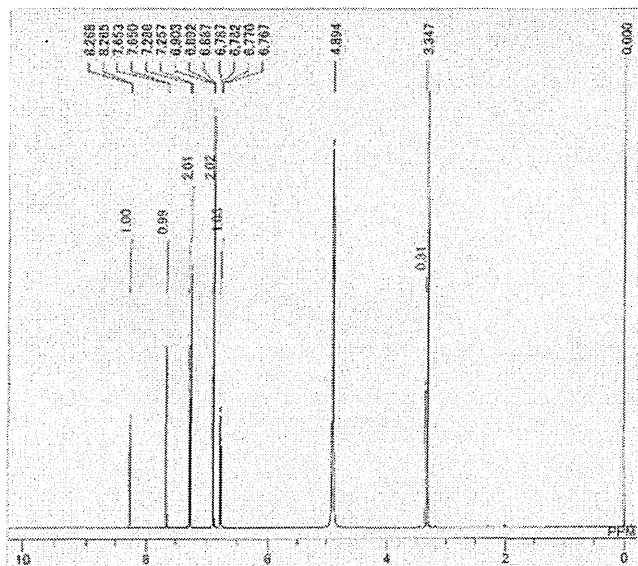


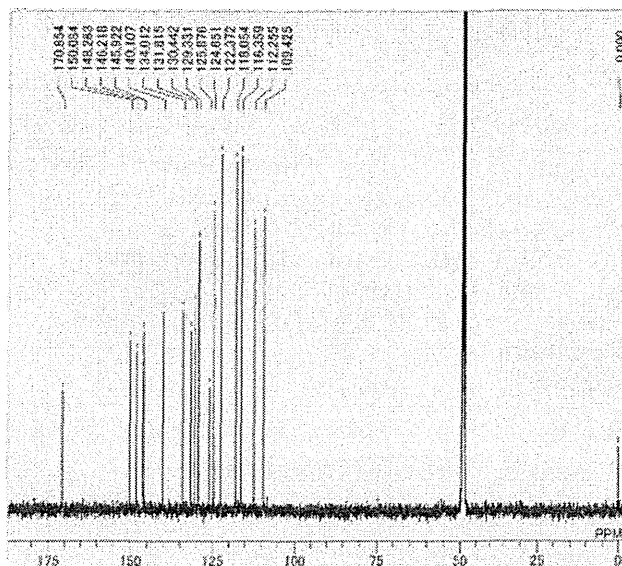
Figure 4

フェノール性化合物と金属の反応によって得られた溶液のLC/PDA クロマトグラム

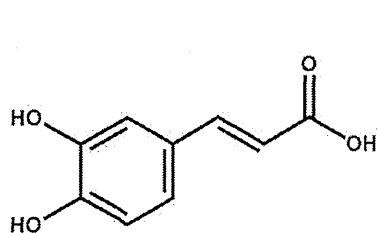
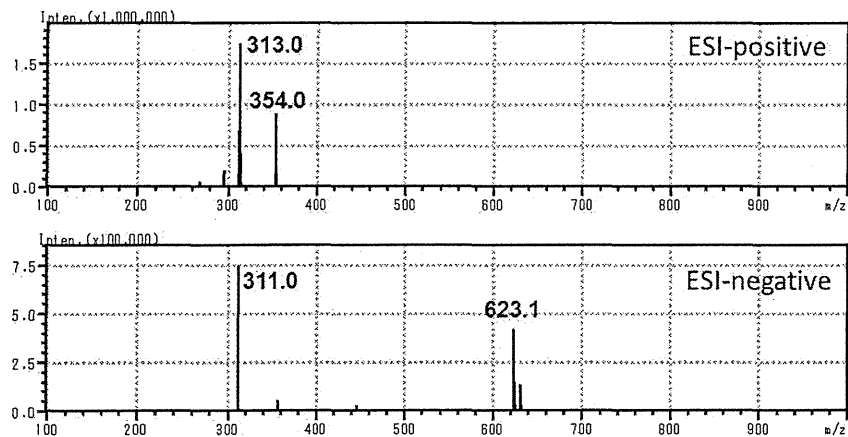
$^1\text{H-NMR}$



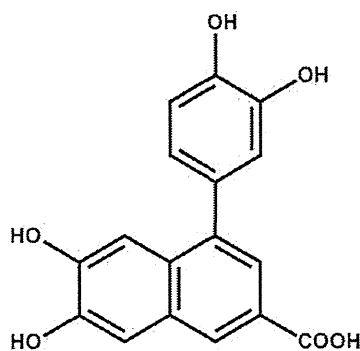
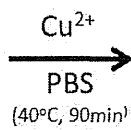
$^{13}\text{C-NMR}$



MSスペクトル



Caffeic acid
Chemical Formula: $\text{C}_9\text{H}_8\text{O}_4$
Exact Mass: 180.04



Compound A
3-Carboxy-6,7-dihydroxy-1-(3',4'-dihydroxyphenyl)-naphthalene
Chemical Formula: $\text{C}_{17}\text{H}_{12}\text{O}_6$
Exact Mass: 312.06

Figure 5

NMR および MS による Compound A の構造解析

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Suzuki, Y., Umemura, T., Hibi, D., Inoue, T., Jin, M., Ishii, Y., Sakai, H., Nohmi, T., Yanai, T., Nishikawa, A., Ogawa, K.	Possible involvement of genotoxic mechanisms in estragole-induced hepatocarcinogenesis in rats.	Arch. Toxicol.	86	1593-1601	2012
Suzuki, Y., Umemura, T., Ishii, Y., Hibi, D., Inoue, T., Jin, M., Ssakai, H., Kodama, Y., Nohmi, T., Yanai, T., Nishikawa, A., Ogawa, K.	Possible involvement of sulfotransferase 1A1 in estragole-induced DNA modification and carcinogenesis in the livers of female mice.	Mutat. Res.	749	23-28	2012
Kuroda, K., Kijima, A., Ishii, Y., Takasu, S., Jin, M., Matsushita, K., Kodama, Y., Umemura, T.	Flumequine enhances the in vivo mutagenicity of MeIQx in the mouse liver.	Arch. Toxicol.			(in press)
Fukuyama, T., Kosaka, T., Hayashi, K., Miyashita, L., Tajima, Y., Wada, K., Nishino, R., Ueda, H., and Harada, T.	Role of regulatory T cells in the induction of atopic dermatitis by immunosuppressive chemicals.	Toxicol Lett,	213	392-401	2012
Fukuyama, T., Kosaka, T., Hayashi, K., Miyashita, L., Tajima, Y., Wada, K., Nishino, R., Ueda, H., and Harada, T.	Immunotoxicity in mice induced by short-term exposure to methoxychlor, parathion, or piperonyl butoxide.	J Immunotoxicol			2012(in press)
Masashi Sekimoto, Shinsuke Sano, Takuomi Hosaka, Kiyomitsu Nemoto and Masakuni Degawa:	Establishment of a stable humancell line, HPL-A3, for use in reporter gene assays of CYP3A inducers.	Biol. Pharm. Bull.,	35	677-685	2012

Y. Iwasaki, K. Mochizuki, Y. Nakano, N. Maruya, M. Goto, Y. Maruyama, R. Ito, K. Saito, H. Nakazawa,	Comparison of fluorescence reagents for simultaneous determination of hydroxylated phenylalanine and nitrated tyrosine by high-performance liquid chromatography with fluorescence detection.	Biomed. Chromatogr.,	26(1)	41-50	2012
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Possible involvement of genotoxic mechanisms in estragole-induced hepatocarcinogenesis in rats

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Abstract Estragole (ES) is a natural organic compound used frequently as a flavoring food additive. Although it has been reported to be tumorigenic and induce DNA adducts in the mouse liver, there have been no reports regarding ES hepatocarcinogenicity in rats. In the current study, we therefore examined potent carcinogenicity, DNA adduct formation and in vivo genotoxicity of ES in the livers of wild and reporter gene-carrying F344 rats. Males were administered 600 mg/kg bw ES by gavage and sequentially sacrificed at weeks 4, 8 and 16 for GST-P and PCNA immunohistochemistry and measurement of ES-specific DNA adducts by LC-MS/MS in the livers. GST-P-positive foci increased with time in ES-treated rats from week 4, PCNA-labeling indices being similarly elevated at both weeks 4 and 8. ES-specific DNA adducts such as ES-3'-N²-dG, 3'-8-dG and 3'-N⁶-dA were consistently detected, particularly at week 4. In a second study, male F344

gpt delta rats were administered 0, 22, 66, 200 or 600 mg/kg bw ES for 4 weeks. *Gpt* mutant frequency in the liver was increased in a dose-dependent manner, with significance at 200 and 600 mg/kg bw in good correlation with PCNA-labeling indices. Mutation spectra analysis showed A:T to G:C transitions to be predominantly increased in line with the formation of ES-3'-N⁶-dA or 3'-8-dG. These results indicate that ES could be a possible genotoxic hepatocarcinogen in the rat, at least when given at high doses.

Keywords Estragole · Rat · Hepatocarcinogenesis · Genotoxicity

Introduction

Estragole (ES) is an allylbenzene compound, which is a natural constituent of several herbs, including basil, fennel, anise and tarragon. It has found use as a flavoring agent although acting as a hepatocarcinogen in various strains of mice (Drinkwater et al. 1976; Miller et al. 1983; Wiseman et al. 1987). DNA adducts were also detected in livers of mice following intraperitoneal injection of ES (Randerath et al. 1984; Phillips et al. 1984), and ES-specific DNA adducts are strongly implicated in carcinogenesis (Wiseman et al. 1985, Fennell et al. 1985). In in vitro assays, ES was generally non-mutagenic in *Salmonella typhimurium*, *Escherichia coli* WP2 uvrA and *Bacillus subtilis* (Zeiger et al. 1987; Sekizawa and Shibamoto 1982), although mutagenicity was demonstrated in strain TA1535 of *S. typhimurium* without metabolic activation (Swanson et al. 1979) and ES induced DNA damage in an in vitro unscheduled DNA synthesis (UDS) test in rat hepatocytes (Nesslany et al. 2010). In vivo, while the UDS assay in rats was also positive, a rat bone-marrow micronucleus test was

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negative (Nesslany et al. 2010). Although DNA adduct formation has been reported in mouse livers (Randerath et al. 1984; Phillips et al. 1984), equivocal results have been generated in terms of genotoxicity and the mechanism of ES hepatocarcinogenesis is unclear and remains to be elucidated.

In 2001, the Scientific Committee on Food (SCF) of the European Union reported a scientific opinion that ES is both genotoxic and carcinogenic. Accordingly, SCF recommended reduction in exposure levels and restrictions on use (SCF 2001). On the other hand, the expert panel of the Flavor and Extract Manufacturers Association (FEMA) concluded that dietary exposure to ES from spice consumption does not pose a significant cancer risk to humans because several studies clearly established that profiles of metabolism, metabolic activation and covalent binding were dose dependent at high levels but diminished markedly at lower levels of exposure (Smith et al. 2002).

In the present study, to assess the carcinogenic potential of ES in rats, quantitative immunohistochemical analyses of glutathione *S*-transferase placental form (GST-P)-positive hepatocytes were performed in F344 rats given ES at a high dose for up to 16 weeks. ES-specific DNA adducts were analyzed by LC-MS/MS, a method as sensitive as ³²P-post-labeling (Singh and Farmer 2006). Additionally, to understand the molecular mechanisms underlying genotoxicity and carcinogenicity of ES, mutation assays were conducted using the reporter gene-carrying *gpt* delta transgenic rat, considered as a useful tool for estimating in vivo genotoxic and carcinogenic risks of environmental chemicals (Nohmi et al. 2000; Kanki et al. 2005; Umemura et al. 2007; Nishikawa et al. 2008).

Materials and methods

Chemicals and animals

ES (CAS No. 140-67-0) and diethylnitrosamine (DEN, CAS No. 55-18-5) were purchased from Tokyo Kasei (Tokyo, Japan). Five-week-old male F344 rats and *gpt* delta rats carrying approximately 10 tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan), housed in polycarbonate cages with hardwood chips for bedding in a conventional animal facility and maintained under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5 %), air change (12 times per hour) and lighting (12 h light/dark cycle). The animals were given free access to CRF-1 basal diet (Oriental Yeast, Tokyo) and tap water and were used after a 1-week acclimation period.

Animal treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo).

Experiment I After acclimation, eighty F344 male rats were randomly divided into three groups, with 30 rats each in two groups (control and ES-treated groups), and 20 in the DEN-treated group. Control animals received corn oil vehicle alone. ES-treated rats received 600 mg/5 mL/kg body weight (bw) ES in corn oil by gavage 5 days per week. Dose level and frequency of dose were determined based on an NTP technical report on the 3-month toxicity of ES (NTP 2011) in which 600 mg/kg bw ES showed potent carcinogenicity based on the occurrence of one hepatocellular adenoma in the liver of one of 10 male rats. The DEN-treated positive control group was given 10 ppm DEN dissolved in the drinking water for 16 weeks. Five animals each of the control and ES groups were euthanized at week 4 and 8, and the other 20 animals each were euthanized at 16 weeks after the beginning of the experiment. At each time point, removed livers were weighed and portions were immediately fixed in buffered formalin and routinely processed for embedding in paraffin, sectioning, and hematoxylin and eosin (H&E) and immunohistochemical staining. The remaining liver tissues were frozen in liquid nitrogen and stored in a deep freezer at -80 °C until measurement of ES-DNA adducts.

Experiment II The dosage levels, experimental design and dosing method were selected based on the results of experiment I and the NTP report (NTP 2011). Groups of five male *gpt* delta rats were given ES by gavage at concentrations of 0, 22, 66, 200 or 600 mg/5 mL/kg bw 5 days per week for 4 weeks. Controls (0 mg/kg bw) received the corn oil vehicle alone. At the end of the experimental period, the animals were euthanized and livers were immediately removed and weighed. Then, portions were fixed in buffered formalin and processed for routine histological and histochemical examinations similarly to Experiment I. The remaining liver tissues were frozen for measurement of ES-DNA adducts, as for Experiment I and in vivo mutation assays.

Immunohistochemistry for GST-P and PCNA

Immunohistochemistry for GST-P and proliferating cell nuclear antigen (PCNA) was conducted in liver tissue sections of all groups of animals, using anti-GST-P rabbit polyclonal antibodies (Medical and Biological Laboratories, Aichi, Japan) at a dilution of 1:1,000 and an anti-PCNA monoclonal antibody (Dako Cytomation A/S, Glostrup, Denmark) at a dilution of 1:100. A streptavidin-

biotin-peroxidase complex kit (Dako Cytomation) was employed with the chromogen 3,3'-diaminobenzidine for demonstration of binding, followed by counterstaining with hematoxylin. Numbers and areas of GST-P-positive foci consisting of 5 or more nucleated hepatocytes in cross-section were evaluated with the aid of an image analyzer (IPAP, Sumika Technoservice, Hyogo, Japan). For quantification of cell proliferation, sections from five rats in each group were analyzed at 200× magnification, with 5 fields chosen at random. The number of PCNA-positive hepatocytes in at least 2,000 cells in normal-looking areas was counted, and the PCNA-positive index was expressed as the percentage value.

In vivo mutation assay

6-Thioguanine (TG) selection was carried out as previously described (Nohmi et al. 2000). Briefly, genomic DNA was extracted from the liver, and lambda EG10 DNA (48 kb) was rescued as a lambda phage by 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. The *gpt* gene of *E. coli* encodes guanine phosphoribosyltransferase that catalyzes phosphoribosylation of guanine and also 6-TG. Since 6-TG is toxic to cells when incorporated into DNA, *E. coli* cells having wild-type *gpt* gene cannot survive on plates containing 6-TG, but only *gpt* mutants can be selected to form colonies on plates containing 6-TG. The gene name *gpt* is derived from the enzyme guanine phosphoribosyltransferase. 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. For characterizing the mutation spectra of *gpt* mutants, a 739-bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously (Nohmi et al. 2000). For mutation spectrum, *gpt* mutants (6-TG^R and Cm^R colonies) shown in Table 5 were all analyzed. Sequencing analysis was performed at Takara Bio Inc. (Mie, Japan), and then, colonies sharing the same mutation regarded as demonstrating clonal growth were eliminated from the total number of mutant colonies.

Quantification of ES-DNA adduct levels in livers

The level of ES-specific DNA adducts in livers was quantified with our newly established method using

LC-MS/MS (Ishii et al. 2011). Briefly, samples were homogenized with the lysis buffer included in a commercial DNA isolation kit. The mixture was centrifuged, and the deposit was dissolved in enzyme reaction buffer. After treatment with RNase and protease K, a DNA pellet was obtained. The dried DNA pellet was dissolved in surrogate standard containing sodium acetate buffer, pH 4.8, and incubated with nuclease P1. This was followed with the addition of Tris-HCl buffer, pH 8.2, and the solution was incubated with alkaline phosphatase. After the addition of sodium acetate buffer, pH 5.1, samples were injected into the LC-MS/MS. LC-MS/MS analysis was performed using a Quattro Ultima (Micromass, Beverly, MA, USA) coupled to a HEWLETT PACKARD 1100 series (Agilent technologies, Palo Alto, USA). The mass spectrometer was operated using an electrospray ionization source in the positive ion mode (ESI⁺) for multiple reaction monitoring (MRM).

Statistics

The significance of differences in the results of Experiment I was evaluated using Student's *t* test, and in Experiment II with ANOVA, followed by the Dunnett's multiple comparison test or the multiple Student-Welch test with Pharmac Analyst II (Hakuhousha, Tokyo, Japan).

Results

Sequential analysis of rats given 600 mg/kg bw ES for up to 16 weeks

Body and liver weights measured in Experiment I are shown in Fig. 1 and Table 1. Body weights were significantly ($P < 0.01$) decreased in the group treated with ES as compared with the control group from week 1. Absolute liver weights in the ES group were significantly ($P < 0.01$) decreased at week 4, but increased at week 16. Relative liver weights in the ES group were significantly ($P < 0.01$)

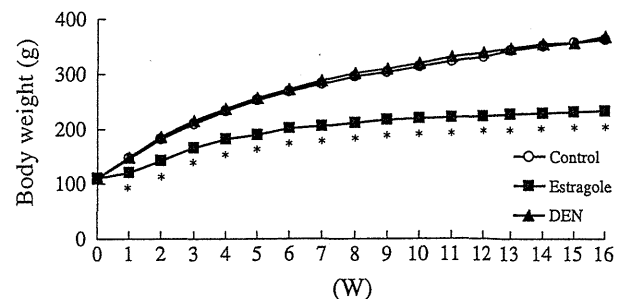


Fig. 1 Growth curves for F344 rats treated with 600 mg/kg estragole or 10 ppm DEN for 16 weeks. * $P < 0.01$ versus control

Table 1 Mean body and liver weights of F344 rats treated with 600 mg/kg bw estragole or 10 ppm DEN for 4, 8 or 16 weeks

Group	Rats	Body weights (g)	Absolute liver weights (g)	Relative liver weights (g %)
4 weeks				
Control	5	231.3 ± 11.8 ^a	8.40 ± 0.54	3.63 ± 0.12
Estragole	5	176.6 ± 8.1* (−24 %)	6.99 ± 0.51* (−17 %)	3.95 ± 0.13* (+9 %)
8 weeks				
Control	5	297.5 ± 8.2	9.80 ± 0.50	3.30 ± 0.12
Estragole	5	211.9 ± 13.4* (−29 %)	9.18 ± 0.89	4.33 ± 0.16* (+31 %)
16 weeks				
Control	20	361.6 ± 17.1	10.48 ± 0.66	2.90 ± 0.08
Estragole	20	228.3 ± 22.3* (−37 %)	21.29 ± 2.77* (+103 %)	9.33 ± 0.84* (+222 %)
DEN	20	366.8 ± 17.5	12.16 ± 0.75* (+16 %)	3.31 ± 0.09* (+14 %)

Values in the parenthesis indicate percentage of change against the mean control values (+: increase, −: decrease)

* $P < 0.01$ versus control

^a Means ± SD

Table 2 Histopathological findings for the livers of F344 rats treated 600 mg/kg bw estragole

Week	4	8	16
No. of animals examined	5	5	20
Lymphocytic aggregation (±/+ ++)	5 (5/0/0)	5 (1/4/0)	20 (0/5/15)
Hepatocyte hypertrophy (++)		0	20
Oval cell proliferation (±/+)		0	1 (1/0)
Bile duct proliferation (±/+)		0	1 (1/0)
Cholangiofibrosis (±/+ +++)		0	1 (1/0/0)

±: minimal, +: mild, ++: moderate

increased compared with the control group at weeks 4, 8 and 16. The absolute and relative liver weights in the DEN group were also significantly ($P < 0.01$) increased at week 16.

Histopathological findings in the liver are summarized in Table 2. Mild to moderate lymphocytic aggregation was observed in livers of ES-treated animals throughout the experiment, becoming more severe over time. Centrilobular hepatocellular hypertrophy was evident only at week 16. Oval cell and bile duct proliferation was noted in one of five and sixteen of twenty ES-treated rats at weeks 8 and 16, respectively. Cholangiofibrosis was detected in one and twelve ES-treated rats at weeks 8 and 16, respectively. The results regarding GST-P positive liver cell foci are shown in Table 3. At weeks 4 and 8, GST-P positive foci were induced in the ES group. At week 16, the mean number and area of GST-P positive foci were significantly ($P < 0.01$) increased in the ES or DEN groups as compared to those in the control group. Data for immunohistochemical expression of the cell proliferation marker PCNA are summarized

in Fig. 2. PCNA-positive indices (%) of the normal-looking hepatocytes in the control and ES-treated livers were 1.15 ± 0.49 and 5.25 ± 1.14 at week 4; and 0.73 ± 0.10 and 5.34 ± 1.01 at week 8, respectively. Values were significantly ($P < 0.01$) increased in the ES group as compared with the control group at both time points.

ES-specific DNA adducts, 3'-8-dG, 3'-N²-dG and 3'-N⁶-dA, were detected by the LC-MS/MS method in the rat liver treated with 600 mg/kg bw ES for 4 and 8 weeks (Fig. 3). No ES-specific DNA adducts were detected in the livers of control group. The level of DNA adducts detected at week 4 was significantly ($P < 0.01$) higher than that seen at week 8.

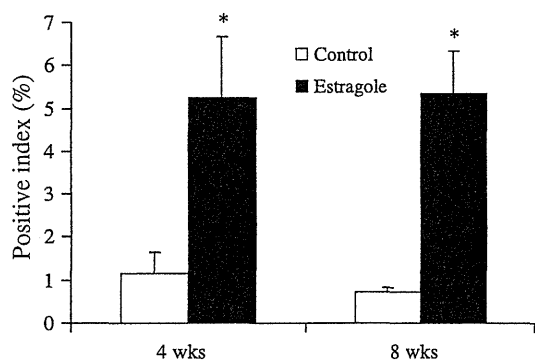
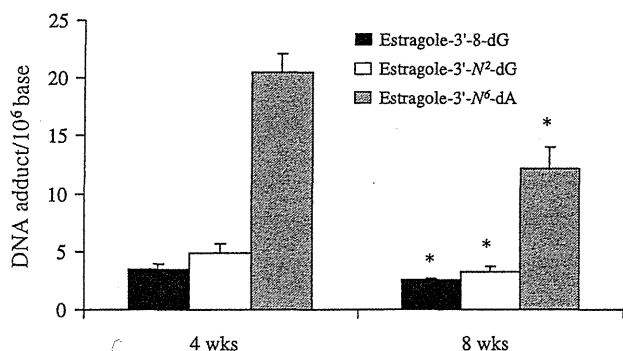
Dose–response analysis of rats given 22, 66, 200 or 600 mg/kg bw ES for 4 weeks

Three of 5 rats in the 600 mg/kg ES group died with severe body weight loss at 1 or 2 weeks after the start of experiment. The cause of deaths could not be clarified because of autolysis or cannibalism although somewhat higher susceptibility to liver toxicity of *gpt* delta rats than wild F344 rats might be suggested. Therefore, data for the 600 mg/kg ES group were excluded from the effective animal numbers. Data for final body weights and absolute and relative liver weights measured in Experiment II are summarized in Fig. 4 and Table 4. Body weights were significantly ($P < 0.01$) decreased in the 200 mg/kg bw treated group as compared with the control group from week 3. Relative liver weights were significantly ($P < 0.01$) increased in the 200 mg/kg bw treated group as compared with the control group. Final body weights and liver weights were comparable between control and 66 mg/kg bw or lower ES-treated groups.

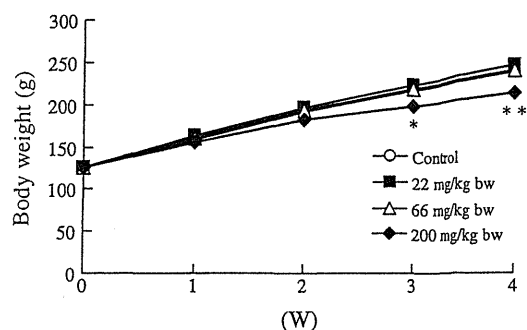
Table 3 Quantitative data for GST-P positive liver cell foci found in F344 rats given estragole or DEN

	4 weeks	8 weeks	16 weeks
Number (No./cm ²)			
Control	ND	ND	0.08 ± 0.19
Estragole	9.48 ± 2.67 ^a	60.57 ± 10.91	84.83 ± 34.41*
DEN	– ^b	–	34.91 ± 10.10*
Area (mm ² /cm ²)			
Control	ND	ND	0.00 ± 0.00 ^c
Estragole	0.03 ± 0.02	0.33 ± 0.08	4.27 ± 3.50*
DEN	–	–	0.02 ± 0.01*

ND not detected

* $P < 0.01$ versus control^a Means ± S.D^b Not examined^c Actual value was about 0.0003 ± 0.0007 mm²/cm²**Fig. 2** PCNA-positive indices (%) of normal-looking hepatocytes in the livers of F344 rats treated with 600 mg/kg bw estragole for 4 and 8 weeks (mean ± SD). * $P < 0.01$ versus control**Fig. 3** Hepatic estragole-DNA adduct levels in F344 rats treated with 600 mg/kg bw estragole for 4 and 8 weeks. Each point represents the mean ± SD data from five rats. * $P < 0.01$ versus 4 weeks

Histopathologically, minimal lymphocytic aggregation was observed in three of five animals of the 200 mg/kg bw group. Immunohistochemical expression for the cell proliferation marker PCNA is summarized in Fig. 5. PCNA-

**Fig. 4** Growth curves for *gpt delta* rats treated with 0, 22, 66 or 200 mg/kg estragole for 4 weeks. *, ** $P < 0.01, 0.05$ versus control

positive indices (%) in the liver were significantly ($P < 0.01$) increased in the 200 mg/kg bw group as compared to the control group. Rats dosed with less than 66 mg/kg showed no increase in cell proliferative activity caused by treatment.

The ES-specific DNA adducts, ES-3'-8-dG, 3'-N²-dG and 3'-N⁶-dA, in the rat liver treated with 0, 22, 66 or 200 mg/kg bw ES for 4 weeks were also assessed by the same method as in Experiment I. ES-DNA adducts were found in ES-treated rats, but were not detected in controls (Fig. 6). 3'-N⁶-dA was predominant in the groups given more than 66 mg/kg bw, whereas 3'-N²-dG was predominant only in the 22 mg/kg bw group. While 3'-8-dG and 3'-N⁶-dA in the ES-treated rats were increased in a clear dose-dependent manner, 3'-N²-dG was increased without dose dependence.

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Table 5. The MFs ($1.09 \pm 0.26 \times 10^5$) in the 200 mg/kg bw group were significantly ($P < 0.01$) higher than the control value ($0.49 \pm 0.18 \times 10^5$) but in a dose-dependent manner. As a reference data, *gpt* MFs from survived 2 rats in the 600 mg/kg bw group were much higher (2.96×10^5 and 2.89×10^5) as compared to the other groups. The characterization of *gpt* mutations induced by ES exposure was analyzed by DNA sequencing of mutant colonies (Table 6). In the 200 mg/kg bw groups, A:T base pair was the preferable site for mutation, accounting for 43.5 % (10/23) of the mutations. Specific mutation frequencies of AT:GC transitions were significantly ($P < 0.05$) higher in the 200 mg/kg bw group than in the control group.

Discussion

In the present study, GST-P-positive liver cell foci were increased in ES-treated rats in the early period, correlating well with increase in relative liver weights. Because GST-P-positive foci are established rat liver preneoplastic lesions detectable for the medium-term assay (Ito et al. 1988; Tsuda et al. 2003), our results suggest that ES may

Table 4 Mean body and liver weights of *gpt* delta rats treated with estragole for 4 weeks

Dose (mg/kg bw)	Rats	Body weights (g)	Absolute weights (g)	Relative weights (g%)
0	5	236.9 ± 10.6 ^a	9.67 ± 0.53	4.09 ± 0.19
22	5	243.8 ± 12.1	10.43 ± 1.04	4.27 ± 0.24
66	5	237.0 ± 6.4	10.20 ± 0.30	4.30 ± 0.16
200	5	212.1 ± 5.0* (−11 %)	9.60 ± 0.35	4.53 ± 0.13* (+11 %)

Values in the parenthesis indicate the percentage of change against the mean control values (+: increase, -: decrease)

* $P < 0.01$ versus control

^a Means ± SD

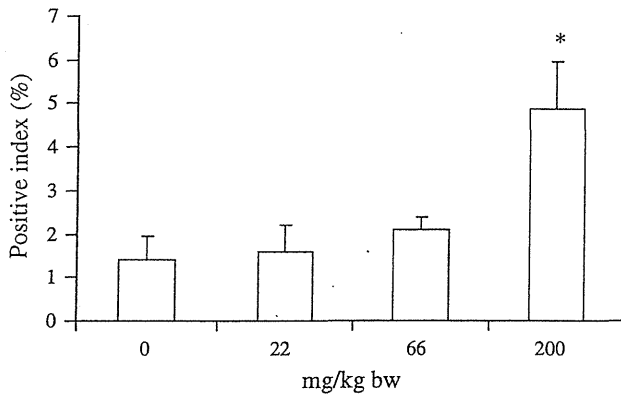


Fig. 5 PCNA-positive indices (%) in the livers of *gpt* delta rats treated with 0, 22, 66 or 200 mg/kg bw estragole for 4 weeks (mean ± SD). * $P < 0.01$ versus control

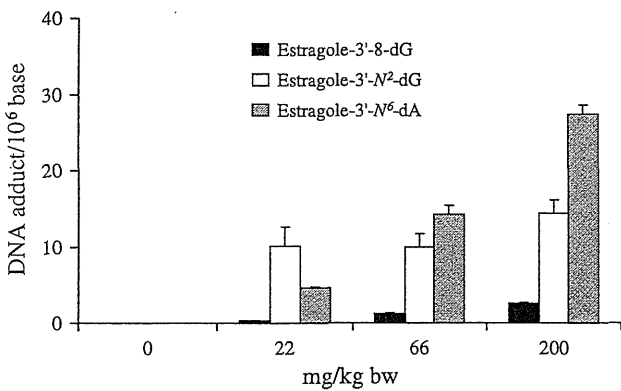


Fig. 6 Hepatic estragole-DNA adduct levels in *gpt* delta rats treated with 0, 22, 66 or 200 mg/kg bw estragole for 4 weeks. Each point represents the mean ± SD of data from five rats

also exert hepatocarcinogenicity in rats, in addition to mice. The fact that the cell proliferation activity assessed using PCNA immunohistochemistry was increased in the present study also indicates that ES could exhibit tumor-promotion activity. Interestingly, PCNA-positive cell ratios at week 4 were almost the same as those at week 8, implying sustained cell proliferation at least during this period. At the same time, ES-specific DNA adducts such as

ES-3'-8-dG, 3'-N²-dG and predominantly 3'-N⁶-dA were consistently detected in rat livers treated with ES, suggestive of genotoxic initiation activity. Since 8-week-treated rats demonstrated lower DNA adduct levels than 4-week-treated rats, DNA adduct might not accumulate over time, rather being repaired.

The overall results in the present study using wild F344 rats are in good agreement with those in the NTP 3-month toxicity study of ES (NTP 2011) in terms of liver weight and histopathological changes. However, in comparison with wild-type rats, it is likely that *gpt* delta rats may be somewhat more susceptible to toxicity of ES judging from lethality, liver weight change and cell proliferative activity. It can be said that the duration of exposure is not long enough to fully assess the carcinogenicity, and the dose was somewhat too high to evaluate systemic toxicity. However, the medium-term bioassay for rat hepatocarcinogenesis using GST-P immunohistochemistry is aimed at screening potent hepatocarcinogenicity but not strictly assessing repeated dose toxicity. Therefore, it is suggested that ES could be a possible hepatocarcinogen.

In the *in vivo* genotoxicity assay with *gpt* delta rats, MFs were significantly increased in rats treated with 200 mg/kg bw ES in a dose-dependent manner. Because normal ranges are relatively stable compared to the other models as reported previously (OECD 2011), statistically significant twofold increase in MFs in the 200 mg/kg was considered to be biologically meaningful. This is important, considering that ES-specific DNA adduct formation is implicated in hepatocarcinogenesis (Wiseman et al. 1985; Fennell et al. 1985). It is well known that DNA adducts can be repaired rather than becoming fixed as mutations. However, if the balance between DNA adduct formation and DNA damage removal is disturbed, the DNA adducts accumulate and may be fixed as mutations (de Vries et al. 1997). Taken together with the results of the mutation spectrum analysis and the quantitative ES-DNA adduct analysis, it is conceivable that increased MF in the 200 mg/kg bw group was due to the increase in AT-base pair mutation resulting from the ES-3'-N⁶-dA adduct formation. This fact

Table 5 *gpt* mutant frequencies in the livers of *gpt* delta rats treated with estragole for 4 weeks

Dose (mg/kg bw)	Animal no.	Cm ^R colonies (×10 ⁵)	6-TG ^R and Cm ^R colonies	Mutant frequency ^a (×10 ⁻⁵)	Mean ± SD
0	101	3.29	2	0.61	0.49 ± 0.18 ^b
	102	6.98	3	0.43	
	103	ND	ND	ND	
	104	3.83	1	0.26	
	105	4.59	3	0.65	
22	201	5.76	3	0.52	0.75 ± 0.25
	202	4.41	5	1.13	
	203	7.29	4	0.55	
	204	3.65	3	0.82	
	205	6.93	5	0.72	
66	301	4.05	4	0.99	0.82 ± 0.33
	302	7.61	3	0.39	
	303	3.24	3	0.93	
	304	6.89	4	0.58	
	305	3.29	4	1.22	
200	401	3.11	3	0.97	1.09 ± 0.26*
	402	5.31	8	1.51	
	403	4.05	4	0.99	
	404	3.65	3	0.82	
	405	4.28	5	1.17	

ND not detected

* $P < 0.01$ versus control^a Mutant frequency = the number of 6-TG^R and Cm^R colonies/the number of Cm^R colonies^b Means ± SD**Table 6** Mutation spectra of *gpt* mutant colonies detected in *gpt* delta rat livers given estragole for 4 weeks

	Control		22 mg/kg bw		66 mg/kg bw		200 mg/kg bw	
	No.	Mutation frequency ^a (×10 ⁻⁵)	No.	Mutation frequency (×10 ⁻⁵)	No.	Mutation frequency (×10 ⁻⁵)	No.	Mutation frequency (×10 ⁻⁵)
Base substitutions								
Transversions								
GC:TA	3 ^b	0.13 ± 0.15 ^c	9	0.35 ± 0.18	1	0.03 ± 0.06	4	0.21 ± 0.13
GC:CG	0	0.00 ± 0.00	0	0.00 ± 0.00	3	0.13 ± 0.21	3	0.13 ± 0.21
AT:TA	0	0.00 ± 0.00	0	0.00 ± 0.00	1	0.03 ± 0.06	3	0.13 ± 0.18
AT:CG	0	0.00 ± 0.00	1	0.05 ± 0.10	0	0.00 ± 0.00	0	0.00 ± 0.00
Transitions								
GC:AT	2	0.13 ± 0.15	6	0.22 ± 0.06	8	0.36 ± 0.19	3	0.16 ± 0.15
AT:GC	2	0.13 ± 0.15	1	0.03 ± 0.06	3	0.15 ± 0.26	7	0.34 ± 0.10*
Deletions								
Single bp	2	0.10 ± 0.13	1	0.03 ± 0.08	2	0.12 ± 0.17	1	0.05 ± 0.10
Over 2 bp	0	0.00 ± 0.00	1	0.05 ± 0.10	0	0.00 ± 0.00	0	0.00 ± 0.00
Insertions	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00
Complexes	0	0.00 ± 0.00	1	0.03 ± 0.06	0	0.00 ± 0.00	2	0.08 ± 0.17

* $P < 0.05$ versus control^a Specific mutation frequency = the number of specific mutations/the number of Cm^R colonies^b The number of colonies with independent mutations after clonal correction^c Means ± SD

is in line with statistically significant increase in MFs in the 200 mg/kg. Moreover, cell proliferation is thought to play an important role in fixation of DNA lesions into mutations (de Vries et al. 1997). Based on the results of histopathological analysis and PCNA immunohistochemical analysis, it is considered that the enhancement of cell proliferation subsequent to liver parenchymal damage due to ES treatment may have contributed to the significant increase in MF at higher doses of ES.

It has been reported that ES-3'-N²-dG is a major adduct derived from ES exposure (Punt et al. 2007; Wiseman et al. 1985; Phillips et al. 1981), but in the current experiment, hepatic levels did not clearly correlate with the dose of ES. Rather, the liver DNA adduct level of ES-3'-N⁶-dA was much higher than that of ES-3'-N²-dG in the 200 mg/kg bw ES-treated rat, clearly indicating that ES-3'-N⁶-dA is a predominant adduct formed under the present experimental conditions. However, the possibility that this difference may result from variation in the experimental design cannot be ruled out. Namely, previous studies used mouse livers after treatment with a single intraperitoneal injection of ES and assessed by a ³²P-post-labeling assay (Randerath et al. 1984; Phillips et al. 1984), whereas herein our study was investigated in rat livers repeatedly treated by gavage and then evaluated by an LC-MS/MS method. ES-3'-8-dG was consistently lower in level than other DNA adducts, but the dose-dependent increase may suggest some role in vivo genotoxicity.

Although MFs of the *gpt* gene were not significantly increased at less than 66 mg/kg bw ES treatments, MFs tended to be elevated dose-dependently, suggesting possible genotoxicity even at low doses, given the DNA adduct levels clearly showed a dose-dependent increase in the prevailing paradigm of the absent threshold for genotoxic carcinogens. Interestingly, PCNA immunohistochemistry showed that proliferation was not affected by ES treatment in the lower-dose groups. It was similarly reported that DNA adduct formation but no mutations were detected in rats given a genotoxic carcinogen MeIQx at low doses (Fukushima et al. 2009). It is well known that DNA adducts and/or mutated cells are removed by the kinetics of DNA repair and apoptosis during cell turnover (Kuraoka 2008). Therefore, genotoxicity of ES at such lower doses may not be enough to cause mutation at least in 4-weeks administration.

When extrapolating the data from high-dose animal experiments to the low-dose human situation, it is important to take species differences in metabolism and metabolic activation into account (Rietjens et al. 2005). It was reported that there was a minor influence of species differences between human and rat on the ultimate overall bioactivation of ES to its ultimate carcinogenic DNA reactive metabolite 1'-sulfooxyestradiol (Punt et al. 2009).

The estimated average human daily intake of ES is evaluated as 0.01–0.07 mg/kg bw in FEMA and SCF. It has been reported that ES-specific DNA adduct formation is predicted to be linear with increasing dose of ES by a physiologically based biodynamic model (Paini et al. 2010). Taken together with our data showing that ES-specific DNA adduct levels were shown in a dose-dependent manner at 22 mg/kg bw to 200 mg/kg bw, ES-DNA adducts may be formed even in very low-dose human situation. However, the possible involvement of other factors in the fixation of DNA lesions to give mutations must be taken into consideration for human risk assessment. In conclusion, the results in the present study suggest that ES may be a possible genotoxic hepatocarcinogen in rats at high doses. However, further studies are needed to critically assess the human risk of ES-genotoxicity and/or carcinogenicity.

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Conflict of interest We declare that we have no conflict of interest.

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Possible involvement of sulfotransferase 1A1 in estragole-induced DNA modification and carcinogenesis in the livers of female mice

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ABSTRACT

Estragole (ES), a natural organic compound, is frequently used as a flavoring in food even though it is a hepatocarcinogen in mice. Although formation of ES-specific DNA adducts following conversion from ES to the nucleophilic metabolite by sulfotransferase 1A1 (SULT1A1) has been reported, the modes of action underlying ES-induced hepatocarcinogenesis remain uncertain because conventional genotoxicity tests for ES yield negative results. In the present study, taking notice of the fact that there is a sex difference in SULT1A1 activity in the mouse liver, we assessed the frequency of micronuclei in polychromatic erythrocytes and the mutant frequency (MF) of reporter genes in female *gpt* delta mice treated with ES at doses of 0 (corn oil), 37.5, 75, 150 or 300 mg/kg body weight (bw) by gavage for 13 weeks. Results were compared with those obtained in males. Since one female was found dead at week one, the highest dose was reduced to 250 mg/kg bw in females from week two. As reported previously in C57BL/6 mice, the mRNA levels of *Sult1a1* in female *gpt* delta mice were significantly higher than those in the males. The levels of ES-specific DNA adducts in the females were higher than those in the males at all doses except the highest dose. In addition, MFs of the *gpt* gene were significantly increased from doses of 75 mg/kg bw of females, but the increment was observed only at the highest dose in males. There were no changes in the micronucleus test among the groups. Thus, the overall data suggest that specific DNA modifications by the SULT1A1-mediated carbocation formation and the resultant genotoxicity are key events in the early stage of ES-induced hepatocarcinogenesis of mice.

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1. Introduction

Estragole (ES) is an allylbenzene compound that is a natural constituent of several herbs, including basil, fennel, anise and tarragon and has been used as a flavoring agent. However, it has been found to be a hepatocarcinogen in various strains of mice [1–3]. Following intraperitoneal injections of ES, specific DNA adducts were detected in the livers of adult CD-1 female mice or newborn B6C3F₁ male mice [4,5]. It has been reported that allylbenzene analogues are mainly catalyzed by CYP1A2 and sulfotransferase 1A1 (SULT1A1), in the liver and metabolized to the ultimate carcinogenic metabolite that is capable of binding covalently to DNA [6,7]. Nevertheless, ES has been shown to be non-mutagenic in reverse

mutation assays using *Salmonella typhimurium*, *Escherichia coli* WP2 *uvrA* and *Bacillus subtilis* [8,9] except for TA1535 of *S. typhimurium* without metabolic activation [10]. In *in vivo* assays, it was found that the UDS (unscheduled DNA synthesis) assay in rats was positive while a rat bone marrow micronucleus test was negative [11]. Thus, ES genotoxicity has remained equivocal. However, the fact that a potent sulfotransferase inhibitor, pentachlorophenol (PCP), strongly inhibits hepatocarcinogenicity in mice along with covalent binding of the ultimate carcinogenic metabolite to hepatic macromolecules [12,13] suggests a possible participation of DNA modification in ES-induced hepatocarcinogenesis.

We used *gpt* delta mice in the present study. The assays with this transgenic mouse permit quantitative measurements of mutant frequencies (MFs) of the two transgenes, *i.e.* *gpt* and *red/gam*, in multiple tissues/organs and molecular analysis of induced and spontaneous mutations by DNA sequencing analysis [14]. Additionally, since the assays can include *in vivo* metabolism, it may be a

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useful tool to explore *in vivo* mutagenicity of chemicals requiring metabolic activation to exert their genotoxicities. Moreover, the levels of ES-specific DNA adducts in the livers can be quantified with our established method using LC–MS/MS [15] together with an *in vivo* mutation assay.

In the present study, to clarify the modes of action underlying ES-induced hepatocarcinogenesis, male and female *gpt* delta mice were given ES at carcinogenic doses for 13 weeks. We assessed the quantitative levels of ES-specific DNA modifications with LC–MS/MS methods, MFs of reporter genes and frequencies of micronuclei in polychromatic erythrocytes in the bone marrows of mice. Considering that there are sex differences in sulfotransferase activity in mouse liver, we compared the results obtained in male and female mice.

2. Materials and methods

2.1. Chemicals and animals

ES (CAS No. 140-67-0) was purchased from Tokyo Kasei (Tokyo, Japan). Male and female B6C3F₁ *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome status were raised by mating C57BL/6 *gpt* delta and non-transgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Fifty male and 50 female B6C3F₁ *gpt* delta mice were each randomized by weight into five groups. They were housed in a room with a barrier system, and maintained under the following constant conditions: temperature, 23 ± 2 °C; relative humidity, 55 ± 5%; ventilation frequency, 18 times/h; 12 h light:12 h dark cycle, with free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo) and tap water.

2.2. Animal treatment and sample collection

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Starting at six weeks of age, the mice received ES in corn oil by gavage at doses of 37.5, 75, 150, or 300 mg/10 mL/kg body weight (bw) (250 mg/10 mL/kg bw from week two in the females) five days per week for 13 weeks. Vehicle control animals (0 mg/10 mL/kg bw) received corn oil only. The doses of 150 and 300 mg/kg bw were carcinogenic doses for female mice reported in a 20-month carcinogenicity study [2]. All mice were euthanized 24 h after the last administration. Ten *gpt* delta mice were used to compare the toxicity of ES to that in the wild type mice observed in the previous report [16]. For other analyses (DNA adducts, micronuclei, *gpt* and Spi⁻ mutations, hepatic *Cyp1a2* and *Sult1a1* mRNA levels), the first five animals each group were selected.

The livers were immediately removed and weighed; slices were fixed in buffered formalin for hematoxylin and eosin (H&E) staining. Histopathological examination was performed using non-coded slides. A part of the liver was preserved with an ISOGEN kit (Nippon Gene, Tokyo) and stored at –80 °C until used to isolate total RNA. Remaining pieces of the liver were frozen with liquid nitrogen and stored at –80 °C until performance of mutation assays and DNA adduct measurements. Bone marrow, from epiphyseal cartilage decapitated femurs, was flushed out with fetal bovine serum (Invitrogen, Carlsbad, CA) for the micronucleus (MN) test.

2.3. Quantitative real time RT-PCR

Total RNA from the liver was isolated with the ISOGEN kit according to the manufacturer's instructions. RNA reverse transcription with random hexamers was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR was performed on an Applied Biosystems 7900HT FAST Real-Time PCR Systems (Applied Biosystems) with TaqMan[®] Fast Universal PCR Master Mix (Applied Biosystems), TaqMan[®] Gene Expression Assays (Applied Biosystems) and TaqMan[®] Rodent *GAPDH* control reagents (Applied Biosystems). The assayed genes included cytochrome P450 family 1, subfamily A, polypeptide 2 (*Cyp1a2*) and sulfotransferase family 1A, phenol-preferring, member 1 (*Sult1a1*). Rodent glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. The expression levels of the target genes *Cyp1a2* and *Sult1a1* were evaluated by a relative standard curve method.

2.4. Quantification of ES-DNA adduct levels in livers

The levels of ES-specific DNA adducts in livers were quantified with our newly established method using LC–MS/MS [15]. Briefly, samples were homogenized with the lysis buffer included in a commercial DNA isolation kit. The mixture was centrifuged, and the deposit was dissolved in enzyme reaction buffer. After treatment with RNase and protease K, a DNA pellet was obtained. The dried DNA pellet was dissolved in surrogate standard solution containing sodium acetate buffer, pH 4.8, and incubated with nuclease P1. This was followed with the addition of Tris–HCl buffer, pH 8.2, and the solution was incubated with alkaline phosphatase. After the

addition of sodium acetate buffer, pH 5.1, 1 μL of digested samples were diluted with an equal volume of methanol and injected into the LC–MS/MS. One millimolar solutions of ES-3'-C8-dG, ES-3'-N²-dG and ES-3'-N⁶-dA were prepared in methanol and immediately diluted with methanol/HPLC grade water (50/50, v/v) to 10 μM (stock solution). Working solutions for calibration (0.1–10 nM) were prepared by the addition of an adequate amount of surrogate standard and diluted with methanol/HPLC grade water (50/50, v/v) to appropriate concentrations. LC–MS/MS analysis was performed using a Quattro Ultima (Micromass, Beverly, MA, USA) coupled to a HEWLETT PACKARD 1100 series (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer was operated using an electrospray ionization source in the positive ion mode (ESI⁺) for multiple reaction monitoring (MRM).

2.5. *In vivo* mutation assays

6-Thioguanine (TG) and Spi⁻ selections were performed as previously described [14,17–21]. Briefly, genomic DNA was extracted from the livers, and lambda EG10 DNA (48 kb) was rescued as the lambda phage by *in vitro* packaging. For 6-TG selection, the packaged phages were incubated with *E. coli* YG6020, which expresses 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, 9000-fold diluted phages were used to infect YG6020, and poured on the plates containing chloramphenicol without 6-TG. The plates were then incubated at 37 °C for selection of 6-TG-resistant colonies. Positively selected colonies were counted on day three and collected on day four. The MF was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For the Spi⁻ selection, the packaged phages were 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 where XL-1 Blue MRA, XL-1 Blue MRA P2 or WL95 P2 strains were spread with soft agar. Real Spi⁻ mutants, which made clear plaques on every plate, were counted.

To characterize the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously [19]. Sequencing analysis was done at Takara Bio Inc. (Mie, Japan).

2.6. *In vivo* mouse bone marrow MN test

The MN test was performed as described by Hayashi et al. [22]. Briefly, bone marrow was collected by mild centrifugation in centrifuge tubes, and pelleted marrow cells were resuspended in a small amount of serum, spread on clean microscope slides, air-dried, methanol fixed, and were finally stained with acridine orange (Wako Pure Chemical Industries, Ltd., Osaka, Japan). To determine MN frequencies, 2000 polychromatic erythrocytes (PCEs) were analyzed for each treatment and animal. PCEs are developmentally immature erythrocytes still containing ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes (NCEs) by selective ribosome staining. In addition, 1000 total (immature and mature) erythrocytes were scored for PCE frequency for each treatment and animal.

2.7. Statistical evaluation

The significance of differences in the body and liver weights and the results for the mutation assays were evaluated with ANOVA, followed by Dunnett's multiple comparison test. MN–PCEs were compared using Fisher's exact test. PCE frequencies were analyzed using Dunnett's *t*-test for group mean comparisons. Significant differences of RT-PCR analysis were evaluated by Student's *t*-test.

3. Results

3.1. Body weights, liver weights

Final body weights and liver weights are shown in Table 1. In the females treated with 300 mg/kg bw, a dead mouse appeared at week one and body weights decreased from week two. Therefore, the highest dose in females was reduced to 250 mg/kg bw from week two. One each dead mouse appeared in that group at weeks two, four, or 11. One male in the 37.5 mg/kg bw group died accidentally at week one. Body weights in the 150 and 250 mg/kg bw female group were significantly decreased as compared with the control group. Absolute and relative liver weights at doses greater than 75 mg/kg bw in males and 250 mg/kg bw females were significantly increased.

Table 1
Body and liver weights of B6C3F₁ *gpt* delta mice treated with estragole for 13 weeks.

	Dose (mg/kg bw)	Number of mice	Final body weights (g)	Liver weights	
				Absolute (g)	Relative (g%)
Male	0	10	32.5 ± 1.7 ^d	1.41 ± 0.07	4.33 ± 0.13
	37.5	9 ^b	31.6 ± 1.7	1.45 ± 0.11	4.59 ± 0.15
	75	10	33.1 ± 2.4	1.57 ± 0.09**	4.74 ± 0.23*
	150	10	31.6 ± 1.3	1.53 ± 0.10*	4.85 ± 0.28**
	300	10	30.9 ± 1.9	1.57 ± 0.13**	5.09 ± 0.26**
Female	0	10	25.8 ± 1.6	1.08 ± 0.07	4.17 ± 0.07
	37.5	10	25.7 ± 1.5	1.03 ± 0.09	4.00 ± 0.13
	75	10	24.6 ± 1.0	1.03 ± 0.07	4.18 ± 0.19
	150	10	24.4 ± 1.2*	1.08 ± 0.07	4.42 ± 0.30
	250 ^a	6 ^c	23.5 ± 0.5**	1.25 ± 0.05**	5.29 ± 0.17**

^a The highest dose was changed from 300 to 250 mg/kg bw after week two.

^b One mouse was found dead during week one accidentally.

^c Four mice were found dead during weeks one, two, four or 11.

^d Mean ± S.D.

* $p < 0.05$ vs. control (0 mg/kg bw).

** $p < 0.01$ vs. control (0 mg/kg bw).

3.2. Histopathological changes in the livers

No histopathological changes in the livers were found in males. In females, very slight hepatocellular hypertrophy was observed in two of 10, five of 10 and five of six animals in the 75, 150 and 250 mg/kg bw groups, respectively. These results are in good agreement with those in the NTP 3-month toxicity study of ES using wild-type B6C3F₁ mice [16].

3.3. Comparison of mRNA expression between male and female livers

Expression levels of *Cyp1a2* and *Sult1a1* in the livers of male and female mice are shown in Fig. 1. Expression levels of *Cyp1a2*

were not affected by sex differences, but those of *Sult1a1* were significantly higher in female groups as compared to male groups at the same doses of administration (except the highest dose). Expression levels were not affected in any of the treated groups in either sex.

3.4. The levels of ES-DNA adducts in mouse livers

The levels of ES-DNA adducts, ES-3'-8-dG, 3'-N²-dG and 3'-N⁶-dA, in liver DNA were measured by the LC-MS/MS method after ES-treating mice for 13 weeks (Fig. 2). The DNA adducts were detected in ES-treated livers, whereas no ES-DNA adducts were detected in controls. ES-specific DNA adduct formation from ES-treated mice increased in a linear dose-dependent manner, except

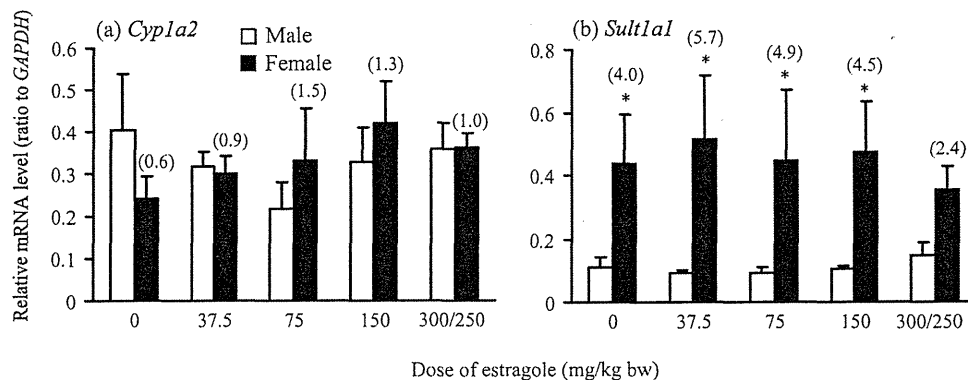


Fig. 1. *Cyp1a2* (a) and *Sult1a1* (b) mRNA expression determined by real-time RT-PCR in the livers of male and female mice. Data in the parentheses are shown as the fold change in the female mice relative to the expression levels of male mice. * $p < 0.01$, significantly different from corresponding male values.

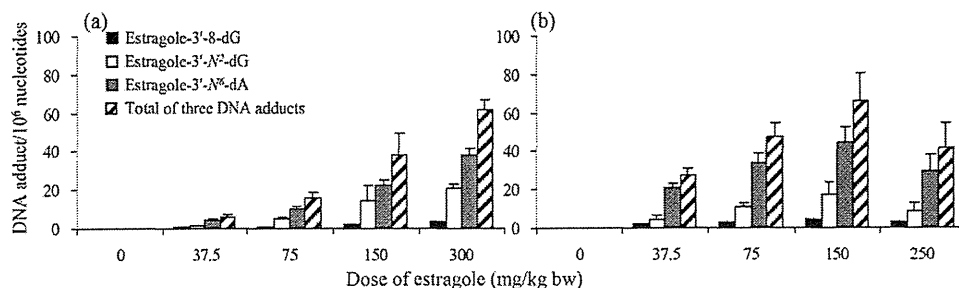


Fig. 2. Hepatic estragole DNA adduct levels in *gpt* delta (a) male and (b) female mice treated with 0, 37.5, 75, 150 and 300 or 250 mg/kg bw for 13 weeks. Each point represents the mean ± S.D. of five mice.

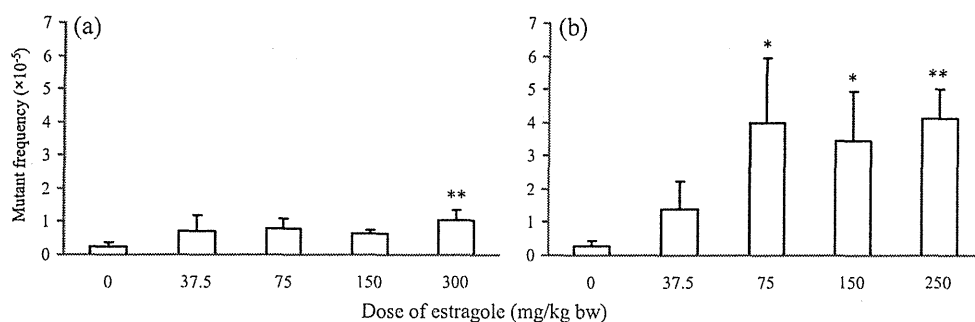


Fig. 3. Data for *gpt* mutant frequencies in livers of *gpt* delta (a) male and (b) female mice given estragole at concentrations of 0, 37.5, 75, 150 and 300 or 250 mg/kg bw for 13 weeks. Values are mean \pm S.D. of data for five mice. * $p < 0.05$, ** $p < 0.01$, significantly different from controls (0 mg/kg bw groups in males and females).

in the 250 mg/kg bw group in females. DNA adduct levels in females were higher than those of males treated with the same doses except at the highest dose.

3.5. In vivo mutation assays

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Fig. 3. The MFs in males at the highest doses and in females at doses greater than 75 mg/kg bw were significantly higher than in the respective control group. In males, the MFs in the highest group were significantly higher than those in the control group, but with a slight change. The MF values of females were higher than those of males treated with the same doses. To characterize *gpt* mutations in both sexes at the highest doses due to ES exposure, the mutant colonies were analyzed by DNA sequencing (Table 2). In male and female mice, G:C mutations were predominant, accounting for 62.1% (18/29) and 58.5% (69/118) of the mutations, respectively. In males, the specific mutation frequency of GC:TA transversion was significantly higher than in the control group. In females, the specific mutation frequency of GC:TA and GC:CG transversions, GC:AT transitions and complex mutations were significantly higher than the control group.

As shown in Fig. 4, while there were no significant differences in the Spi⁻ MFs between the male groups, the MF in the 250 mg/kg bw treated females was 1.16 ± 0.72 , which was significantly higher than the control value (0.28 ± 0.11). Since inactivation of the red/gam protein requires a disruption spanning the two

genes, a positive result in the Spi⁻ assay is indicative of an increase in deletion mutations [14], ES might cause deletion mutations as well as point mutations in the mouse liver DNA.

3.6. MN test in mouse bone marrow

The results are tabulated in Table 3. No significant increases in the frequencies of micronucleated PCEs were observed in male or female mice, and no significant changes in PCE frequency were shown, which indicates that ES induces no chromosomal aberration in the bone marrow. This is consistent with the fact that the bone marrow is not implicated as a target organ.

4. Discussion

ES is a natural constituent and human exposure level is estimated to be 0.01–0.07 mg/kg/day [23,24]. Although it has been reported that ES is hepatocarcinogenic and is capable of forming specific DNA adducts in the liver [1–5], genotoxic tests for ES have been equivocal [8–11] and the mechanism of ES-induced hepatocarcinogenicity is unclear. However, in our previous study, ES could be a possible genotoxic hepatocarcinogen in the rat, at least when given at high doses [25]. It has been proposed that ES carcinogenicity is linked to its metabolic conversion [1–3]. ES is metabolized to 1'-hydroxyestragole mainly by cytochrome P450 enzymes [6] followed by formation of 1'-sulfoxyestragole, the ultimate carcinogenic metabolite, by sulfotransferases

Table 2
Mutation spectra of *gpt* mutant colonies.

	Male		Female		Female		Female	
	0 mg/kg bw		300 mg/kg bw		0 mg/kg bw		250 mg/kg bw	
	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)
Base substitution								
Transversions								
GC:TA	2 ^a	0.04 ± 0.05^b	9	$0.37 \pm 0.23^*$	4	0.05 ± 0.07	32	$1.06 \pm 0.44^{**}$
GC:CG	2	0.04 ± 0.05	1	0.02 ± 0.04	0	0.00 ± 0.00	15	$0.40 \pm 0.24^{**}$
AT:TA	0	0.00 ± 0.00	2	0.09 ± 0.12	2	0.02 ± 0.03	11	$0.29 \pm 0.21^*$
AT:CG	0	0.00 ± 0.00	0	0.00 ± 0.00	1	0.01 ± 0.03	3	0.10 ± 0.17
Transitions								
GC:AT	5	0.08 ± 0.08	8	0.26 ± 0.29	9	0.12 ± 0.10	22	$0.68 \pm 0.30^{**}$
AT:GC	0	0.00 ± 0.00	2	0.04 ± 0.09	0	0.00 ± 0.00	14	$0.58 \pm 0.47^*$
Deletion								
Single bp	1	0.02 ± 0.04	2	0.06 ± 0.08	0	0.00 ± 0.00	4	0.19 ± 0.20
Over 1 bp	0	0.00 ± 0.00	1	0.07 ± 0.15	0	0.00 ± 0.00	0	0.00 ± 0.00
Insertion	0	0.00 ± 0.00	1	0.05 ± 0.11	2	0.02 ± 0.03	4	0.08 ± 0.14
Complex	1	0.01 ± 0.03	3	0.09 ± 0.12	3	0.04 ± 0.06	13	$0.46 \pm 0.33^*$

^a The number of colonies with independent mutations.

^b Mean \pm S.D.

* $p < 0.05$ vs. control (0 mg/kg bw).

** $p < 0.01$ vs. control (0 mg/kg bw).

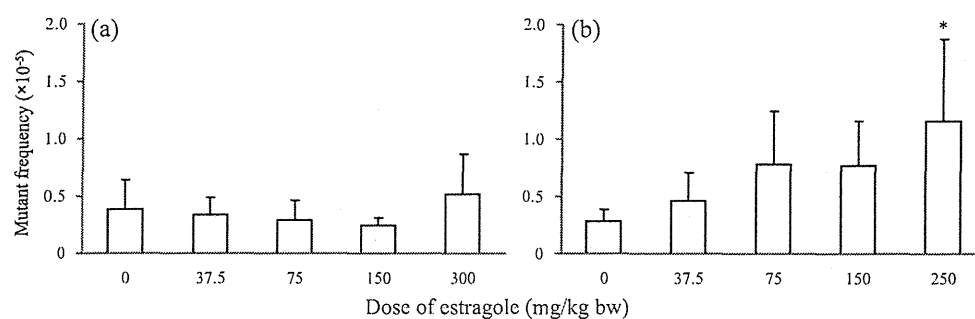


Fig. 4. Data for Spi⁻ mutant frequencies in livers of *gpt delta* mice given estragole at concentrations of 0, 37.5, 75, 150 and 300 or 250 mg/kg bw in (a) males and (b) females for 13 weeks. Values are mean \pm S.D. of data for five mice. * $p < 0.05$, significantly different from the controls (0 mg/kg bw groups in males and females).

Table 3
Frequency of micronuclei in bone marrow of mice following treatment with estragole by gavage for 13 weeks.

	Dose (mg/kg bw)	Number of mice	Micronucleated PCEs/PCEs (%)	PCEs/PCEs + NCEs (%)
Male	0	5	0.18 \pm 0.08 ^a	46.72 \pm 11.65
	37.5	5	0.13 \pm 0.08	46.52 \pm 7.74
	75	5	0.19 \pm 0.07	40.28 \pm 4.65
	150	5	0.15 \pm 0.05	40.28 \pm 5.71
	300	5	0.27 \pm 0.16	43.00 \pm 15.32
Female	0	5	0.07 \pm 0.06	50.54 \pm 7.62
	37.5	5	0.14 \pm 0.10	52.08 \pm 12.31
	75	5	0.14 \pm 0.07	49.58 \pm 5.83
	150	5	0.12 \pm 0.08	57.94 \pm 8.90
	250	5	0.11 \pm 0.07	59.42 \pm 10.63

NCE: normochromatic erythrocyte; PCE: polychromatic erythrocyte.

^a Mean \pm S.D.

(SULT). 1'-Sulfooxyestragole is unstable and subjected to degradation in an aqueous environment, consequently leading it to form a reactive carbocation that is capable of binding covalently to DNA [6,26,27]. As SULT activity and mRNA expression were reported to vary with sex [28,29], we made an attempt to compare changes in various parameters relating to carcinogenesis between male and female mice following ES exposure. Certainly, the present study demonstrated that mRNA levels of *Sult1a1* in female *gpt delta* mice were significantly higher than those in the males, in line with other strains of non-transgenic mice [28].

Quantification of DNA adduct levels by LC-MS/MS clearly showed that the levels of ES-specific DNA adducts in females were higher than those in the males. The expression levels of *Cyp1a2* catalyzing ES into proximate carcinogenic metabolites were comparable between females and males. Thus, it is highly probable that the sex difference of ES-specific DNA adduct levels resulted from the differences in SULT activity, supporting the proposed etiology of ES-specific DNA modifications. In addition, it has been reported that pre-exposure of mice to pentachlorophenol, a known inhibitor of SULT, inhibited ES-induced hepatocarcinogenicity as well as DNA adduct formation [12,13]. In the present *gpt* assay, the MFs of females were significantly higher at the low dose while those of males increased significantly only at the highest dose. Therefore, the overall data strongly suggest that ES-specific DNA adducts are formed by SULT, and consequent metabolites contribute to ES genotoxicity.

Although the recent *in vitro* data demonstrated that the amount of DNA adduct at dG was more abundant than that at dA [30,31], the present *in vivo* study showed that the predominant DNA adduct in any group in both sexes was ES-3'-N⁶-dA and the predominant mutations in the *gpt* assay were GC base-pair mutations. Considering that point mutations occur mainly at bases opposite to the modified one [32,33], it is likely that ES-3'-N⁶-dA could induce mutations at AT base-pairs. Thus, the amount of DNA adducts did not always result in mutations at the site. Additionally, in females, obvious increases in MF values were observed in all dosing groups

except at the lowest dose in which DNA adduct formation appeared to some extent, but MF values in males showed no change even though DNA adduct level of 150 mg/kg males was comparable to 75 mg/kg females. Also, the MF of males receiving the highest doses was significantly increased, but obviously lower than that of 150 mg/kg females in spite of equivalent DNA adduct levels. Therefore, it is suggested that ES-induced mutagenicity is related not only to DNA adduct formation mediated by SULT metabolic activation but also to other factors. For example, the cell proliferation progressed the cell cycle or Y-family polymerase required the error-prone replication of damaged DNA [32,34].

It has been reported that ES is hepatocarcinogenic in adult female mice [1–3] and SULT activity may contribute to the hepatocarcinogenicity [4,5]. In the present study, females (having higher *Sult1a1* expression level) showed higher levels of DNA adducts and MFs of the *gpt* gene compared to males. Therefore, it is suggested that the genotoxic mechanism is related to the carcinogenic mechanism of ES. It is likely that ES-specific DNA adduct formation by SULT mediated metabolites is responsible for its genotoxicity.

Conflict of interest statement

The authors declare no conflict of interests.

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