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DNA modifications that do not cause gene mutations confer the potential for mutagenicity by combined treatment with food chemicals

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A B S T R A C T

Cell proliferation plays a key role in fixing mutations induced by DNA damage. We clarified whether this phenomenon occurred after combined treatment with chemicals in food. The effects of antibiotic flumequine (FL), a residue of veterinary medicinal products in foodstuffs, on mutagenicity in the liver were examined in mice treated with estragole (ES), a natural food flavouring compound. *Gpt* delta mice were orally administered 10 or 100 mg/kg/day ES and simultaneously fed a diet containing 0.4% FL for 4 weeks. Proliferating cell nuclear antigen-positive cells and cell cycle-related genes were additively increased in the livers of combined treatment groups as compared with high-dose ES or FL groups. Mutant frequencies (MFs) in *gpt* after cotreatment with low-dose ES and FL were significantly increased, although treatment with ES alone increased MFs only in the high-dose group. *Sult1a1* mRNA levels were unchanged after FL treatment. Liquid chromatography with tandem-mass spectrometry analysis showed that FL did not affect the amount of ES-specific DNA adducts in the livers, indicating that FL treatment did not influence metabolic pathways of ES. Thus, enhancement of the mutagenic potential of a chemical by chemical-induced cell proliferation may occur as a result of the combined effects of chemicals in food.

1. Introduction

Chemical-specific DNA adducts can be a trigger for gene mutations and therefore play a critical role in chemical carcinogenesis (Swenberg et al., 1985). However, DNA adduct formation does not always lead to gene mutation. DNA modifications are known to be detected in various organs other than the carcinogenic target site (Bieler et al., 2007; Zuo et al., 2014), indicating that progression from DNA modification to gene mutation depends on the microenvironment. Moreover, various chemicals in foods, including carcinogens, are present as unexpected products generated during food processing or as contaminants. Therefore, investigation of the effects of co-exposure to chemicals in foods having the potential for modification of the microenvironment is essential for confirming the safety and security of food.

Cell proliferation is believed to play an important role in fixing mutations from DNA damage through replicative DNA synthesis (Bielas and Heddle, 2000; Tombolan et al., 1999). Additionally, co-exposure to chemicals inducing genotoxicity and enhancing cell proliferation may cause synergistic deleterious effects. However, there have been few reports demonstrating this phenomenon using animal models because of potential interactions of the metabolic pathways of chemicals. Indeed, chemicals in foods have various properties, including effects on metabolising enzymes (Abdull Razis et al., 2012; Lnenickova et al., 2017; Zhang et al., 1992). These effects can influence experimental conditions, resulting in differences in the actual level of exposure to the treatment chemical among groups (Kuroda et al., 2013).

Flumequine (FL), an antibacterial quinolone agent, induces hepatotoxicity in mice, resulting in hepatocyte vacuolation, inflammatory cell infiltration, and induction of compensatory hepatocyte proliferation (Yoshida et al., 1999; Kuroiwa et al., 2007). Based on the negative results of various genotoxicity tests, FL is thought to be a nongenotoxic hepatocarcinogen. Compensatory regeneration resulting from its hepatotoxicity may be responsible for induction of liver tumours in mice (JECFA, 2004). In particular, because FL is suspected to persist in the edible tissues of domestic animals and fish (Choma et al., 1999; Karbiwnyk et al., 2007; Gajda et al., 2012), we have evaluated the role of FL as a modifier of the microenvironment around cells initiated by carcinogens contaminated in food. In our previous studies, we reported

Abbreviations: dA, deoxyadenosine; dG, deoxyguanosine; ES, estragole; FL, flumequine; HD, high dose; HE, hematoxylin and eosin; LD, low dose; MeIQx, 2-amino-3,8-diemethylimidazo[4,5-f]quinoxaline; MF, mutant frequency; SULT, sulfotransferase

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that co-exposure to FL plus 2-amino-3,8-diemethylimidazo [4,5-*f*]quinoxaline (MeIQx), a heterocyclic amine, enhances MeIQx genotoxicity (Kuroda et al., 2013). However, gene expression analysis revealed that FL treatment also altered the expression of several enzymes involved in MeIQx metabolism, indicating that the actual level of MeIQx exposure could differ between experimental groups as described above.

Estragole (ES), an allylbenzene compound, is a natural flavour in many herbs and spices. Therefore, despite showing hepatocarcinogenicity in rodents (Miller et al., 1983; Suzuki et al., 2012a), ES is an unintentional contaminant of foods. Although equivocal results have been generated in several *in vitro* and *in vivo* mutagenicity tests (Zeiger et al., 1987; Sekizawa and Shibamoto, 1982; Swanson et al., 1979; Swanson et al., 1979, 1979), our previous study clearly showed that specific DNA modifications generated by sulfotransferase (SULT) 1A1-mediated carbocation formation and the resulting genotoxicity are key events in the early stages of ES-induced hepatocarcinogenesis in mice (Suzuki et al., 2012b). In addition to the lack of effect of FL treatment on SULT1A1 status (Kuroda et al., 2013), our established analytical method for ES-specific DNA adducts could be used to confirm actual ES exposure levels (Ishii et al., 2011). Therefore, ES and FL may be an appropriate combination to investigate the effects of microenvironment alterations on genotoxicity as a result of the combined effects of chemicals in foods.

In the current study, in order to investigate the effects of co-exposure to FL on the *in vivo* mutagenicity of ES in the mouse liver, *gpt* delta mice (Nohmi et al., 1996; Masumura et al., 2015) were given ES and FL at several doses for 4 weeks. Mouse livers were used for histopathological examination, proliferating cell nuclear antigen (PCNA) immunostaining, gene expression analysis, ES-specific DNA adduct analysis, and reporter gene mutation assays.

2. Materials and methods

2.1. Chemicals and reagents

FL, a white crystalised powder (purity: 99.3%), was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). ES (purity: > 98%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). ES-specific DNA adducts were synthesised from acetylated 1-hydoroxy ES, deoxyguanosine (dG), and deoxyadenosine (dA), as described in our previous report (Ishii et al., 2011).

2.2. Animals and treatments

The protocols for this study were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (approval no. 367). B6C3F1 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome were generated by mating C57BL/6 *gpt* delta and nontransgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Thirty male B6C3F1 *gpt* delta mice were randomised by weight into six groups. Animals were housed in polycarbonate cages (five mice per cage) with hardwood chips for bedding in a conventional animal facility and maintained under a controlled temperature (23 ± 2 °C), humidity (55% ± 5%), and lighting (12-h light/dark cycle) with regular air changes (12 times/h). The animals were given free access to a CRF-1 basal diet (Oriental Yeast, Tokyo, Japan) and tap water and acclimated for 1 week prior to the experimental period.

Starting at 6 weeks of age, *gpt* delta transgenic mice were administered 10 or 100 mg/kg/day ES in corn oil by oral gavage once daily with/without feeding a diet containing 0.4% FL for 4 weeks. Animals in the control and FL only groups were administered corn oil by oral gavage and given the basal diet or a diet containing 0.4% FL, respectively, for 4 weeks. General signs were observed daily, and body weight and food consumption per cage were measured once weekly. At necropsy, livers were weighed, and a portion of the left lateral lobe was fixed in 10% neutral formalin. Fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) or used for immunohistochemical staining. The remaining liver tissues were stored at −80 °C for *in vivo* mutation assays, DNA adduct analysis, and gene expression analysis.

2.3. Immunohistochemical staining for PCNA

Paraffin-embedded sections from groups of five animals were used. Immunohistochemical staining for PCNA was performed using monoclonal anti-mouse PCNA antibodies (1:100; Dako Denmark A/S, Glostrup, Denmark) followed by incubation with a high polymer stain (HISTOFINE Simple Stain, Nichirei Bioscience Inc., Tokyo, Japan). At least 2000 intact hepatocyte cells in the liver were counted for each animal, and labelling indices were calculated as the percentage of cells positive for PCNA staining.

2.4. In vivo mutation assays (gpt assays)

6-Thioguanin (6-TG) and Spi$^-$ selections were performed as previously described (Nohmi et al., 2000). 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 phage was incubated with *Escherichia 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. To determine the total number of rescued plasmids, phages diluted 3000-fold were used to infect YG6020 and poured on 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 3, and the colonies were collected on day 4. The mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the number of rescued phages. To characterise the mutation spectra of *gpt* mutants, a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene was amplified by polymerase chain reaction (PCR) using two primers (primer 1 [forward]: 5′-TACCACTTTATCCCGCGTCAGG-3′, primer 2 [reverse]: 5′-ACAGGGTTTGGCTCAGGTTTGC-3′; Eurofins Genomics Inc., Louisville, KY, USA) as described previously (Nohmi et al., 2000). PCR amplification was carried out in a 40-μL volume containing 5 pmol of each primer, 200 mM of each dNTP, and 1 U *Taq* DNA polymerase (TaKaRa Ex Taq; Takara Bio Inc., Shiga, Japan). Bacterial colonies were used as a source of template DNA. Briefly, 6-TG-resistant cells were transferred from the cell pellet to the reaction mixture using a toothpick. Amplification was performed using 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 120 s. PCR products were analysed by agarose gel electrophoresis to determine the amount of product generated. DNA sequencing was carried out at Takara Bio Inc. The specific MF was calculated by dividing the number of each mutation except clonal expansion of a single mutant by the number of rescued phages.

2.5. In vivo mutation assays (Spi$^-$ assays)

For Spi$^-$ selection, packaged phage was incubated with *E. coli* XL-1 Blue MRA and *E. coli* XL-1 Blue MRA P2 for survival titration and mutant selection, respectively. Infected cells were mixed with molten lambda-trypticase soft agar and poured onto lambda-trypticase agar plates. The following day, plaques (Spi$^-$ candidates) were collected with sterilised glass pipettes, and the agar plugs were suspended in SM buffer. To confirm the Spi$^-$ phenotype of the candidates, suspensions were spotted on three types of plates on which XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. Spi$^-$ mutants that produced clear plaques on all three plate types were counted.

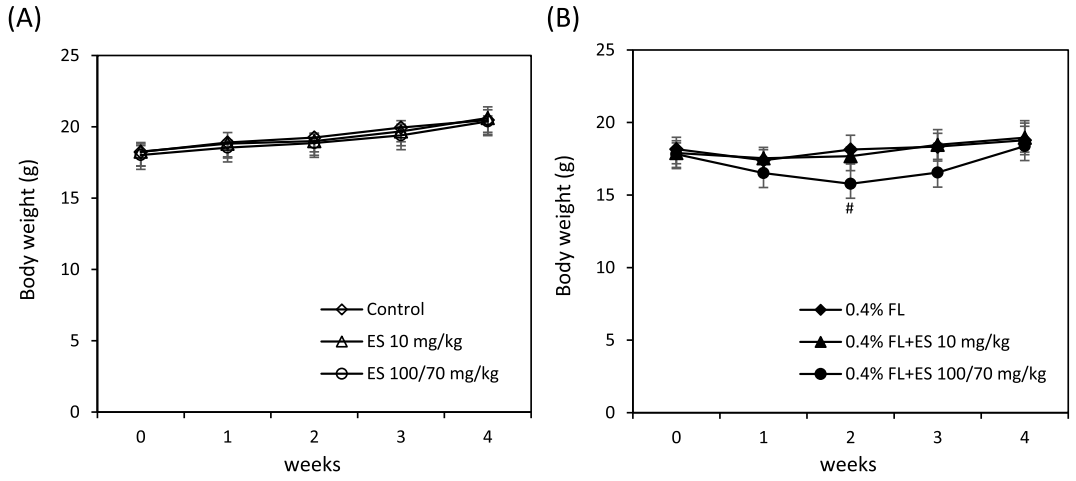

Fig. 1. Body weights for B6C3F1 *gpt* delta mice in the control, LD-ES, and HD-ES groups (A) and FL, LD-ES + FL, and HD-ES + FL groups (B). Data represent means ± SDs (n = 4 or 5). #: $p < 0.05$ versus the 0.4% FL + vehicle group.

Table 1
Filial body and liver weights in B6C3F1 *gpt* delta mice treated with ES with/without FL.

Item	Basal diet			0.4% FL		
	Vehicle	ES 10 mg/kg	ES 100/70 mg/kg	Vehicle	ES 10 mg/kg	ES 100/70 mg/kg
No. of animals	5	5	5	5	5	4
Body weight (g) Absolute (g)	20.5 ± 0.3[a]	20.6 ± 0.8	20.4 ± 0.8	18.8 ± 1.2	19.0 ± 0.8	18.4 ± 1.8
Liver	0.89 ± 0.05	0.92 ± 0.05	0.95 ± 0.02	0.99 ± 0.11	0.93 ± 0.04	1.03 ± 0.06
Relative (g%) Liver	4.37 ± 0.22	4.45 ± 0.11	4.65 ± 0.17	5.26 ± 0.30[$$]	4.98 ± 0.09[$]	5.60 ± 0.34[$$]

[$, $$]: $p < 0.05$, 0.01 vs. Each basal diet groups.
[a] Data represent means ± SD ($n = 4$ or 5).

2.6. RNA isolation and quantitative real-time PCR for mRNA expression

Total RNA was extracted using ISOGEN (Nippon Gene Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions. The concentration and quality of total RNA were analysed by UV-VIS spectrophotometry (Nanodrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). cDNA copies reverse transcribed from total RNA were obtained using a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed with primers for mouse *Ccnb1* (Mm03053893_gH), *Ccnd1* (Mm00432359_m1), *Ccne1* (Mm01266311_m1), *Tnf* (Mm00443258_m1), *Il1b* (Mm00434228_m1), *Cyp1a2* (Mm00487224_m1), *Sult1a1* (Mm00467072_m1), and *Ugt1a1* (Mm02603337_m1). TaqMan Rodent GAPDH Control Reagents were used as an endogenous reference. PCR was carried out in an Applied Biosystems 7900HT FAST Real-Time PCR System using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays (Thermo Fisher Scientific). The expression levels of target genes were calculated using the relative standard curve method and were determined by normalisation to *GAPDH* expression. Data are presented as fold-change of treated samples relative to controls.

2.7. Analysis of ES-specific DNA adducts

Levels of ES-specific DNA adducts in livers were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS), as previously described (Ishii et al., 2011). Briefly, frozen liver tissues were homogenised with lysis buffer included in the DNA Extractor WB kit (FUJIFILM Wako Pure Chemical Corp., Japan). The mixture was centrifuged, and the resulting pellet was dissolved in enzyme reaction buffer. After treatment with RNase and protease K, the resulting dried DNA pellet was resuspended in surrogate standard solution containing sodium acetate buffer (pH 4.8) and incubated with nuclease P1. After addition of Tris-HCl buffer (pH 8.2), the solution was incubated with alkaline phosphatase. Sodium acetate buffer (pH 5.1) was then added, and 1 mL of the digested samples was diluted with an equal volume of methanol and injected into the LC-MS/MS. ES-3′-C8-dG and ES-3′-N^2-dG and ES-3′-N^6-dA solutions (1 mM) were prepared in methanol and immediately diluted with methanol/high-performance liquid chromatography (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 diluting with methanol/HPLC-grade water (50%/50%, v/v) to the indicated concentration. LC-MS/MS analysis was performed using a Quattro Ultima Pt triple stage quadrupole mass spectrometer (Waters Micromass, Milford, MA, USA) equipped with an Agilent 1100 LC system (Agilent Technologies, Palo Alto, CA USA). The mass spectrometer was operated using an electrospray ionisation source in the positive ion mode for multiple reaction monitoring.

2.8. Statistical evaluation

The significance of differences between body and liver weights, *gpt* and Spi$^-$ MFs, specific MFs, PCNA-positive indices, and gene expression levels was evaluated. For comparisons between groups treated with ES or FL alone, each parameter was evaluated for homogeneity using Bartlett's tests. For homogenous and heterogeneous data, one-way analysis of variance and Kruskal-Wallis tests, respectively, were applied. When statistically significant differences were indicated, Dunnett's multiple tests were used to compare results for the control and treatment groups. For comparisons between groups treated with and without FL, differences in each parameter measured for FL-treated and untreated groups were evaluated using Tukey's test.

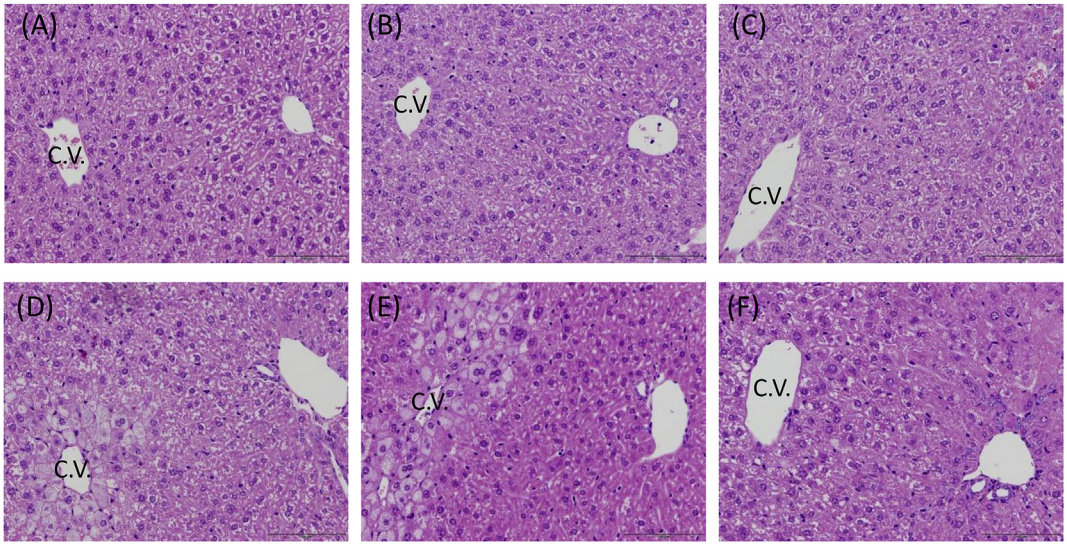

Fig. 2. Histopathological changes in the livers of *gpt* delta mice in the control (A), LD-ES (B), HD-ES (C), FL (D), LD-ES + FL (E), and HD-ES + FL (F) groups. C.V., central vein.

Table 2
gpt MFs in the livers of*gpt* delta mice treated with ES for 4 weeks.

Treatment		Animal No.	Cm^R colonies (x 10^5)	6-$TG^R$and Cm^R colonies	Mutant frequency (x 10^{-5})	Mean ± SD (x 10^{-5})
Basal diet	ES0 mg/kg/day	101	–	–	–	0.49 ± 0.25[a]
		102	4.7	4	0.85	
		103	17.8	5	0.28	
		104	21.2	8	0.38	
		105	10.8	5	0.46	
	ES 10 mg/kg/day	201	15.2	8	0.53	0.48 ± 0.15
		202	13.3	3	0.23	
		203	11.4	6	0.52	
		204	14.3	9	0.63	
		205	12.2	6	0.49	
	ES 100/70 mg/kg/day	301	12.5	11	0.88	1.11 ± 0.55*
		302	7.2	10	1.38	
		303	6.7	13	1.94	
		304	11.7	9	0.77	
		305	10.4	6	0.58	
0.4% FL in diet	ES10 mg/kg/day	401	16.9	5	0.30	0.55 ± 0.26
		402	7.9	5	0.63	
		403	5.4	5	0.92	
		404	4.9	6	0.61	
		405	9.6	6	0.31	
	ES 10 mg/kg/day	501	11.7	12	1.03	1.04 ± 0.19
		502	9.0	9	1.00	
		503	8.3	9	1.08	
		504	7.6	10	1.31	
		505	9.1	7	0.77	
	ES 100/70 mg/kg/day	601	6.1	16	2.63	1.88 ± 0.73##, $
		602	–	–	–	
		603	12.0	27	2.26	
		604	5.2	5	0.97	
		605	9.6	16	1.67	

*: $p < 0.05$ vs. Basal diet + Vehicle group.
##: $p < 0.01$ vs. 0.4% FL + Vehicle group.
$: $p < 0.05$ vs. Eachbasal diet groups.
[a] Data represent means ± SD ($n = 4$ or 5).

3. Results

3.1. General signs, body weight, and liver weight

All FL-treated mice showed reduced body weight gain (Fig. 1). Animals in the high-dose (HD)-ES + FL group showed severe body weight loss, and one animal died at 2 weeks. Therefore, beginning at week 3, the HD was reduced from 100 mg/kg/day to 70 mg/kg/day ES in both experimental groups. In all groups treated with FL, the final body weight was clearly decreased, and the relative liver weight was significantly increased compared with those in the control and ES alone groups, respectively (Table 1).

3.2. Histopathological changes in the liver

Histopathological analysis showed hypertrophy of centrilobular

Table 3
Mutation spectra of gpt mutants in the livers ofgpt delta mice treated with ES for 4 weeks.

Specific mutation frequencies ($xl0^{-5}$)						
	Basal diet + ES (mg/kg/day)			0.4% FL in diet + ES (mg/kg/day)		
	0	10	100/70	0	10	100/70
Base substitution Transversion						
G:C-T:A	0.04 ± 0.07[a]	0.09 ± 0.06	0.18 ± 0.10*	0.06 ± 0.09	0.33 ± 0.06#$	0.54 ± 0.26*##$$
−G:C-C:G	0	0	0.02 ± 0.04	0	0.05 ± 0.06	0.21 ± 0.25
A:T-T: A	0.05 ± 0.11	0.03 ± 0.05	0.08 ± 0.13	0	0.02 ± 0.05	0.18 ± 0.14##
A:T-C:G	0	0.03 ± 0.06	0	0	0.02 ± 0.06	0
Transition						
G:C-A:T	0.20 ± 0.06	0.18 ± 0.14	0.43 ± 0.22	0.27 ± 0.23	0.30 ± 0.11	0.55 ± 0.40
A:T-G:C	0.01 ± 0.02	0.01 ± 0.03	0.17 ± 0.12	0.04 ± 0.09	0.12 ± 0.12	0.09 ± 0.11
Deletion						
Single bp	0.07 ± 0.08	0.09 ± 0.06	0.10 ± 0.06	0.15 ± 0.16	0.13 ± 0.09	0.07 ± 0.08
Over 2bp	0	0.02 ± 0.04	0.08 ± 0.11	0	0	0.12 ± 0.15
Insertion	0.12 ± 0.21	0.03 ± 0.05	0	0.03 ± 0.06	0.02 ± 0.05	0.04 ± 0.08
Complex	0	0	0.06 ± 0.13	0	0.04 ± 0.06	0.08 ± 0.16

*: $p < 0.05$ vs. Basal diet + Vehicle group.
#, ##: $p < 0.05, 0.01$ vs. 0.4% FL + Vehicle group.
$, $$: $p < 0.05, 0.01$ vs. Each basal diet groups.
[a] Data represent means ± SD ($n = 4$ or 5).

Table 4
Spr MFs m the livers *gpt* delta mice treated with ES for 4 weeks.

Treatment		Animal No.	Plaques within XL-1 Blue MRA ($xl0^5$)	Plaques within WL95 (P2)	Mutant frequency (x 10^{-5})	Mean ± SD
Basal diet	ES 0 mg/kg/day	101	–	–	–	0.26 ± 0.13[a]
		102	6.3	1	0.16	
		103	27.7	5	0.18	
		104	32.2	8	0.25	
		105	13.5	6	0.44	
	ES 10 mg/kg/day	201	34.3	3	0.09	0.22 ± 0.10
		202	22.3	8	0.36	
		203	21.2	6	0.28	
		204	22.4	4	0.18	
		205	25.0	5	0.20	
	ES 100/70 mg/kg/day	301	21.8	3	0.14	0.22 ± 0.13
		302	26.8	3	0.11	
		303	21.3	5	0.23	
		304	24.8	5	0.20	
		305	13.6	6	0.44	
0.4% FL in diet	ES 0 mg/kg/day	401	19.4	3	0.16	0.30 ± 0.12
		402	8.8	4	0.45	
		403	4.5	1	0.22	
		404	3.6	1	0.28	
		405	13.6	5	0.37	
	ES 10 mg/kg/day in diet	501	26.4	15	0.57	0.22 ± 0.13
		502	23.4	6	0.26	
		503	17.1	3	0.18	
		504	22.1	3	0.14	
		505	20.0	7	0.35	
	ES 100/70 mg/kg/day	601	4.1	2	0.49	0.30 ± 0.12
		602	–	–	–	
		603	13.7	1	0.07	
		604	17.6	6	0.29	
		605	8.6	2	0.44	

[a] Data represent means ± SD ($n = 4$ or 5).

hepatocytes with vacuolation in liver tissues from groups treated with FL but not those exposed to ES alone (Fig. 2). Slight cell infiltration was also observed in the FL-treated groups.

3.3. Effects of FL treatment on ES-induced gene mutations

The results of *gpt* and Spi^- assays are indicative of increases in point mutations and deletion mutations, respectively. Compared with untreated control animals, *gpt* MFs were significantly increased in the HD-ES group, but not the low-dose (LD)-ES group (Table 2). In the HD-ES + FL group, *gpt* MFs were further increased, and this increase was statistically significant compared with those in the FL and HD-ES groups. Additionally, *gpt* MFs were clearly increased in the LD-ES + FL group compared with those in the FL and LD-ES groups. Spectrum analysis for *gpt* mutants showed increases in G:C-T:A transversions and G:C-A:T transition mutations in the HD-ES group (Table 3). These mutations were further increased in the HD-ES + FL group, and the increase in G:C-T:A transversions was statistically significant compared with those in the FL and HD-ES groups. A statistically significant increase in G:C-T:A transversions was also observed in the LD-ES + FL group compared with those in the FL and LD-ES groups. However, we detected no changes in Spi^- MFs among the experimental groups using

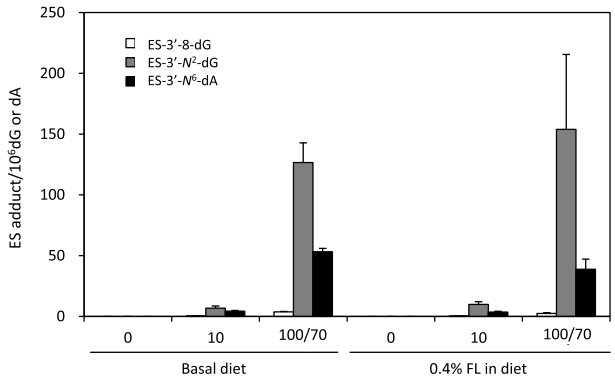

Fig. 3. Levels of the ES-specific DNA adducts ES-3′-8-dG, ES-3′-N^2-dG and ES-3′-N^6-dA in the livers of *gpt* delta mice treated with ES and/or FL for 4 weeks. Data represent means ± SDs (n = 4 or 5).

Spi$^-$ assays (Table 4). These results indicated that point mutations were dominantly induced by ES.

3.4. Effects of FL treatment on ES-induced DNA damage

ES-specific DNA adducts in the livers of ES-treated *gpt* delta mice were quantified by isotope-diluted LC-MS/MS analysis. ES-3′-N^2-dG, ES-3′-8-dG, and ES-3′-N^6-dA were detected in the livers of mice in all ES-treated groups (Fig. 3). DNA adduct levels were dose-dependently increased in all experimental groups and were not affected by combined treatment with ES and FL.

3.5. Effects of FL treatment on hepatocyte proliferation

The number of PCNA-positive hepatocytes was significantly increased in the HD-ES and HD-ES + FL groups compared with those in the control and FL groups, respectively, and were significantly increased in all FL-treated groups compared with those in the control, HD-ES, and LD-ES groups (Fig. 4A). The expression levels of the cell cycle progression factors *Ccnb1* and *Ccnd1* were significantly increased in the HD-ES and HD-ES + FL groups compared with those in the control and FL groups, and there were significant differences between the two groups (Fig. 4B–D). In the LD-ES + FL group, the expression levels of *Ccnb1*, *Ccnd1*, and *Ccne1* were significantly increased compared with those in the LD-ES group. In addition, *Ccnb1* and *Ccne1* expression levels were significantly increased in the FL group compared with those in the control group.

3.6. Effects of FL treatment on the expression of genes encoding inflammatory cytokines and metabolic enzymes

In our previous study, we analysed representative genes that showed altered expression in response to FL treatment or genes related to ES metabolism by quantitative real-time RT-PCR (Kuroda et al., 2013). The expression levels of the cytokine *Tnf* were significantly increased in the HD-ES + FL and LD-ES + FL groups compared with those in the HD-ES and LD-ES groups, whereas no changes in *Il1b* expression were observed in all experimental groups (Fig. 5). In an analysis of the expression of genes encoding enzymes involved in ES metabolism, *Cyp1a2* levels were significantly increased in the HD-ES group compared with those in the control group and were significantly increased in the FL and LD-ES + FL groups compared with those in the control and LD-ES groups, respectively (Fig. 6A). The expression of *Ugt1a1* was significantly decreased in the FL and HD-ES + FL groups compared with those in the control and HD-ES groups, respectively (Fig. 6B). *Sult1a1* expression was similar among all experimental groups (Fig. 6C).

4. Discussion

In this study, we found that a 4-week treatment with HD-ES caused significant increases in *gpt* MFs in the livers of *gpt* delta mice. Mutation spectrum analysis of *gpt* mutant colonies showed a characteristic

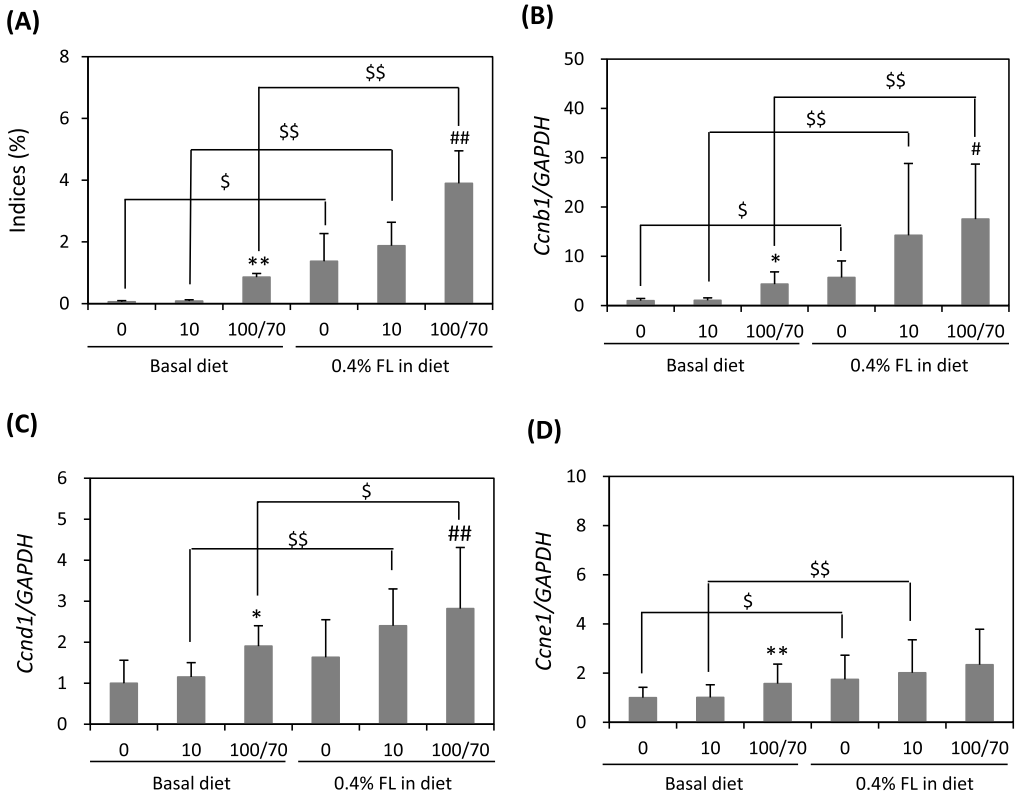

Fig. 4. Changes in cell cycle-related parameters in livers of *gpt* delta mice treated with ES and/or FL for 4 weeks. (A) Proliferating cell nuclear antigen (PCNA)-positive indices (%) of control hepatocytes and mRNA levels of *Ccnb1* (B), *Ccnd1* (C), and *Ccne1* (D). Data represent means ± SDs (n = 4 or 5). *, **: $p < 0.05$, 0.01 versus the basal diet + vehicle group. #, ##: $p < 0.05$, 0.01 versus the 0.4% FL + vehicle group. $, $$: $p < 0.05$, 0.01 versus each basal diet group.

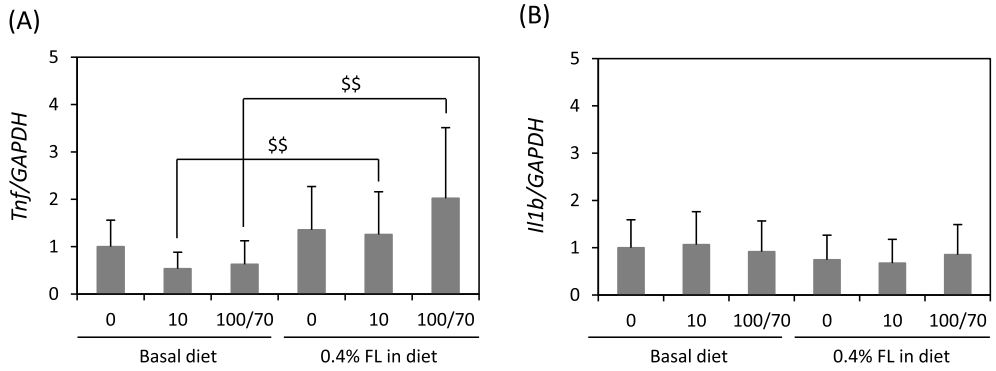

Fig. 5. Changes in mRNA levels of the cytokines *Tnf* (A) and *Il1b* (B) in the livers of *gpt* delta mice treated with ES and/or FL for 4 weeks. Data represent means ± SDs (n = 4 or 5). $, $$: $p < 0.05, 0.01$ versus each basal diet group.

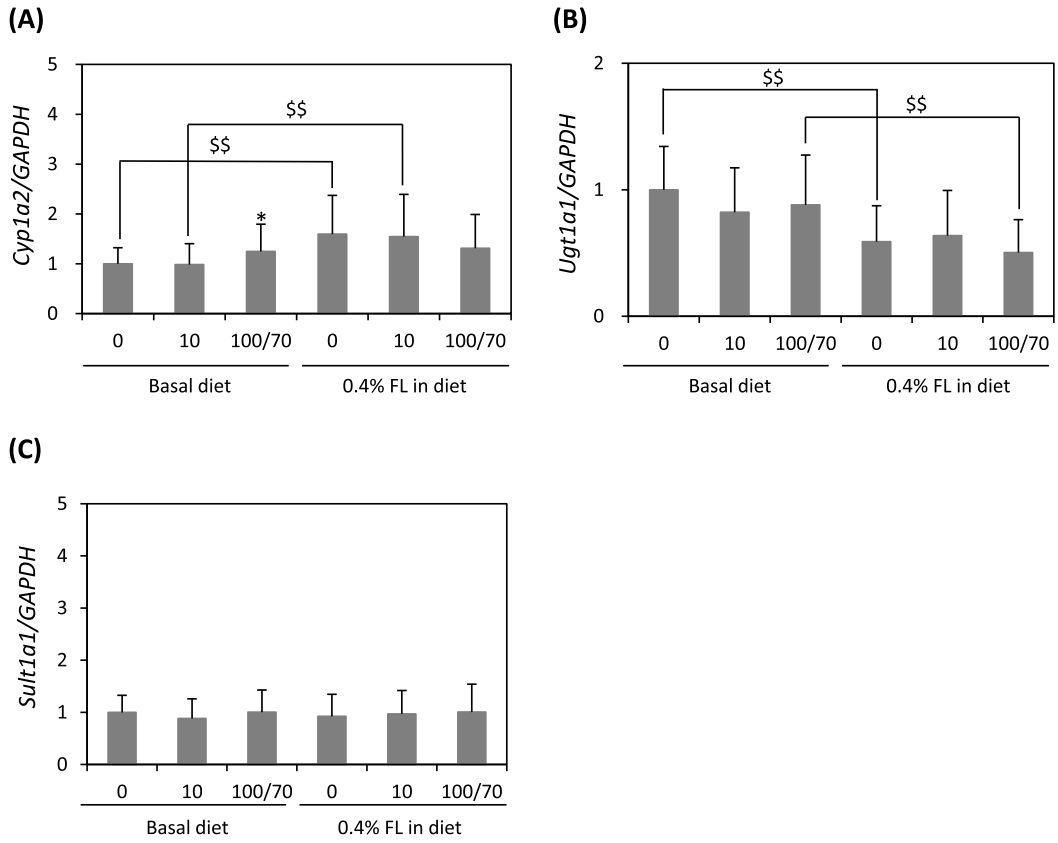

Fig. 6. Changes in mRNA levels of the metabolic enzymes *Cyp1a2* (A), *Ugt1a1* (B), and *Sult1a1* (C) in the livers of *gpt* delta mice treated with ES and/or FL for 4 weeks. Data represent means ± SDs (n = 4 or 5). *: $p < 0.05$ versus the basal diet + vehicle group. $$: $p < 0.01$ versus each basal diet group.

mutation spectrum induced by ES exposure, including base substitution at G:C base pairs that was consistent with previous reports (Suzuki et al., 2012a). Combined treatment with FL and HD-ES was able to further increase *gpt* MFs with statistical significance as compared with that in the HD-ES-treated group. Cotreatment with FL and LD-ES tended to increase *gpt* MFs as compared with those in mice treated with LD-ES alone, which failed to elevate *gpt* MFs. In addition, MFs of G:C-T:A transversion mutations, which are ES-specific mutations, as described above, were significantly increased in the combined treatment group as compared with those in mice given LD-ES. Because no changes in *gpt* MFs were observed in mice treated with FL alone, these results indicated that FL enhanced mutagenicity in the presence of ES exposure.

ES is metabolically activated by Cyp1A2, followed by Sult1A1-mediated reactions to generate the nucleophilic form; this leads to the formation of ES-specific DNA adducts, such as ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA (Jeurissen et al., 2007; Suzuki et al., 2012b). Comprehensive DNA adduct analysis indicated the formation of five ES-specific DNA adducts, including three dG adducts, one dA adduct, and one deoxycytidine adduct (unpublished data). In particular, two dG and one dA adducts were detected as major adducts and identified as ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA, respectively. The amounts of unidentified dG and dC adducts were much lower than the amounts of identified adducts. Therefore, it is highly probable that ES-3′-N^2-dG, ES-3′-C8-dG, and ES-3′-N^6-dA are responsible for ES-induced gene mutations, such as G:C-T:A, A:T-T:A transversions, and A:T-G:C transitions (Ishii et al., 2017; Suzuki et al., 2012b). Our previous data demonstrated that FL did not affect sulfotransferase activity; however, some metabolising enzymes, such as Cyp1A2 and Ugt1a1, were slightly modified (Kuroda et al., 2013). Because Cyp1A2 and Ugt1a1 are key enzymes involved in activation and elimination of ES, respectively, it is important to determine whether combined treatment with FL affects the metabolism of ES. Therefore, we used real-time RT-PCR to measure

the gene expression levels of *Cyp1A2*, *Ugt1A1*, and *Sult1A1*; the protein products of these genes (i.e., Cyp1A2, Ugt1A1, and Sult1A1 proteins) contribute to the metabolism of ES. As in previous studies, FL-treatment slightly affected the expression of *Cyp1A2* and *Ugt1A1* genes, but not the *Sult1A1* gene (Kuroda et al., 2013). However, we observed no changes in the amounts of DNA adducts between the corresponding ES-treated groups. These data implied that the experimental conditions in the current study were appropriate to achieve the aim of this study. Based on these results, we concluded that the actual ES exposure levels in the ES-treated group were almost identical to those in the ES- and FL-treated groups. Therefore, it is likely that FL was able to change the microenvironment surrounding ES-initiated cells in order to enhance its mutagenicity. In particular, we noted a relationship between the amount of DNA adducts and their potential to induce gene mutations in LD-ES-treated groups. Data for these groups implied that the amount of DNA adducts lacking the potentials for inducing gene mutations were able to acquire its power by the alterations of microenvironment.

In the current study, FL treatment was found to cause significant increases in the number of PCNA-positive hepatocytes and expression of cell cycle-related genes, including *Ccnb1* and *Ccne1* (encoding cyclin B1 and E1). Based on findings for hepatocyte injury-related gene expression, these results regarding cell proliferation may represent a compensatory response, as suggested by our previous study (Kuroda et al., 2013). Our recent study showed that inactivation of protein phosphatase 2 A may be responsible for ES-induced cell proliferation (Ishii et al., 2017). Although it is unknown whether FL is able to inactivate this protein, simple analyses of *Ccnb1* expression and PCNA-positive cell numbers showed that the combined values were far in excess of the additive values. Therefore, it is likely that FL exhibited synergistic enhancement of cell proliferation induced by ES. At any rate, induction of hepatocyte proliferation by FL through alteration of the microenvironment resulted in enhancement of ES-induced mutagenicity. Lower doses of ES failed to induce cell proliferation and did not cause gene mutations, despite formation of ES-specific DNA adducts to some extent. Therefore, it is likely that ES-induced gene mutations following formation of ES-specific DNA adducts require cell proliferation. Accordingly, our current results demonstrated that cell proliferation, as a prerequisite for ES-induced mutagenicity, was able to be promoted by other chemicals. Additionally, these combined effects occurred when using a combination of chemicals in food.

Recently, highly sensitive DNA adduct analysis using ultra-performance LC-MS/MS revealed that DNA adducts induced by methyleugenol, an alkoxylbenzene derivative similar to ES, could be detected in human tissue samples (Monien et al., 2015). The data implied that DNA adducts were formed in human tissues by chemicals that unintentionally contaminated foods. In addition, our data suggested that not only chronic inflammation but also microenvironmental modifiers of chemicals in foods could increase the risk of genotoxicity from chemicals in foods.

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Transparency document

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