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

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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.

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) from 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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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 (×10²/μl)	54.1 ± 5.5[a]	46.1 ± 7.7*	46.5 ± 5.8*
RBC (×10⁴/μl)	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 (×10⁴/μl)	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 (×10²/μl)	34.9 ± 5.6	34.6 ± 10.6	44.8 ± 8.9*
RBC (×10⁴/μl)	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 (×10⁴/μl)	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; PI, 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).

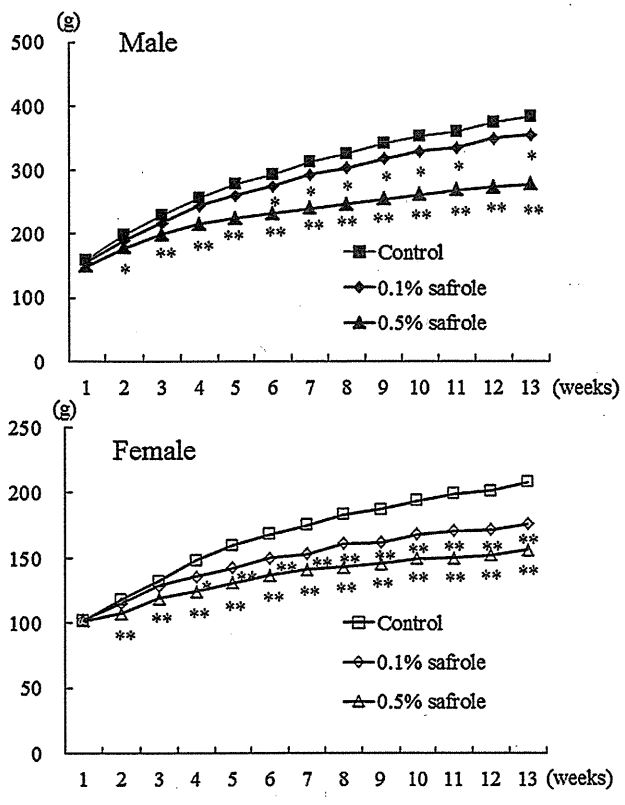

Fig. 1. Body weight curves for F344 *gpt* delta rats given safrole for 13 weeks. *,**: significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

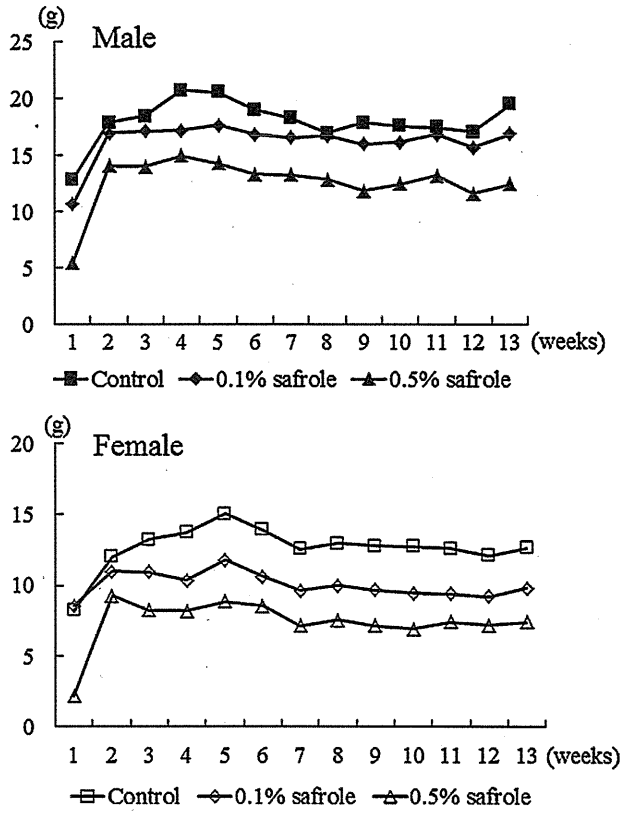

Fig. 2. Daily food intake for F344 *gpt* delta rats given safrole for 13 weeks. *,**: significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

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, liver, 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/10^5 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 (/cm^2) and areas (mm^2/cm^2) of the GST-P-positive foci (>0.1 mm^2) 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 No. of animal	Control 10	0.1% safrole 10	0.5% safrole 10
Body weight	369.5 ± 24.5[a]	341.1 ± 20.0**	264.4 ± 13.0**
Absolute (g)			
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 No. of animal	Control 9	0.1% safrole 10	0.5% safrole 10
Body	202.1 ± 7.8[a]	168.7 ± 10.3**	150.1 ± 8.6**
Absolute (g)			
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	Male			Female		
		Groups	Control	0.1% safrole	0.5% safrole	Control	0.1% safrole	0.5% safrole
		No. of animals	10	10	10	9	10	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)**
	Micrograniuloma		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

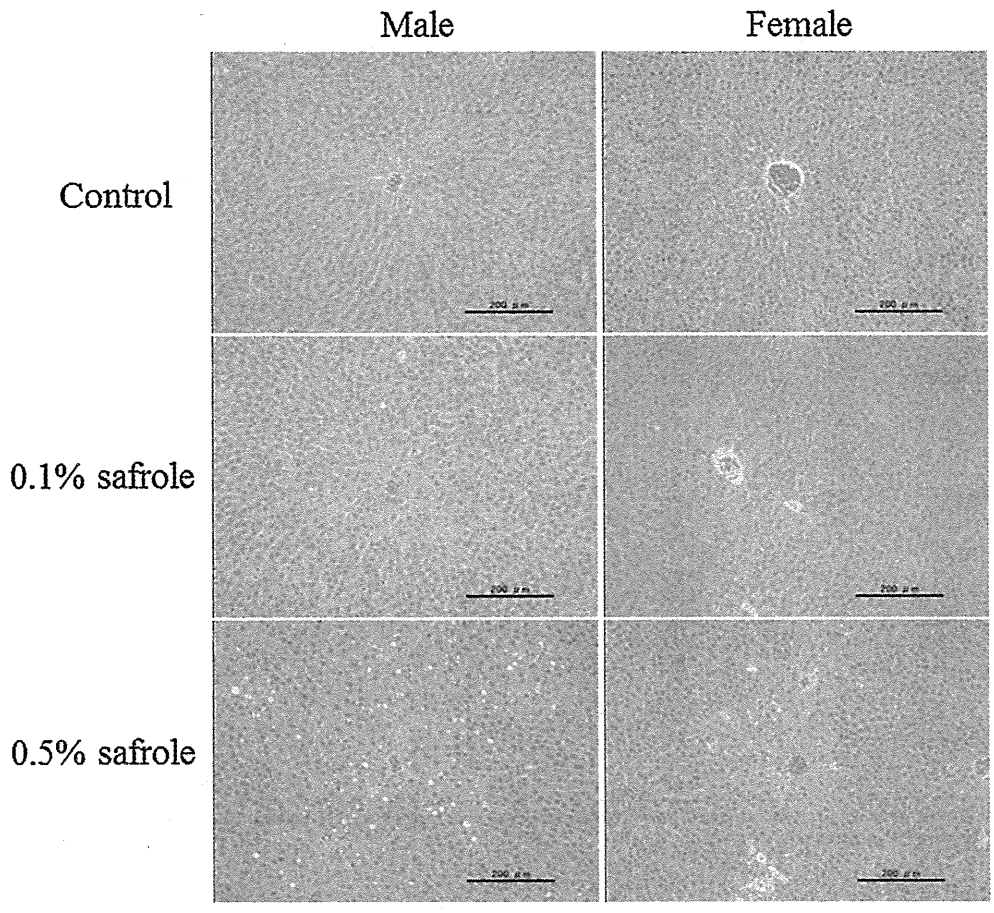

Fig. 3. Histopathological features in the livers of F344 *gpt* delta rats given safrole for 13 weeks. Centrilobular hypertrophy and vacuolar degeneration of hepatocytes are evident in the safrole-treated rats. HE stain. Bar represents 200 μm.

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 $T = 1.77$; $p < 0.05$).

Table 9
Mutation spectra of *gpt* mutant colonies in the livers of F344 *gpt* delta rats given safrole for 13 weeks.

Sex	Base substitution	Control		0.1% safrole		0.5% safrole	
		Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})
Male	Transversions						
	GC-TA	2[a] (25.0)	0.06 ± 0.12	4(20.0)	0.15 ± 0.21	6(17.1)	0.30 ± 0.30
	GC-CG	1(12.5)	0.05 ± 0.09	2(10.0)	0.09 ± 0.20	4(11.4)	0.26 ± 0.31
	AT-TA.	0	0	2(10.0)	0.07 ± 0.10	5(14.3)	0.29 ± 0.33
	AT-CG	0	0	0	0	1(2.9)	0.08 ± 0.17
	Transitions						
	GC-AT	4(50.0)	0.11 ± 0.12	8(40.0)	0.30 ± 0.25	5(14.3)	0.19 ± 0.18
	AT-GC	0	0	3(15.0)	0.12 ± 0.17	10(28.6)	0.54 ± 0.40**
	Deletion						
	Single bp	1(12.5)	0.05 ± 0.10	1(5.0)	0.04 ± 0.10	4(11.4)	0.19 ± 0.21
	Over 2 bp	0	0	0	0	0	0
	Insertion	0	0	0	0	0	0
	Complex	0	0	0	0	0	0
	Total	8	0.26 ± 0.21	20	0.77 ± 0.63	35	1.89 ± 0.67
Female	Transversions						
	GC-TA	4(19.0)	0.11 ± 0.19	10(26.3)	0.27 ± 0.17	5(25.0)	0.33 ± 0.45
	GC-CG	3(14.3)	0.08 ± 0.12	2(5.7)	0.04 ± 0.06	1(5.0)	0.06 ± 0.13
	AT-TA	1(4.8)	0.04 ± 0.09	6(15.8)	0.18 ± 0.19	2(10.0)	0.12 ± 0.28
	AT-CG	1(4.8)	0.04 ± 0.08	3(7.9)	0.08 ± 0.14	0	0
	Transitions						
	GC-AT	12(57.1)	0.38 ± 0.22	8(21.1)	0.18 ± 0.15	6(15.0)	0.37 ± 0.34
	AT-GC	0	0	8(21.1)	0.21 ± 0.05	5(25.0)	0.31 ± 0.31
	Deletion						
	Single bp	0	0	1(2.6)	0.03 ± 0.06	1(5.0)	0.07 ± 0.15
	Over 2 bp	0	0	0	0	0	0
	Insertion	0	0	0	0	0	0
	Complex	0	0	0	0	0	0
	Total	21	0.65 ± 0.39	38	0.98 ± 0.39	20	1.26 ± 1.04

[a] Number of colonies with independent mutations.
** Significantly different from the control group at $p < 0.01$.

calcification and interstitial cell infiltration in the kidney were significantly increased in males of the treated groups. The overall data indicated that safrole is a nephrotoxicant as well as a hepatotoxicant. In previous studies, the suppression of body weight gain and liver enlargement were also observed in safrole-treated rats (Homburger et al., 1962; Hagan et al., 1965). These results show that the *gpt* delta rat has a similar sensitivity to safrole in comparison to non-transgenic wild rats. This implies that the *gpt* delta rat model can be used to investigate general toxicities of agents.

Safrole forms safrole-specific DNA adducts through hepatic cytochrome P450 biotransformation and subsequent conjugation by sulfotransferase (Miller and Miller, 1983). Alternatively, safrole can be biotransformed though the methylenedioxy ring-opening to hydroxychavicol. Hydroxychavicol could be biotransformed to o-quinone through 2-electron oxidation, and this redox-active quinone is considered to induce oxidative damages (Klungsoyr and Scheline, 1983; O'Brien, 1991). In fact, the levels of 8-OHdG were significantly increased in both sexes of the safrole-treated groups as compared to those of the control group. To the best of our knowledge, there are no reports demonstrating the significant increase of 8-OHdG levels in livers of rats treated with a low dose (half of a carcinogenic dose) for 13 weeks. However, the genotoxicity of safrole remained unknown in conventional genotoxicity tests such as the Ames test, sister chromatid exchanges (SCE) test and micronucleus test in spite of its hepatocarcinogenicity being clear (Green and Savage, 1978; Swanson et al., 1979; Baker and Bonin, 1985; Bradley, 1985; Gocke et al., 1981). The present study demonstrated that an increase or increasing tendency of the *gpt* MFs was observed in both sexes in the 0.5% group, a carcinogenic dose, despite the Spi^- MFs being unchanged. These results suggested that safrole has a potential to be genotoxic *in vivo* in the livers of rats. In the mutation spectra, the AT:GC transitions were significantly induced by safrole in males of the 0.5% group. If has been reported that 8-OHdG is capable to form a base pair with adenine and subsequently produce a GC:AT transversion mutation. In addition, recent studies suggest that 8-OHdG can cause large deletion mutations associated with double strand break during base excision repair by *OGG1* (Umemura

Table 10
8-OHdG, PCNA and GST-P levels in the livers of F344 *gpt* delta rats given safrole for 13 weeks.

Sex	Treatment	Control	0.1% safrole	0.5% safrole
Male	8-OHdG	0.27 ± 0.02[a]	0.35 ± 0.05*	0.52 ± 0.06**
	PCNA-positive ratio	0.27 ± 0.12	0.54 ± 0.17*	0.51 ± 0.14*
	GST-P (number/cm^2)	0.00 ± 0.00	0.88 ± 0.55	9.42 ± 3.51**
	GST-P (mm^2/cm^2)	0.00 ± 0.00	0.005 ± 0.004	0.134 ± 0.070**
Female	8-OHdG	0.36 ± 0.04	0.51 ± 0.06**	0.62 ± 0.06**
	PCNA-positive ratio	0.20 ± 0.07	0.66 ± 0.09	1.06 ± 0.55**
	GST-P (number/cm^2)	0.00 ± 0.00	0.44 ± 0.46*	2.78 ± 1.22**
	GST-P (mm^2/cm^2)	0.00 ± 0.00	0.004 ± 0.005	0.026 ± 0.016**

[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).

et al., 2007). Thus, in the light of the type of mutations induced by safrole, it is unlikely that 8-OHdG formation contributes to safrole-induced genotoxicity, although there is a possibility of any other oxidized DNA damages being involved. The present data showing a significant increase in PCNA-positive hepatocytes might suggest the possible participation of oxidative stress in cell proliferation.

It is well known that the results of bioassay using GST-P-positive foci show good correlation with those of the 2-year cancer bioassay (Ito et al., 2000; Ogiso et al., 1985). Therefore, it has been widely accepted that the analysis of GST-P positive foci may be a useful indicator to predict carcinogenicity of agents. In the present study, the number and the area of GST-P positive foci were significantly increased in both sexes in the 0.5% group. The data on quantitative analysis for GST-P foci using *gpt* delta rats are also in agreement with the carcinogenicity data previously reported by Long et al. (1963).

In conclusion, the present medium-term animal model using F344 *gpt* delta rats confirmed previous reports of the hepatotoxicity and hepatocarcinogenicity of safrole. The genotoxicity of safrole, which remained unknown, so far, was clearly demonstrated in the target organ by this *in vivo* model. Thus, this animal model might be a promising tool for investigating comprehensive toxicities of agents. The acquisition of additional data on key events in chemical carcinogenesis, such as base modification and cell proliferation, could assist in understanding the modes of action. Applications of this model should be further expanded in future studies.

Conflict of interest statement

None.

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Regular article

Modulatory Effects of Capsaicin on N-diethylnitrosamine (DEN)-induced Mutagenesis in Salmonella typhimurium YG7108 and DEN-induced Hepatocarcinogenesis in gpt Delta Transgenic Rats

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Capsaicin from the red chili pepper is a prospective chemopreventive agent. To explore the possible antigenotoxic effects of capsaicin on N-diethylnitrosamine (DEN)-induced mutagenesis in vitro, we conducted bacterial mutation assays with Salmonella typhimurium YG7108, a sensitive strain to mutagenic alkylating agents. Capsaicin was not mutagenic either with or without S9 activation. Unexpectedly,it enhanced the mutagenicity of DEN in the presence of S9 activation significantly. Capsaicin also enhanced the mutagenicity of 2-aminoanthracene and benzo[a]pyrene in the presence of S9 activation and benzo[a]pyrene diolepoxide in the absence of S9 activation. However, it reduced the mutagenicity of ethylnitrosourea in the absence of S9 activation. To examine whether capsaicin modulates DEN-induced mutagenesis and hepatocarcinogenesis in vivo, we took advantage of gpt delta rats, transgenic rodents that carry reporter genes for mutations. Female gpt delta rats were given drinking water containing 40 ppm DEN for five weeks. They were fed diets containing capsaicin at doses of 0, 100 or 500 ppm for seven weeks, starting one week before the DEN treatment. Samples were collected at weeks 7 and 32, respectively, for mutagenicity and carcinogenicity assays. DEN enhanced gpt mutant frequency more than 200 fold in the liver. However, capsaicin displayed no modulating effects on the mutagenesis. Rather, it reduced the number of liver neoplasms, especially liver cell adenomas, in a dose-dependent manner although the reduction in hepatocellular carcinoma was statistically insignificant. These results suggest that chemopreventive effect of capsaicin against DEN-induced hepatocarcinogenesis is slight and that the effect is not due to antimutagenesis. The results also caution that chemopreventive effects of chemicals should be examined not only in vitro but also in vivo with multiple indexes, e.g., in vitro and in vivo mutations and pathological examinations.

Key words: capsaicin, chemoprevention, N-diethylnitrosamine, hepatocarcinogenicity, gpt delta transgenic rats

Introduction

Capsaicin is the principal pungent constituent of hot red chili peppers, which are the most frequently consumed spices in the world (1). In addition to a spicy dietary ingredient, capsaicine is known to exhibit various biological activities, such as inhibition of CYP-depenent xenobiotic metabolism, inhibition of cellular signal transduction and induction of apoptosis (1–6). Thus, it is expected that capsaicin can be chemopreventive against tumors via antigenotoxic mechanisms. In fact, it is reproted that dietary exposure to capsaicin suppresses azoxymethane-induced colon tumors in rats (7). Capsaicin also inhibits DNA binding of aflatoxin B1 in the presence of *in vitro* metabolic activation by S9 enzymes (8). Several reports suggest, however, that capsaicin itself is mutagenic in Ames bacterial mutation assays (9–11), V79 mammalian gene mutation assays (12) and micronucleus assays *in vivo* (13), and one report suspects the carcinogenicity (14). It remains elusive, there-

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fore, whether capsaicin is chempreventive and if so whether antigenotoxic mechanisms are involved in the chemopreventive action.

To examine the possible chemopreventive effects of capsaicin against *N*-diethylnitrosamine (DEN), we conducted *in vitro* and i*n vivo* mutation assays. We chose DEN as a target carcinogen because it induces hepatocarcinoma effectively in a genotoxic manner (15). In addition, liver cancer is one of the most prevalent cancer diseases world wide (16). We employed *Salmonella typhimurium* YG7108 (17,18), a sensitive strain to mutagenicity of various alkylating agents, for *in vitro* mutation assays and Fischer 344 *gpt* delta transgenic rats for *in vivo* assays (19). The strain *S. typhimurium* YG7108 is sensitive to mutagenicity of alkylating agents because it lacks DNA repair enzymes of O^6-alkylguanine alkyltransferases encoded by the ada_{ST} and ogt_{ST} genes (17,18). *gpt* delta transgenic rats carry reporter genes for *in vivo* mutations (20). Point mutations and deletions can be identified in any organs or tissues of F344 rats and the mutations are analyzable at the sequence level (19). Chemopreventive effects of capsaicin against tumor induction was histopathologically evaluated in the liver of DEN-treated F344 *gpt* delta rats. Glutathione *S*-transferase placenta form (GST-P) positive foci are frequently used as an indicator of pre-neoplastic lesions of liver of rats because this bioassay shows good correlations with long-term carcinogenicity results (21). The results suggest that capcaisin suppresses DEN-induced heptatocarcinogenesis slightly. However, the chemopreventive effect is not due to antigenotoxic mechanims because capsaicin displayed no antimutgenic activity against DEN-induced mutations *in vivo*. Capsaicin is not mutagenic *in vitro* and *in vivo*. Because of the complex properties, chemopreventive effects of capsaicin should be further evaluated via multiple indexes such as mutations and proliferating lesions (preneoplasms and neoplasms) induced by other genotoxic carcinogens.

Materials and Methods

Materials: Capsaicin (synthetic, *N*-vanillylnonanamide, CAS: 2444-46-4) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The purity was >96%. DEN (CAS No.: 55-18-5) and ethylnitrosourea (ENU; CAS: 759-73-9) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). 2-Aminoanthracen (2-AA; CAS No.: 103404-81-5), benzo[*a*]pyrene (BP; CAS No.: 50-32-8), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1; CAS No.: 67730-11-4), and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Benzo[*a*]pyrene-dihydrodiol epoxide (BPDE; CAS No.: 60268-85-1) was purchased from Midwest Research Institute Global (Missouri, MO, USA). S9 prepared from male Sprague-Dawley rats pretreated with phenobarbital and 5,6-benzoflavone was purchased from Kikkoman Cooperation, Chiba, Japan.

Bacterial reverse mutation test (Ames test): Modulating effects of capsaicin against DEN-induced mutagenesis were assayed in a bacterial reverse mutation assay using *S. typhimurium* tester strains YG7108 (17,18), as TA1535 but is $\Delta ada_{ST}\Delta ogt_{ST}$, in the presence of S9 enzymes. The test was conducted by the pre-incubation method with modifications (22). Briefly, capsaicin dissolved in DMSO was mixed with S9 mix for 5 min on ice. Then, DEN dissolved in distilled water was added, followed by addition of overnight culture of *S. typhimurium* YG7108. The mixture was incubated for 20 min at 37°C and poured onto agar plates with soft agar. The plates were incubated for 2 days at 37°C. Assays were performed on triplicate. When the antimutagenic effects of capsaicin on other chemicals were examined, the test conditions, i.e., *S. typhimurium* strains and requirements for S9 mix, were as follows: 2-AA and Glu-P-1, TA98, +S9 mix; BP, TA100, +S9 mix; BPDE, TA100, −S9 mix; ENU, YG7108, −S9 mix.

Animals, diet and housing conditions: Female six-week-old F344 *gpt* delta transgenic rats (19) were obtained from Japan SLC and housed three or four animals per polycarbonate cage under specific pathogen-free standard laboratory conditions: room temperature, 23±2°C; relative humidity, 60±5%; with a 12:12-h light-dark cycle and free access to CRF-1 basal diet (Oriental Yeast Company, Tokyo, Japan) and tap water.

Treatments of animals: The protocol for this study was approved by the Animal Care and Utilization Committee of Kanazawa Medical University. Fifty-four rats were randomly divided into five groups (Fig. 1). Groups

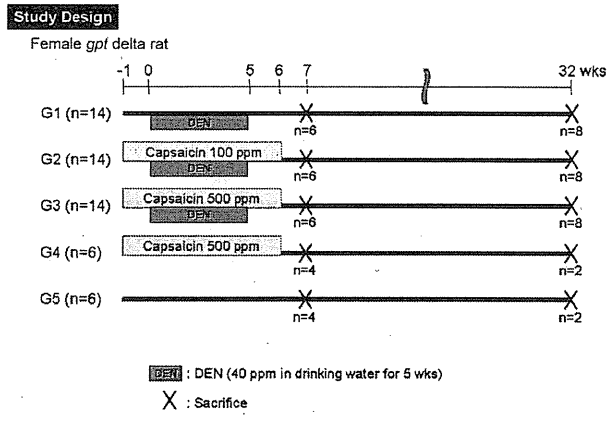

Fig. 1. Experimental protocol to examine *in vivo* modulating effects of capsaicin against DEN-induced mutagenesis and hepatocarcinogenesis using F344 *gpt* delta rats.

1 through 3 were treated with 40 ppm DEN in drinking water for five weeks. Group 2 was fed diets containing 100 ppm capsaicin, and Groups 3 and 4 were fed diets containing 500 ppm capsaicin for seven weeks, starting one week before DEN administration. Group 5 served as an untreated control. All rats were carefully observed for clinical welfare and weighed weekly, and experimental diet consumption was recorded. The experiment was terminated at 32 weeks after the start of DEN administration. During the study, animals were killed at week 7 to determine the effects of capsaicin on the mutation frequency. At autopsy, liver, kidneys and spleen were macroscopically examined for the presence of pathologic lesions, and then isolated. Tissues were fixed in 10% buffered formalin and processed to hematoxylin and eosin (HE) stained sections. Neoplastic lesions of liver were histopathologically classified into adenomas and hepatocellular carcinomas (HCC). Left lobes of the livers from rats sacrificed at week 7 were excised and frozen in liquid nitrogen for mutation assay. Then, remaining livers were fixed in 10% buffered formalin, embedded in paraffin, sectioned, stained by HE and histopathologically examined.

DNA isolation, in vitro packaging and gpt mutation assay: High-molecular-weight genomic DNA was extracted from the liver using the RecoverEase DNA Isolation Kit (Stratagene by Agilent Technologies, Santa Clara, CA, USA). λ EG10 phages were rescued using Transpack Packaging Extract (Stratagene). The *gpt* assay was conducted according to previously published methods (23). The mutant frequencies of the *gpt* gene (*gpt* MFs) in the liver were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of rescued plasmids.

Immunohistochemical procedures: Liver sections of 3 μm thickness from short period groups were treated with rabbit anti-rat GST-P antibody (1:1,000; Medical & Biological Laboratories, Nagoya, Japan). Immunohistochemical staining was done by the avidin-biotin complex method (ABC) using the Vectastain Elite ABC system (Vector Laboratories, Inc., Burlingame, CA, USA). Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody at a dilution of 1:200. Sections were lightly counterstained with hematoxylin for microscopic examination. Areas and numbers of GST-P-positive foci larger than 0.1 mm in diameter of the liver sections were quantitatively measured with an image processor for analytical pathology (IPAP-WIN; Sumika Technos Company, Osaka, Japan).

Statistical analysis: The statistical significance of the difference in the value of MFs between the treated groups and negative controls was analyzed by Student's *t*-test. A p value less than 0.05 denoted the presence of a statistically significant difference. Variances in values for body weight, organ weight and pathological data were examined by Dunnett and Tukey multiple comparison post tests using GraghPad InStat (GraphPad Software, Inc., La Jolla, CA, USA) to compare the differences. The tumor incidence was examined by Yates $m \times n$ χ^2 test.

Results

Capsaicin enhanced mutagenicity of DEN in S. typhimurium YG7108: To examine modulating effects of capsaicin on DEN-induced mutagenicity, bacterial mutation assays with *S. typhimurium* YG7108 were performed (Fig. 2). Capsaicin itself was not mutagenic with or without S9 activation in *S. typhimurium* TA98, TA100 and YG7108. DEN itself at a dose of 200 μg/plate induced about 500 His^+ revertants per plate in the presence of S9 activation. When capsaicin was added in the reaction mixture, it enhanced the mutagenicity of DEN in a dose-dependent manner, and the number of His^+ revertants per plate reached about 4,000 at a dose of 50 μg capsaicin/plate. We also examined the modulating effects with 2-AA, BP and Glu-P-1 in the presence of S9 activation, and with ENU and BPDE in the absence of S9 activation. We used *S. typhimurium* strains TA98 or TA100 when the test chemicals were not alkylating agents. Capsaicin substantially enhanced mutations induced by 2-AA and BP in a dose-dependent manner as in the case of DEN. It enhanced mutations induced by BPDE dose-dependently at doses less than 100 μg of capsaicin per plate. Capsaicin slightly enhanced the mutagenicity of Glu-P-1 at lower doses and then reduced it at higher doses. Capsaicin reduced the mutagenicity of ENU.

General observation of in vivo study: To reveal modulating effects of capsaicin *in vivo*, genotoxicity assay and carcinogenesis study were conducted with *gpt* delta rats. Consumptions of capsaicin-mixed diets were 25% lower than those of normal diet at the first experimental week (Groups 2 to 4, data not shown). It recovered, however, to the level similar to the control group at the second experimental week. During drinking administration of 40 ppm DEN, growth of body weight of DEN-treated animals was slightly reduced (Groups 1 to 3, data not shown). At week 7, their body weight except for Group 3, which received DEN plus 500 ppm capsaicin (Suppl. Table 1, available at http://www.j-ems.org/journal/) did not differ from that of the control group. This difference was, however, not observed at week 32 (Suppl. Table 2, available at http://www.j-ems.org/journal/). Organ weight did not show any differences among the groups (Suppl. Table 1 and Suppl. Table 2).

Capsaicin did not affect formation of preneoplastic hepatocellular lesions: DEN induced small GST-P positive foci in liver at week 7 (Group 1, Table 1).

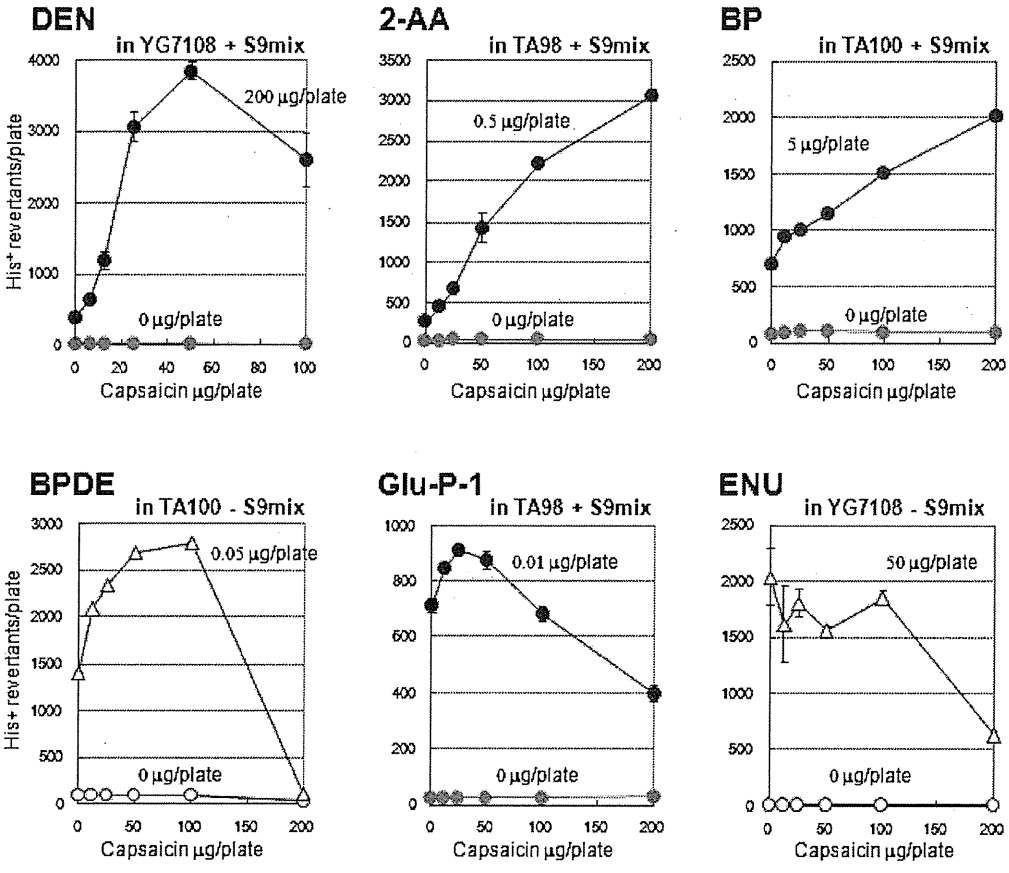

Fig. 2. Modulating effects of capsaicin on *in vitro* mutagenesis induced by DEN, 2-AA, BP, BPDE, Glu-P-1 and ENU. Doses of each chemical, 0.01–200 μg/plate, in the condition with chemical were indicated on the graph and doses of capsaicin were indicated on the X axis. Strains and S9 mix conditions are indicated on each panel.

Table 1. Quantification of GST-P positive foci at week 7

Group	Treatment		No. of rats	No. of foci [No./cm²]	Area of foci [mm²/cm²]
	DEN	Test chemical			
1	+	—	6	15.425±7.233*	0.445±0.280
2	+	Capsaicin 100 ppm	6	16.553±10.543	0.538±0.429
3	+	Capsaicin 500 ppm	6	20.405±14.939	0.718±0.816
4	−	Capsaicin 500 ppm	4	0.000±0.000	0.000±0.000
5	−	—	4	0.000±0.000	0.000±0.000

*Mean±SD. Experimental period was 7 weeks. Values were compared with Group 1 by Student's *t*-test.

Quantitative analysis (number and area) of the lesions did not show any difference among the DEN-treated groups, i.e., Groups 1, 2 and 3 (Table 1). These preneoplastic lesions did not develop in the liver of rats treated without DEN (Groups 4 and 5).

Capsaicin did not affect gene mutations in vivo: DEN treatments enhanced *gpt* MF in the liver 200 times over the control levels (Table 2 and Suppl. Table 3, available at http://www.j-ems.org/journal/). Capsaicin was non-genotoxic (Group 4). Unlike the *in vitro* results, capsaicin treatments did not show any substantial effects on DEN-induced mutagenesis *in vivo*. The *gpt* MFs of Groups 2 and 3, which received DEN plus 100 and 500 ppm capsaicin, respectively, were not different from that of Group 1 that received DEN alone (Table 2).

Capsaicin slightly suppressed hepatocarcinogenesis: The incidence and multiplicity of tumors at week 32 were slightly reduced in the capsaicin treated groups (Groups 2 and 3, Table 3). The treatment with capsaicin

decreased the incidence and multiplicity of liver tumors by 20–40% and 47–64%, respectively. The number of adenomas per rat was significantly decreased by the capsaicin treatment ($p<0.05$), although the reduction of HCCs was statistically insignificant.

Discussion

In this study, we examined the modulating effects of capsaicin on DEN-induced mutagenesis *in vitro* and *in vivo*. We could not confirm previously reported mutagenicity of capsaicin in *S. typhimurium* TA98 and TA100 in the presence of S9 activation (9,11,13). Capsaicin was not mutagenic not only *in vitro* (Fig. 2) but also *in vivo* (Table 2). Purity of the samples may account for the different findings (24). Instead, we found that capsaicin effectively enhanced mutagenicity of DEN, 2-AA and BP in the presence of S9 activation *in vitro* (Fig. 2). At first, we assumed that capsaicin might modulate the activities of CYP enzymes involved in metabolic activation of the xenobiotics, thereby enhancing the mutagenesis. In fact, it is suggested that metabolites of capsaicin bind microsomal proteins, such as CYP enzymes (25–27). However, in this study capsaicin also enhanced mutations induced by BPDE without S9 activation. Therefore, we suggested that capsaicin modulated not only metabolic activation but also mutagenesis and/or DNA repair. Interestingly, capsaicin displayed opposite modulating effects on mutagenesis, i.e., reduction of mutagenesis by Glu-P-1 in the presence of S9 enzymes and by ENU in the absence of S9 (Fig. 2). These results suggest that capsaicin can suppress metabolic activation by S9 enzymes and mutagenesis/DNA repair in some cases. It is puzzling, however, why capsaicin enhanced mutations induced by DEN but reduced those induced by ENU, although both DEN and ENU induce mutagenic O^6-ethylguanine in DNA. One possible explanation for the complex modulating effects is that capsaicin might enhance membrane permeability of bacteria to chemical carcinogens, thereby displaying various modulating effects on the mutagenicity of chemicals. Complex modulating effects of capsaicin *in vitro* have been described by Huynh and Teel (28). They have reported that capsaicin at doses of 0.25 μmol (76.3 μg) and 0.5 μmol (152.7 μg) per plate reduced mutations in *S. typhimurium* TA98 induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and Glu-P-1, but enhanced those by Trp-P-2 in the presence of S9 enzymes (28). Collectively, our results illustrated in Fig. 2 along with other reports (2,29,30) suggest that capsaicin has ability to modulate multiple steps leading to mutations at least *in vitro*.

In contrast, capsaicin neither affected development of DEN-induced preneoplastic lesions, GST-P positive foci (Table 1) nor mutagenesis in the liver of rats (Table 2). These results were unexpected because capsaicin substantially enhanced the mutagenicity of DEN *in vitro* (Fig. 2) and it is reported that capsaicin inhibits metabolism of carcinogens, including dimethylnitrosamine (DMN), which are preferentially activated by CYP2E1 (26,27). In the report, capsaicin at 0.25 μmol (76.3 μg) per plate reduces the mutagenicity of DMN in

Table 2. Mutant frequency in Liver at week 7

Group	Treatment		No. of rats	Mutant frequency ($\times 10^{-6}$)
	DEN	Test chemical		
1	+	—	6	225.31±52.51*
2	+	Capsaicin 100 ppm	6	254.76±83.47
3	+	Capsaicin 500 ppm	6	245.10±114.52
4	−	Capsaicin 500 ppm	4	1.10±1.09
5	−	—	4	1.49±1.90

*Mean±SD. Values were examined by Student's *t*-test. Significant differences among groups 1, 2 and 3 were not observed.

Table 3. Pathological findings in liver at week 32

Group	Treatment		No. of rats	Incidence			Multiplicity		
				No. of rats with tumors			No. of tumors/ rats		
	DEN	Test chemical		Total	AD	HCC	Total	AD	HCC
1	+	—	8	8 (100%)	7 (88%)	5 (63%)	2.75±2.12*	1.75±1.28	1.00±1.07
2	+	Capsaicin 100 ppm	8	6 (75%)	5 (63%)	4 (50%)	1.38±1.30	0.75±0.71	0.63±0.74
3	+	Capsaicin 500 ppm	8	5 (63%)	5 (63%)	3 (38%)	1.13±1.13	0.63±0.52†	0.50±0.76
4	−	Capsaicin 500 ppm	2	0 (0%)	0 (0%)	0 (0%)	0.00±0.00	0.00±0.00	0.00±0.00
5	−	—	2	0 (0%)	0 (0%)	0 (0%)	0.00±0.00	0.00±0.00	0.00±0.00

AD, adenoma; HCC, hepatocellular carcinoma. *Mean±SD. †Significantly different from group 1 by Dunnett multiple comparison test ($p<0.05$).

S. typhimurium TA100 by more than 50% (26). A possible reason for the discrepancy between *in vitro* and *in vivo* findings is that, unlike *in vitro* where capsaicin can interact with S9 enzymes at high concentrations, the local concentration (tissue distribution) in the liver of rats might not be high enough to modulate the activity of CYP enzymes. In fact, there is a report suggesting that capsaicin did not inhibit any of CYP enzyme activities at concentrations occurring after ingestion of chili peppers (31). Direct inhibition may be observed at much higher concentrations. Although exact concentrations in the liver are unavailable in this study, the negative modulating effects of capsaicin in the liver of rats caution that chemopreventive effects of chemicals should be evaluated not only *in vitro* but also *in vivo*.

Interestingly, capsaicin slightly suppressed the incidence and multiplicity of hepatocellular tumors at week 32. Although the reduction in the multiplicity of liver cell carcinomas did not reach the statistical significance, the value of liver cell adenomas was significantly reduced by feeding with capsaicin at a dose level of 500 ppm ($p<0.05$, Table 3). Capsaicin was previously demonstrated to be chemopreventive against azoxymethane-induced colon carcinogenesis (7). Capsaicin has multiple biological activities, such as block of signal transduction pathways leading to carcinogenesis, induction of apoptosis, cell-cycle delay and anti-inflammation (1,3,5,6,32). It is unclear which biological activities are involved in the slight reduction of DEN-induced hapatocarcinogenesis. However, we suggest that antimutagenesis does not play roles in the weak chemopreventive ability because of the negative modulating effects on DEN-induced mutagenesis *in vivo*.

In summary, we revealed that capsaicin was slightly chemopreventive against liver cell tumors induced by DEN in rats through mechanisms other than antigenotoxicity. Our study highlights the importance of employment of multiple biological parameters, such as mutations and pathological biomarkers, to investigate the mechanisms underlying the chemopreventive effects of chemicals. In this regard, F344 *gpt* delta rats (19) are quite useful, because mutations *in vivo* as well as pathological alterations (incidences and multiplicities of tumors and preneoplasms) can be analyzed at the same time in target tissues of the same rats that received carcinogens and/or test agents.

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