ, it can be used to investigate the mutagenic potential of test compounds by independent laboratories. Further studies, however, are required to validate this transgenic rodent mutation assays for use in evaluating *in vivo* mutagenicity in the target organ.

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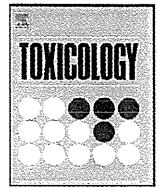
# Comprehensive toxicity study of safrole using a medium-term animal model with *gpt* delta rats

M. Jin[a], A. Kijima[a], Y. Suzuki[a], D. Hibi[a], T. Inoue[a], Y. Ishii[a], T. Nohmi[b], A. Nishikawa[c], K. Ogawa[a], T. Umemura[a,*]

[a] Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Sciences of Genome Safety, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[c] Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

## ARTICLE INFO

## ABSTRACT

In order to investigate a medium-term animal model using reporter gene transgenic rodents in which general toxicity, genotoxicity and carcinogenicity are evaluated, F344 *gpt* delta rats were given a diet containing 0.1% and 0.5% (a carcinogenic dose) safrole for 13 weeks. Serum biochemistry and histopathological examinations revealed overt hepatotoxicity of safrole, in line with previous reports. In the current study, safrole treatment possibly resulted in renal toxicity in male rats. In the *in vivo* mutation assays, an increase or a tendency to increase of the *gpt* mutant frequencies (MFs) was observed in both sexes at the carcinogenic dose. The number and area of foci of glutathione *S*-transferase placental form (GST-P) positive hepatocytes, ratio of proliferating cell nuclear antigen (PCNA)-positive hepatocytes and 8-hydroxydeoxyguanosine (8-OHdG) levels in liver DNA were significantly increased in both sexes of the 0.5% group. The overall data suggested that the present model might be a promising candidate for investigating comprehensive toxicities of the agents. In addition, data demonstrating the base modification and cell proliferation due to exposure to safrole could contribute to understanding safrole-induced hepatocarcinogenesis, which imply expanding in application of this model.

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## 1. Introduction

According to Environmental Health Criteria; 240 (EHC240) by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the safety assessment of food additive should be evaluated based on data such as various genotoxicity, repeated dose toxicity and carcinogenicity tests. However, these studies are time-intensive and require the extensive use of laboratory animals. Thus, the development of a new medium-term animal model in which general toxicity, genotoxicity and carcinogenicity in target organs can be collectively evaluated is desired in terms of the 3R's (Reduction of experimental animals, Refinement of pain of experimental animal and Replacement of animal experiments) of animal testing.

The *gpt* delta rat is one of the reporter gene transgenic rats and carries approximately five tandem copies of the transgene lambda EG10 per haploid genome. It is well recognized that an *in vivo* mutation assay using *gpt* delta rats can detect not only point mutations by 6-TG selection, but also deletion mutations by Spi⁻ selection (Hayashi et al., 2003; Umemura et al., 2009; Masumura et al., 2003). In addition, the glutathione S-transferase placental (GST-P)

positive preneoplastic hepatic foci can be analyzed in the *gpt* delta rats (Toyoda-Hokaiwado et al., 2010). Accordingly, the use of *gpt* delta rats enables us to examine 90-day repeated dose toxicity, *in vivo* genotoxicity and carcinogenicity tests as analysis of preneoplastic changes in a single study.

Safrole (4-allyl-1,2-methylenedioxybenzene) is a natural plant constituent found in the essential oils of sassafras, sweet basil, cinnamon and spices (Furia and Bellanca, 1975; Leung, 1980; Ioannides et al., 1981). Safrole is a hepatocarcinogen in mice and rats fed as 0.5–1% of the diet of mice and rats (Borchert et al., 1973; IARC, 1976; Wislocki et al., 1977). In addition, exposure of safrole resulted in liver toxicity in short-term studies using rats (Hagan et al., 1965). It was reported that safrole-specific DNA adducts were formed following the hepatic cytochrome P450 biotransformation of safrole to 1′-hydroxy-safrole (Daimon et al., 1998) and that high doses of safrole induced oxidative DNA damage in the livers of Sprague Dawley rats (Liu et al., 1999). However, safrole was not mutagenic in *Salmonella typhimurium* TA98 (Dorange et al., 1978; To et al., 1982). In other studies, conventional genotoxicity tests such as the Ames test, sister chromatid exchange (SCE) test and micronucleus test were positive (Natarajan and Darroudi, 1991). Accordingly, it is unclear whether safrole has an *in vivo* genotoxicity, and genotoxic mechanisms are involved in its hepatocarcinogenesis.

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* Corresponding author. Tel.: +81 03 3700 9819; fax: +81 03 3700 1425.
  *E-mail address:* umemura@nihs.go.jp (T. Umemura).

**Table 1**
Final body weight and diet consumptions and test substance intake in F344 *gpt* delta rats given diet containing safrole for 13 weeks.

| Sex | Groups | No. of animals | Final body weight (g) | Diet consumption (g/rat/day) | Daily intake of test substance (mg/kg/day) |
|---|---|---|---|---|---|
| Male | Control | 10 | 369.5 ± 24.5[a] | 18.0 ± 2.0 | 0.0 ± 0.0 |
| | 0.1% safrole | 10 | 341.1 ± 20.0** | 16.2 ± 1.8 | 60.6 ± 13.4 |
| | 0.5% safrole | 10 | 264.4 ± 13.0** | 12.6 ± 2.4 | 274.5 ± 61.9 |
| Female | Control | 9 | 202.1 ± 7.8 | 12.7 ± 1.6 | 0.0 ± 0.0 |
| | 0.1% safrole | 10 | 168.7 ± 10.3** | 10.0 ± 0.9 | 69.1 ± 13.9 |
| | 0.5% safrole | 10 | 150.1 ± 8.6** | 7.4 ± 1.7 | 275.6 ± 77.9 |

[a] Mean ± SD.
** Significantly different from the control group at the levels of $p < 0.01$ (Dunnett's test).

In the present study, to confirm availability of a medium-term animal model using *gpt* delta rats, we examined repeated dose toxicity, *in vivo* genotoxicity and possible carcinogenicity in *gpt* delta rats given safrole at two doses, including a carcinogenic dose, for 13 weeks together with measurements of oxidative DNA damage and cell proliferation in the liver.

## 2. Materials and methods

### 2.1. Chemicals

Safrole and corn oil were purchased from Tokyo Kasei (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Alkaline phosphatase was obtained from Sigma Chemical (St. Louis, MO, USA) and nuclease P1 was from Yamasa Shoyu (Chiba, Japan).

### 2.2. Animals, diet and housing conditions

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan). Five-week-old male and female F344 *gpt* delta rats carrying approximately five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan). They were housed in polycarbonate cages (three or four rats per cage) with hardwood chips for bedding in a conventional animal facility. Animals were maintained under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5 °C), air change (12 times per hour), and lighting (12 h light/dark cycle).

**Table 2**
Hematological data for F344 *gpt* delta rats given diet containing safrole for 13 weeks.

| | Groups | | |
|---|---|---|---|
| | Control | 0.1% safrole | 0.5% safrole |
| **Males** | | | |
| No. of animals examined | 10 | 10 | 10 |
| WBC ($\times 10^2/\mu$l) | 54.1 ± 5.5[a] | 46.1 ± 7.7* | 46.5 ± 5.8* |
| RBC ($\times 10^4/\mu$l) | 976.7 ± 37.3 | 964.1 ± 42.1 | 919.1 ± 33.9** |
| Hb (g/dl) | 15.6 ± 0.2 | 15.6 ± 0.2 | 15.4 ± 0.5 |
| Ht (%) | 51.1 ± 2.1 | 51.1 ± 2.1 | 50.0 ± 1.8 |
| MCV (fl) | 52.2 ± 0.4 | 53.0 ± 0.4** | 54.3 ± 0.3** |
| MCH (pg) | 16.2 ± 0.6 | 16.2 ± 0.6 | 16.7 ± 0.4** |
| MCHC (g/dl) | 30.5 ± 1.0 | 30.5 ± 1.0 | 30.8 ± 0.7 |
| Plt ($\times 10^4/\mu$l) | 72.8 ± 4.2 | 66.1 ± 4.2** | 71.6 ± 5.7 |
| Differential leukocyte counts (%) | | | |
| Band form neutrophils | 1.4 ± 0.9 | 2.3 ± 2.0 | 1.7 ± 0.6 |
| Segmented neutrophils | 36.7 ± 7.8 | 31.9 ± 4.9 | 37.6 ± 6.6 |
| Eosinophils | 1.9 ± 0.9 | 1.1 ± 0.9 | 1.4 ± 1.2 |
| Basophils | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Lymphocytes | 59.5 ± 8.2 | 61.0 ± 13.8 | 59.2 ± 7.0 |
| Monocytes | 0.6 ± 0.4 | 0.3 ± 0.3 | 0.1 ± 0.2** |
| Reticulocytes | 1.8 ± 1.6 | 0.4 ± 0.9 | 0.6 ± 1.3 |
| **Females** | | | |
| No. of animals examined | 9 | 10 | 10 |
| WBC ($\times 10^2/\mu$l) | 34.9 ± 5.6 | 34.6 ± 10.6 | 44.8 ± 8.9* |
| RBC ($\times 10^4/\mu$l) | 923.5 ± 31.9 | 916.5 ± 31.0 | 896.7 ± 29.3 |
| Hb (g/dl) | 15.8 ± 0.6 | 15.5 ± 0.6 | 15.4 ± 0.4 |
| Ht (%) | 51.7 ± 2.0 | 50.9 ± 1.8 | 50.0 ± 1.6 |
| MCV (fl) | 56.0 ± 0.5 | 55.6 ± 0.4* | 55.8 ± 0.4 |
| MCH (pg) | 17.2 ± 0.2 | 17.0 ± 0.2 | 17.2 ± 0.4 |
| MCHC (g/dl) | 30.6 ± 0.5 | 30.5 ± 0.3 | 30.8 ± 0.7 |
| Plt ($\times 10^4/\mu$l) | 74.6 ± 8.1 | 72.4 ± 2.7 | 59.3 ± 3.3** |
| Differential leukocyte counts (%) | | | |
| Band form neutrophils | 1.9 ± 1.1 | 1.0 ± 0.7 | 0.5 ± 0.4* |
| Segmented neutrophils | 28.4 ± 6.0 | 22.2 ± 6.7 | 19.3 ± 47** |
| Eosinophils | 1.6 ± 1.0 | 1.0 ± 0.4 | 0.7 ± 0.5* |
| Basophils | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Lymphocytes | 68.0 ± 7.0 | 75.3 ± 7.0* | 79.2 ± 4.6** |
| Monocytes | 0.2 ± 0.3 | 0.5 ± 0.4 | 0.5 ± 0.3 |
| Reticulocytes | 5.4 ± 3.7 | 2.8 ± 2.8 | 1.0 ± 0.8** |

Abbreviations: WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Pl, platelet.
[a] Mean ± SD.
* Significantly different from the controls at the levels of $p < 0.05$ (Dunnett's test).
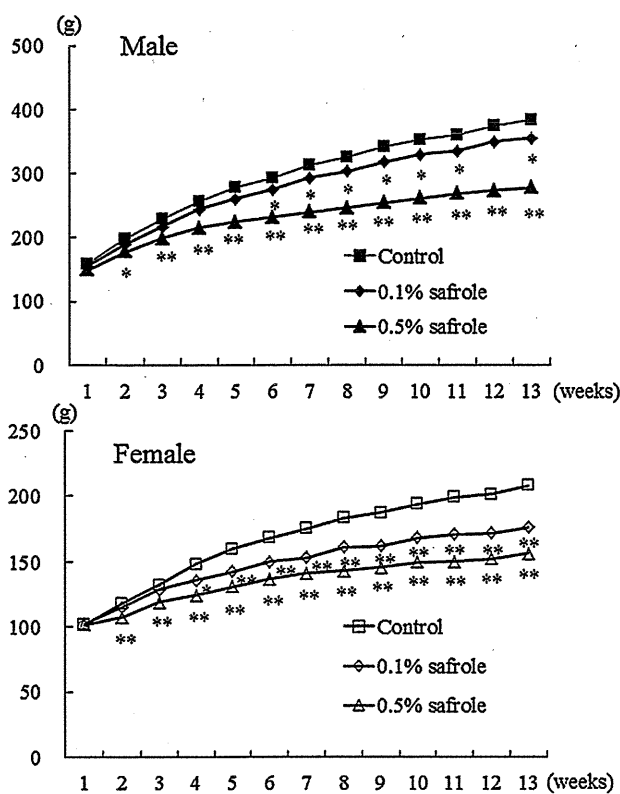** Significantly different from the controls at the levels of $p < 0.01$ (Dunnett's test).

**Fig. 1.** Body weight curves for F344 gpt delta rats given safrole for 13 weeks. *,**: significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.



**Fig. 2.** Daily food intake for F344 gpt delta rats given safrole for 13 weeks. *,**: significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

Animals were given free access to a CRF-1 basal diet (Charles River Japan, Kanagawa, Japan) and tap water.

### 2.3. Experimental design

After a 1-week acclimatization period, animals were divided into 3 groups consisting of 10 male and 10 female F344 gpt delta rats per group, and given a diet containing 0.1%, 0.5% or 0% safrole for 13 weeks.

Clinical signs and general appearance were observed once a day. Body weight and food consumption were measured once a week. At the end of each period, the animals were euthanized under deep anesthesia. Left liver lobes were fixed with neutral-buffered formalin for histopathological and immunohistopathological examination. The remaining liver was stored at −80 °C for 8-OHdG measurements and in vivo mutation assays. At necropsy, blood samples were collected from the abdominal aorta for hematology and serum biochemistry. Relative organ weights were calculated as the values relative to body weights.

Hematological analysis was performed using an automated hematology analyzer, K-4500 (Sysmex Corp., Hyogo, Japan). Differential leukocyte and reticulocyte count were performed with a MICROX HEG-505 (Sysmex Corp.). Parameters for serum biochemistry shown in Table 3 were analyzed at SRL, Inc. (Tokyo, Japan) using sera frozen after centrifugation of whole blood.

At autopsy, weights of brain, heart, lungs, kidneys, spleen, thymus, adrenal glands and testes were measured. In addition to these organs, the artery, bone/marrow, coagulation gland, esophagus, epididymides, large intestine (cecum, colon, and rectum), lymph node, mammary gland, pancreas, peripheral nerve, prostate gland, pituitary gland, thyroid glands, salivary gland, skeletal muscle, skin, small intestine (duodenum, jejunum, and ileum), spinal cord, stomach, urinary bladder, tongue, trachea, vagina, uterus, and ovaries were fixed in 10% neutral buffered formalin. Testes were fixed in Bouin's solution overnight and then transferred into 10% neutral buffered formalin. Tissues that needed decalcification, such as the nasal cavity, spinal cord with bones, sternum, and femur, were treated with a mixture of 10% formic acid and 10% neutral phosphate-buffered formalin. These tissues were routinely embedded in paraffin, sectioned at 3 μm thick for hematoxylin and eosin staining, and examined under light microscopy. Histopathological examinations were carried out for all groups.

### 2.4. In vivo mutation assays

The 6-TG and Spi⁻ (insensitive P2 interference) selection was carried out as previously described (Nohmi et al., 1996, 2000). Briefly, genomic DNA was extracted
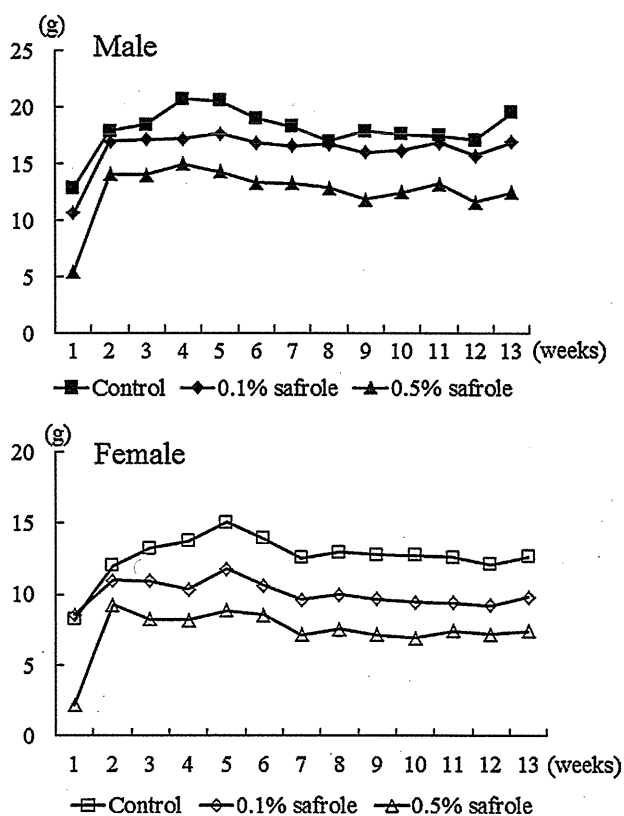
from liver tissue, and lambda EG10 DNA (48 kb) was rescued as the lambda phage through in vitro packaging. For 6-TG selection, the packaged phage was incubated with E. coli YG6020, expressing Cre recombinase, and converted to a plasmid carrying gpt and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, infected cells were also poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37 °C for the selection of 6-TG resistant colonies, and the gpt mutant frequency (MF) was calculated by dividing the number of gpt mutants after clonal correction by the number of rescued phages. To characterize gpt mutations, a 739 bp DNA fragment containing the 456 bp coding region of the gpt gene was amplified by PCR as previously described, and the PCR products were analyzed with Applied Biosystems 3730 × 1 DNA Analyzer (Applied Biosystems Japan Ltd).

For Spi⁻ selection, the packaged phage was incubated with E. coli XL-1 Blue MRA for survival titration and E. coli XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. The next day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipettes and the agar plugs were suspended in SM buffer. In order to confirm the Spi⁻ phenotype of candidates, the suspensions were spotted on three types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and were spread with soft agar. The numbers of mutants that made clear plaques on each plate were counted as confirmed Spi⁻ mutants. The Spi⁻ MF was calculated by dividing the number of Spi⁻ mutants by the number of rescued phages. In all in vivo mutations assays, positive DNA samples were simultaneously applied to ensure the procedures well.

### 2.5. Measurement of nuclear 8-OHdG

In order to prevent 8-OHdG formation as a by-product during DNA isolation (Kasai, 2002), liver DNA was extracted using a slight modification of the method by Nakae et al. (1995). Briefly, nuclear DNA was extracted with a DNA Extractor WB Kit (Wako Pure Chemical Industries) containing an antioxidant NaI solution to dissolve cellular components. For further prevention of auto-oxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical, St. Louis, MO, USA) was added to the lysis buffer. The DNA was digested to deoxynucleotides by treatment with nuclease P1 and alkaline phosphatase and the levels of 8-OHdG (8-OHdG/10⁵ dG) were measured by high-performance liquid chromatography with an electrochemical detection system (Coulochem II; ESA, Bedford, MA, USA).

**Table 3**
Serum biochemistry for F344 *gpt* delta rats given safrole for 13 weeks.

| | Groups | | |
| --- | --- | --- | --- |
| | Control | 0.1% safrole | 0.5% safrole |
| **Males** | | | |
| No. of animals examined | 10 | 10 | 10 |
| TP (g/dl) | 7.0 ± 0.2[a] | 7.0 ± 0.2 | 7.2 ± 0.2 |
| A/G | 2.0 ± 0.1 | 2.2 ± 0.1** | 2.3 ± 0.1** |
| Alb (g/dl) | 4.7 ± 0.1 | 4.8 ± 0.1 | 5.0 ± 0.2** |
| T-Bil (mg/dl) | 0.04 ± 0 | 0.04 ± 0.01 | 0.03 ± 0.01** |
| Glucose (mg/dl) | 159.3 ± 9.2 | 150.9 ± 7.2* | 128.1 ± 3.9** |
| TG (mg/dl) | 132.6 ± 61.8 | 83.3 ± 24.5 | 60.8 ± 23.7** |
| Phospholipid (mg/dl) | 125.1 ± 16.7 | 117.4 ± 9.6 | 147.2 ± 12.5** |
| TC (mg/dl) | 78.1 ± 6.5 | 76.3 ± 6.4 | 105.6 ± 9.8** |
| BUN (mg/dl) | 19.3 ± 1.5 | 20.1 ± 0.7 | 23.6 ± 1.6** |
| CRN (mg/dl) | 0.32 ± 0.01 | 0.34 ± 0.03 | 0.36 ± 0.03** |
| Na (mequiv./l) | 145.9 ± 0.7 | 144.9 ± 0.7* | 145.8 ± 1.1 |
| Cl (mequiv./l) | 106.1 ± 0.9 | 104.5 ± 1.2** | 104.1 ± 1.3** |
| K (mequiv./l) | 4.5 ± 0.2 | 4.4 ± 0.1 | 3.9 ± 0.9* |
| Ca (mg/dl) | 10.8 ± 0.2 | 10.7 ± 0.2 | 11.2 ± 0.2** |
| IP (mg/dl) | 5.7 ± 0.3 | 6.1 ± 0.5 | 6.0 ± 0.4 |
| AST (IU/l) | 98.9 ± 12.1 | 103.1 ± 7.0 | 117.4 ± 12.1** |
| ALT (IU/l) | 54.1 ± 6.1 | 55.9 ± 4.8 | 102.7 ± 16.2** |
| ALP (IU/l) | 497.3 ± 41.2 | 462.9 ± 49.1 | 375.0 ± 30.3** |
| **Females** | | | |
| No. of animals examined | 9 | 10 | 10 |
| TP (g/dl) | 7.2 ± 0.3 | 6.6 ± 0.2** | 6.9 ± 0.2* |
| A/G | 2.7 ± 0.2 | 2.7 ± 0.1 | 2.6 ± 0.1 |
| Alb (g/dl) | 5.2 ± 0.2 | 4.9 ± 0.1** | 5.0 ± 0.1* |
| T-Bil (mg/dl) | 0.06 ± 0.01 | 0.05 ± 0.01* | 0.04 ± 0.01** |
| Glucose (mg/dl) | 130.6 ± 16.2 | 115.7 ± 11.1* | 105.5 ± 10.4** |
| TG (mg/dl) | 27.2 ± 7.5 | 15.3 ± 4.5** | 25.3 ± 3.8 |
| Phospholipid (mg/dl) | 184.9 ± 19.5 | 164.8 ± 10.9* | 247.9 ± 13.8** |
| TC (mg/dl) | 108.2 ± 12.3 | 102.2 ± 6.9 | 186.0 ± 15.0** |
| BUN (mg/dl) | 17.2 ± 2.7 | 17.9 ± 1.3 | 19.0 ± 2.0 |
| CRN (mg/dl) | 0.30 ± 0.03 | 0.31 ± 0.02 | 0.27 ± 0.02** |
| Na (mequiv./l) | 144.1 ± 0.9 | 144.0 ± 1.3 | 143.4 ± 1.3 |
| Cl (mequiv./l) | 106.3 ± 1.9 | 107.0 ± 1.3 | 104.4 ± 1.5* |
| K (mequiv./l) | 4.5 ± 1.7 | 4.0 ± 0.2 | 4.0 ± 0.2 |
| Ca (mg/dl) | 10.6 ± 0.5 | 10.3 ± 0.2 | 10.6 ± 0.1 |
| IP (mg/dl) | 6.7 ± 1.7 | 6.0 ± 0.6* | 5.8 ± 0.3* |
| AST (IU/l) | 82.7 ± 14.2 | 90.5 ± 5.1 | 115.1 ± 21.0** |
| ALT (IU/l) | 42.6 ± 11.7 | 45.6 ± 3.7 | 61.7 ± 12.1** |
| ALP (IU/l) | 343.6 ± 62.9 | 319.6 ± 33.4 | 350.9 ± 32.6 |

Abbreviations: TP, total protein; A/G, albumin/globulin ratio; Alb, albumin; T-Bil, total bilirubin; TG, triglyceride; TC, total cholesterol; BUN, blood urea nitrogen; CRN, creatinine; Na, sodium; Cl, chlorine; K, potassium; Ca, calcium; IP, inorganic phosphate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.
[a] Mean ± SD.
* Significantly different from the controls at the levels of $p < 0.05$ (Dunnett's test).
** Significantly different from the controls at the levels of $p < 0.01$ (Dunnett's test).

### 2.6. Immunohistochemical staining for GST-P and proliferating cell nuclear antigen (PCNA)

Immunohistochemical staining was performed using polyclonal antibodies against GST-P (1:1000 dilution; Medical and Biological Laboratories Co., Ltd., Nagoya, Japan), a marker of preneoplastic lesions in the rat liver, and monoclonal anti-mouse PCNA antibodies (1:100; Dako, Glostrup, Denmark) to evaluate cell proliferation activity using the avidin–biotin peroxidase complex (ABC) method. The numbers (/cm²) and areas (mm²/cm²) of the GST-P-positive foci (>0.1 mm²) and the total areas of each liver section were measured using an IPAP image analyzer (Sumika Technos, Osaka, Japan) (Watanabe et al., 1994). The numbers of PCNA-positive cells per 600–800 intact liver cells from ten different areas per animal were counted to give the PCNA-positive ratio.

### 2.7. Statistics

The data obtained from the measurements of body weight, food and water consumption, organ weights, hematology, serum biochemistry, 8-OHdG levels, GST-P positive foci, PCNA-LI, *gpt* MFs and Spi⁻ MFs were expressed as mean ± SD. The significant differences between the control and treated groups were determined by Dunnett's multiple comparison test (Dunnet, 1955) after ANOVA. The significant differences in incidences of lesions in the histopathological examinations were evaluated using Fisher's exact probability test. *p*-Values of less than 0.05 were considered statistically significant in both analyses.

## 3. Results

### 3.1. General condition, body weight, food consumption

One female from the control group died during the experimental period. However, no changes related to the death were observed in this rat. No remarkable changes in general appearances were observed in the safrole-treated groups during the experimental period. However, there was a marked suppression of body weight gain in the safrole-treated groups after week 2 (Fig. 1). Data for food consumption and safrole intake are summarized in Fig. 2 and Table 1. In both sexes, food consumption was decreased in the group given 0.5% safrole throughout the study, and the mean values for food consumption/animal were significantly lowered compared to the control group.

### 3.2. Hematology and serum biochemistry

The results of hematological measurements are shown in Table 2. White blood cell (WBC) counts and mean corpuscular hemoglobin (MCV) were significantly decreased and increased

**Table 4**
Organ weights in male F344 *gpt* delta rats given safrole for 13 weeks.

| Groups | Control | 0.1% safrole | 0.5% safrole |
|---|---|---|---|
| No. of animal | 10 | 10 | 10 |
| Body weight Absolute (g) | 369.5 ± 24.5[a] | 341.1 ± 20.0** | 264.4 ± 13.0** |
| Liver | 10.01 ± 0.80 | 9.90 ± 0.81 | 9.80 ± 0.86 |
| Lungs | 1.11 ± 0.07 | 1.06 ± 0.04 | 0.90 ± 0.06** |
| Kidneys | 2.21 ± 0.13 | 2.14 ± 0.13 | 2.03 ± 0.12** |
| Brain | 1.94 ± 0.07 | 2.00 ± 0.07 | 2.01 ± 0.35 |
| Spleen | 0.70 ± 0.05 | 0.66 ± 0.05 | 0.60 ± 0.05** |
| Thymus | 0.24 ± 0.02 | 0.21 ± 0.02** | 0.18 ± 0.02** |
| Heart | 0.97 ± 0.06 | 0.88 ± 0.04** | 0.72 ± 0.03** |
| Adrenals | 0.045 ± 0.005 | 0.047 ± 0.004 | 0.048 ± 0.008 |
| Testes | 3.08 ± 0.16 | 3.10 ± 0.30 | 3.08 ± 0.14 |
| Relative (g/100 g B.W.) | | | |
| Liver | 2.71 ± 0.21 | 2.90 ± 0.15* | 3.71 ± 0.37** |
| Lungs | 0.30 ± 0.02 | 0.31 ± 0.01 | 0.34 ± 0.03** |
| Kidneys | 0.60 ± 0.02 | 0.63 ± 0.03 | 0.77 ± 0.06** |
| Brain | 0.53 ± 0.03 | 0.59 ± 0.02 | 0.76 ± 0.11** |
| Spleen | 0.19 ± 0.01 | 0.19 ± 0.01 | 0.23 ± 0.02** |
| Thymus | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.07 ± 0.01 |
| Heart | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.27 ± 0.02 |
| Adrenals | 0.012 ± 0.001 | 0.014 ± 0.001 | 0.017 ± 0.004** |
| Testes | 0.84 ± 0.02 | 0.91 ± 0.06 | 1.17 ± 0.09** |

[a] Mean ± SD.
* Significantly different from the controls at the levels of $p < 0.05$ (Dunnett's test).
** Significantly different from the controls at the levels of $p < 0.01$ (Dunnett's test).

respectively, in the treated groups of males. Conversely, significant increase of WBC counts was observed in the 0.5% group of females. In males, there was a significant decrease in red blood cell (RBC) counts and ratio of monocytes in the 0.5% group. In addition, significant increase of mean corpuscular hemoglobin (MCH) in the 0.5% group and decrease of platelet (Plt) counts in the 0.1% group were observed. In females, Plt counts and proportions of band form neutrophils, segmented neutrophils, eosinophils, reticulocytes showed significant decreases in the 0.5% group and proportions of lymphocytes showed significant increases in the treated group. In addition, a significant decrease of mean corpuscular volume (MCV) was observed in females of the 0.1% group.

Results from serum biochemical analysis are shown in Table 3. There were significant increases of aspartate (AST) and alanine aminotransferase (ALT) in both sexes in the 0.5% group. In males, significant increases of albumin (Alb), albumin/globulin (A/G) ratio,

blood urea nitrogen (BUN), creatinine (CRN) and decreases of Glucose, triglyceride (TG), potassium (K), alkaline phosphatase (ALP) were observed in the 0.1% or 0.5% groups in a dose-dependent manner. In addition, significant increase of BUN, total cholesterol (TC) and calcium (Ca) and decrease of chlorine (Cl) were observed in males of the 0.5% group. In females, total protein (TP), albumin (Alb), total bilirubin (T-Bil), Glucose and inorganic phosphate (IP) were decreased in all treated groups. In addition, significant increase of TC and decrease of CRN and Cl were observed in the 0.5% group.

### 3.3. Organ weights and histopathological examination

Final body weights and the absolute and relative organ weights are shown in Tables 4 and 5. Final body weights were significantly decreased in the safrole-treated groups of both sexes. Absolute liver

**Table 5**
Organ weights in female F344 *gpt* delta rats given safrole for 13 weeks.

| Groups | Control | 0.1% safrole | 0.5% safrole |
|---|---|---|---|
| No. of animal | 9 | 10 | 10 |
| Body Absolute (g) | 202.1 ± 7.8[a] | 168.7 ± 10.3** | 150.1 ± 8.6** |
| Liver | 5.00 ± 0.42 | 4.62 ± 0.27** | 5.65 ± 0.17** |
| Lungs | 0.76 ± 0.03 | 0.73 ± 0.06 | 0.64 ± 0.04** |
| Kidneys | 1.23 ± 0.06 | 1.13 ± 0.07* | 1.09 ± 0.10** |
| Brain | 1.82 ± 0.03 | 1.82 ± 0.08 | 1.76 ± 0.05 |
| Spleen | 0.43 ± 0.02 | 0.41 ± 0.02 | 0.40 ± 0.02** |
| Thymus | 0.19 ± 0.01 | 0.18 ± 0.02 | 0.17 ± 0.02** |
| Heart | 0.60 ± 0.02 | 0.53 ± 0.03** | 0.45 ± 0.02** |
| Adrenals | 0.052 ± 0.004 | 0.052 ± 0.005 | 0.046 ± 0.006** |
| Relative (g/100 g B.W.) | | | |
| Liver | 2.48 ± 0.27 | 2.74 ± 0.10 | 3.77 ± 0.18** |
| Lungs | 0.38 ± 0.03 | 0.44 ± 0.03** | 0.43 ± 0.03** |
| Kidneys | 0.61 ± 0.04 | 0.67 ± 0.02** | 0.73 ± 0.04** |
| Brain | 0.90 ± 0.04 | 1.08 ± 0.04** | 1.17 ± 0.04** |
| Spleen | 0.21 ± 0.01 | 0.25 ± 0.02** | 0.26 ± 0.03** |
| Thymus | 0.10 ± 0.01 | 0.11 ± 0.01 | 0.11 ± 0.02* |
| Heart | 0.30 ± 0.02 | 0.31 ± 0.02 | 0.30 ± 0.01 |
| Adrenals | 0.026 ± 0.003 | 0.031 ± 0.002 | 0.030 ± 0.005 |

[a] Mean ± SD.
* Significantly different from the controls at the levels of $p < 0.05$ (Dunnett's test).
** Significantly different from the controls at the levels of $p < 0.01$ (Dunnett's test).

**Table 6**

Histopathological findings observed in F344 *gpt* delta rats given safrole for 13 weeks.

| Organs | Findings | Sex Groups No. of animals | Male Control 10 | 0.1% safrole 10 | 0.5% safrole 10 | Female Control 9 | 0.1% safrole 10 | 0.5% safrole 10 |
|---|---|---|---|---|---|---|---|---|
| | Survival rate | | 100% | 100% | 100% | 90% | 100% | 100% |
| Liver | | | | | | | | |
| | Centrilobular vacuolar degeneration (large type) | | 1[a] (10[b]) | 6 (60)* | 5 (50) | 0 (0) | 0 (0) | 6 (60)** |
| | Single cell necrosis | | 1 (10) | 9 (90)** | 9 (90)** | 4 (44) | 7 (70) | 6 (60) |
| | Centrilobular hepatocell hypertrophy | | 0 (0) | 10 (100)** | 10 (100)** | 0 (0) | 0 (00) | 10 (100)** |
| | Microgranuloma | | 0 (0) | 4 (40)* | 2 (20) | 0 (0) | 1 (10) | 2 (20) |
| Lung | | | | | | | | |
| | Thrombus formation | | 3 (30) | 1 (10) | 1 (10) | 0 (0) | 0 (0) | 1 (10) |
| | Focal hemorrhage | | 1 (10) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Calcification | | 0 (0) | 1 (10) | 4 (40)* | 0 (0) | 0 (0) | 0 (0) |
| | Foamy cell infiltration | | 0 (0) | 1 (10) | 1 (10) | 1 (11) | 1 (10) | 1 (10) |
| | Inflammatory cell infiltration | | 0 (0) | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 0 (0) |
| | Arteritis | | 0 (0) | 0 (0) | 0 (0) | 1 (11) | 1 (10) | 0 (0) |
| Kidney | | | | | | | | |
| | Tubular hyaline droplets | | 0 (0) | 0 (0) | 10 (100)** | 0 (0) | 0 (0) | 0 (0) |
| | Hyalin cast | | 0 (0) | 2 (20) | 3 (30) | 0 (0) | 0 (0) | 0 (0) |
| | Tubular regeneration | | 1 (10) | 9 (90)** | 10 (100)** | 0 (0) | 0 (0) | 0 (0) |
| | Granular cast | | 0 (0) | 0 (0) | 10 (100)** | 0 (0) | 0 (0) | 0 (0) |
| | Pelvic calcification | | 0 (0) | 0 (0) | 10 (100)** | 0 (0) | 0 (0) | 0 (0) |
| | Interstitial cell infiltration | | 0 (0) | 0 (0) | 6 (60)** | 0 (0) | 0 (0) | 0 (0) |
| Heart | | | | | | | | |
| | Myocardial inflammation | | 9 (90) | 6 (60) | 5 (50) | 2 (22) | 2 (20) | 0 (0) |
| | Focal hemorrhage | | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Tongue | | | | | | | | |
| | Inflammatory cell infiltration | | 1 (10) | 2 (20) | 3 (30) | 0 (0) | 1 (10) | 0 (0) |
| Thyroid gland | | | | | | | | |
| | Lymphoma | | 1 (10) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Parathyroid gland | | | | | | | | |
| | Aberrant craniopharyngeal tissue | | 0 (0) | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 0 (0) |
| Pituitary gland | | | | | | | | |
| | Anterior hyperplasia | | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (10) |
| Stomach | | | | | | | | |
| | Inflammatory cell infiltration | | 1 (10) | 0 (0) | 0 (10) | 0 (0) | 0 (0) | 0 (0) |
| Glandular stomach | | | | | | | | |
| | Inflammatory cell infiltration | | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Papilloma | | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (10) | 0 (0) |
| Pancreas | | | | | | | | |
| | Inflammatory cell infiltration | | 1 (10) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | Acinar atrophy | | 1 (10) | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 0 (0) |
| | Nesidioblastosis | | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (10) | 0 (0) |
| Testis | | | | | | | | |
| | Atrophy | | 0 (0) | 1 (10) | 0 (0) | – | – | – |
| Prostate gland | | | | | | | | |
| | Prostatitis | | 0 (0) | 1 (10) | 0 (0) | – | – | – |
| Uterus | | | | | | | | |
| | Extension | | – | – | – | 1 (11) | 0 (0) | 0 (0) |
| Deferent duct | | | | | | | | |
| | Inflammatory cell infiltration | | 0 (0) | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 0 (0) |
| Bladder | | | | | | | | |
| | Hydrops | | 0 (0) | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 0 (0) |
| Spinal cord cervical | | | | | | | | |
| | Swelling of nerve cells | | 0 (0) | 0 (0) | 0 (0) | 1 (11) | 0 (0) | 0 (0) |

–, not examined.

[a] The number of animals with histopathological lesions.

[b] The incidence (%) of histopathological lesions.

* Significantly different from the controls at the levels of $p < 0.05$ (Dunnett's test).

** Significantly different from the controls at the levels of $p < 0.01$ (Dunnett's test).

weights in the 0.5% group of females and relative liver weights in the treated groups of males and in the 0.5% group of females significantly increased. In males, absolute weights of the lungs, kidneys, spleen, thymus and heart were statistically lower in the 0.5% group compared to the control group. In addition, a significantly decrease was observed in the thymus and heart of the 0.1% group as well. On the contrast, relative weights of the lungs, kidneys, brain, spleen, adrenals and testes were significantly increased in the 0.5% group compared to the control group. In females, significant decrease of the absolute weights of the lungs, spleen, thymus and adrenals were observed at the 0.5% group. But, the relative weights of these organs were significantly increased except for the adrenals. Furthermore,

the absolute weights of the heart and kidneys were significantly decreased and relative weights of the brain and kidneys were significantly increased in the treated groups.

The results of histopathological examinations are shown in Table 6. Histopathologically, the incidence of centrilobular hypertrophy of hepatocytes was significantly increased in males of the treated groups and females in the 0.5% group compared with that in the control group (Fig. 3). Furthermore, in males, the single cell necrosis in the treated groups was significantly increased compared with that in the control group. The significant increase of centrilobular vacuolar degeneration was observed in males in the 0.1% group and in females in the 0.5% group. The incidences
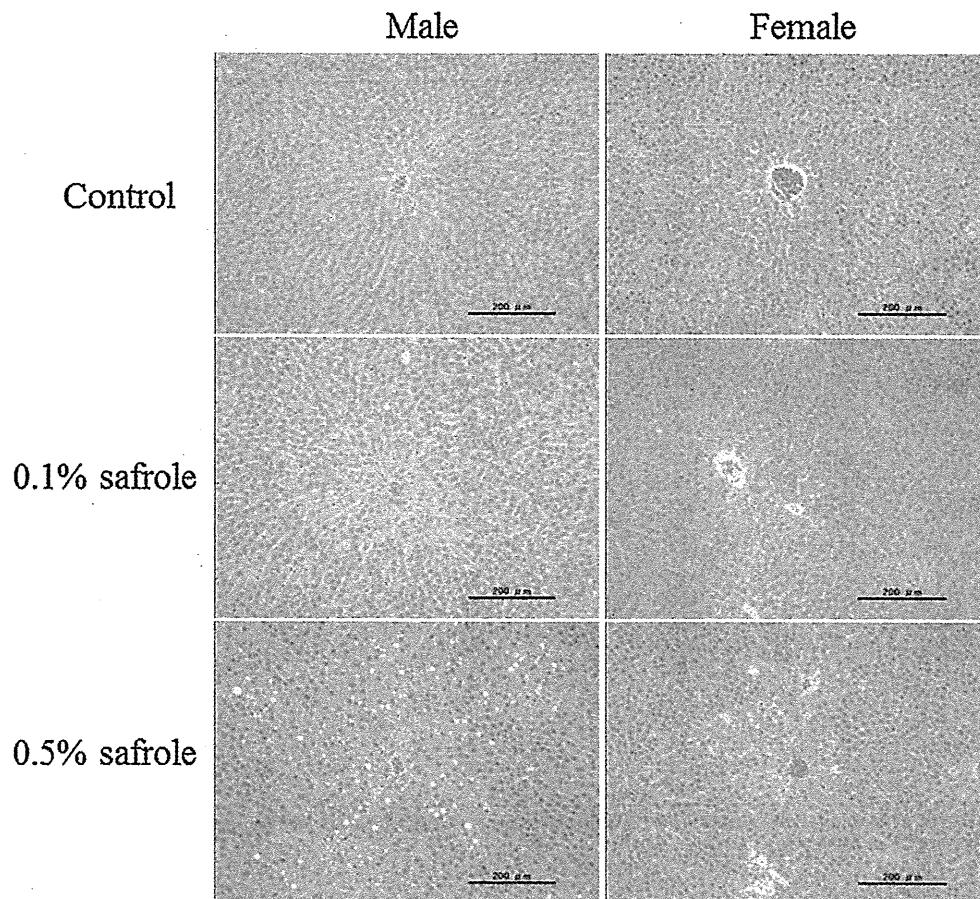
**Fig. 3.** Histopathological features in the livers of F344 *gpt* delta rats given safrole for 13 weeks. Centrilobular hypertrophy and vacuolar degeneration of hepatocytes are evident in the safrole-treated rats. HE stain. Bar represents 200 μm.

of tubular hyaline droplets, granular cast, pelvic calcification and interstitial cell infiltration of the kidney and of calcification of the lung were significantly increased in the males of the 0.5% groups. In addition, tubular regeneration of the kidney was significantly increased in the males of the all treated groups. On the other hand, thrombus formation and foamy cell infiltration of the lung, hyaline cast of the kidney, myocardial inflammation of the heart, and inflammatory cell infiltration of the tongue were observed in the treated rats without significant differences from the control group (10–60%).

### 3.4. In vivo mutation assays in the livers

Data for *gpt* and Spi⁻ MFs in the liver of male and female *gpt* delta rats treated with safrole for 13 weeks are summarized in Tables 7 and 8, respectively. A significant increase of the *gpt* MFs was observed in males of the 0.5% (carcinogenic dose) group. In addition, increased *gpt* MFs were observed in females of the 0.5% group, although the increase was not statistically significant. There were no significant differences in the Spi⁻ MFs among the groups in either sex. In the *gpt* mutant spectra, the predominant type of AT:GC transition was significantly induced by safrole (Table 9).

### 3.5. Oxidative DNA damage in the liver

In order to evaluate whether the oxidative damages to the cellular components occur during the formation of preneoplastic foci, the 8-OHdG levels were measured in liver DNA. The 8-OHdG levels in liver DNA were significantly increased in both sexes of the safrole-treated groups in a dose-dependent manner compared to those of the control groups (Table 10).

### 3.6. Effects of safrole treatment on GST-P positive foci and cell proliferation

Safrole treatment increased both the number and the area of GST-P positive foci in a dose-dependent manner compared with the control groups, although the differences were not statistically significant in males in the 0.1% group (Table 10). In addition, the effect of safrole on cell proliferation was evaluated by immunohistochemistry for PCNA (Table 10). The PCNA-positive ratio of hepatocytes was significantly increased in males of the treated groups and in females of the 0.5% group.

### 4. Discussion

A marked suppression of body weight gain was observed in the safrole-treated groups from week 2 to the end of the experiment. In serum biochemical examinations, there were significant increases of AST and ALT in both sexes of the 0.5% group. BUN and CRN levels significantly increased in males of the 0.5% group. In histopathological examinations, the incidences of centrilobular hypertrophy, centrilobular vacuolar degeneration and single cell necrosis of hepatocytes were significantly increased in males of the treated groups and in females of the 0.5% group. Furthermore, the incidences of tubular hyaline droplets, tubular regeneration, granular cast, pelvic

**Table 7**

$gpt$ MFs in livers of F344 $gpt$ delta rats given safrole for 13 weeks.

| Sex | Groups | Animal no. | $Cm^R$ colonies $(\times 10^5)$ | 6-$TG^R$ and $Cm^R$ colonies | Mutant frequency $(\times 10^{-5})$ | Mean ± S.D. |
|---|---|---|---|---|---|---|
| Male | Control | 1 | 5.3 | 0 | 0.00 | |
| | | 2 | 5.0 | 2 | 0.40 | |
| | | 3 | 8.6 | 4 | 0.47 | |
| | | 4 | 10.4 | 2 | 0.19 | |
| | | 5 | 1.9 | 3 | 1.59[a] | 0.26 ± 0.21 |
| | 0.1% safrole | 11 | 6.3 | 4 | 0.63 | |
| | | 12 | 4.5 | 8 | 1.76 | |
| | | 13 | 5.3 | 5 | 0.95 | |
| | | 14 | 4.7 | 1 | 0.21 | |
| | | 15 | 7.2 | 2 | 0.28 | 0.77 ± 0.63 |
| | 0.5% safrole | 21 | 3.4 | 7 | 2.05 | |
| | | 22 | 2.6 | 7 | 2.68 | |
| | | 23 | 4.0 | 4 | 1.10 | |
| | | 24 | 4.8 | 7 | 1.45 | |
| | | 25 | 4.4 | 10 | 2.29 | 1.89 ± 0.67[**] |
| Female | Control | 36 | 6.9 | 7 | 1.01 | |
| | | 37 | 4.9 | 3 | 0.62 | |
| | | 38 | 5.1 | 0 | 0.00 | |
| | | 39 | 7.7 | 7 | 0.90 | |
| | | 40 | 5.6 | 4 | 0.71 | 0.65 ± 0.39 |
| | 0.1% safrole | 46 | 6.2 | 9 | 1.46 | |
| | | 47 | 7.6 | 10 | 1.32 | |
| | | 48 | 12.8 | 10 | 0.78 | |
| | | 49 | 5.3 | 3 | 0.57 | |
| | | 50 | 7.9 | 6 | 0.76 | 0.98 ± 0.39 |
| | 0.5% safrole | 56 | 2.8 | 3 | 1.06 | |
| | | 57 | 3.3 | 3 | 0.91 | |
| | | 58 | 3.6 | 2 | 0.56 | |
| | | 59 | 3.2 | 10 | 3.09 | |
| | | 60 | 3.0 | 2 | 0.66 | 1.26 ± 1.04 |

[a] Data of animal no. 5 was excluded for the calculation of the MF because of the poor packaging efficiency of the transgene (Smirnov–Grubbs test $T$=1.71; $p$<0.05).
[**] Significantly different from the control group at $p$<0.01.

**Table 8**

Spi⁻ MFs in livers of F344 $gpt$ delta rats given safrole for 13 weeks.

| Sex | Groups | Animal no. | Plaques within XL-1 Blue MRA $(\times 10^5)$ | Plaques within XL-1 Blue MRA (P2) | Mutant frequency $(\times 10^{-5})$ | Mean ± S.D. |
|---|---|---|---|---|---|---|
| Male | Control | 1 | 9.5 | 9 | 0.94[a] | |
| | | 2 | 10.9 | 2 | 0.18 | |
| | | 3 | 10.0 | 3 | 0.30 | |
| | | 4 | 9.2 | 2 | 0.22 | |
| | | 5 | 9.7 | 2 | 0.21 | 0.23 ± 0.51 |
| | 0.1% safrole | 11 | 5.3 | 4 | 0.19 | |
| | | 12 | 6.9 | 0 | 0.00 | |
| | | 13 | 6.9 | 2 | 0.29 | |
| | | 14 | 8.1 | 4 | 0.49 | |
| | | 15 | 12.6 | 8 | 0.64 | 0.32 ± 0.25 |
| | 0.5% safrole | 21 | 4.9 | 1 | 0.21 | |
| | | 22 | 5.7 | 5 | 0.88 | |
| | | 23 | 7.9 | 3 | 0.38 | |
| | | 24 | 6.8 | 1 | 0.15 | |
| | | 25 | 6.0 | 2 | 0.33 | 0.39 ± 0.29 |
| Female | Control | 36 | 7.1 | 1 | 0.14 | |
| | | 37 | 6.7 | 3 | 0.45 | |
| | | 38 | 9.8 | 1 | 0.10 | |
| | | 39 | 8.3 | 4 | 0.48 | |
| | | 40 | 11.5 | 7 | 0.61 | 0.36 ± 0.22 |
| | 0.1% safrole | 46 | 6.9 | 0 | 0.00 | |
| | | 47 | 7.4 | 1 | 0.14 | |
| | | 48 | 11.4 | 5 | 0.44 | |
| | | 49 | 7.1 | 1 | 0.14 | |
| | | 50 | 9.5 | 3 | 0.31 | 0.21 ± 0.17 |
| | 0.5% safrole | 56 | 2.5 | 2 | 0.79 | |
| | | 57 | 5.3 | 0 | 0.00 | |
| | | 58 | 6.0 | 1 | 0.17 | |
| | | 59 | 6.0 | 3 | 0.50 | |
| | | 60 | 8.7 | 0 | 0.00 | 0.29 ± 0.35 |

[a] Data of animal no. 1 was excluded for the calculation of the MF because of the poor packaging efficiency of the transgene (Smirnov–Grubbs test $T$=1.77; $p$<0.05).