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Original Article

In vitro toxicity studies of epoxyoleic acid and diepoxylinoleic acid

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ABSTRACT — Epoxidized fatty acids are generated during food processing and detected in various lipid- and oil-containing foods. Although compounds with an epoxide structure have high reactivity and there is increasing concern about their potential toxic effects such as genotoxicity and cellular damage, information regarding the toxicity of epoxy fatty acids is largely unknown. Therefore, we conducted three *in vitro* genotoxicity studies (bacterial reverse mutation assay, *in vitro* micronucleus test, and p53R2-dependent luciferase reporter gene assay) and HepG2 cytotoxicity assays for epoxyoleic acid (EOA) and diepoxylinoleic acid (DELA). The *in vitro* genotoxicity results of EOA and DELA were uniformly negative. In the cytotoxicity assay, EOA and DELA induced weak cytotoxicity at high concentrations, but the effect was similar to those of oleic and linoleic acids at low concentrations. Considering the existing toxicity information on epoxidized soybean oil, which is a similar compound, there might be little concern concerning the health effects of epoxy fatty acids at low concentrations.

Key words: Epoxy fatty acids, Genotoxicity, Cytotoxicity, Food contaminant

INTRODUCTION

Food processing, such as heating, baking, and frying, is an essential component of human life that improves the digestibility and bioavailability of nutrients, increases appetite by generating characteristic flavors and textures, extends food storage, and prevents food poisoning (Hidalgo and Zamora, 2017; Hoffman and Gerber, 2015). A wide variety of compounds are generated during food processing or storage through various processes (e.g., degradation, oxidation, and bacterial digestion) (Hidalgo and Zamora, 2017; Fu *et al*., 2019). Among them, lipid oxidation is a major cause of quality deterioration for lipid-containing meals leading to nutrient loss and off-flavor generation as well as the formation of potentially toxic compounds such as highly reactive lipid peroxidation products (Liu *et al*., 2018).

In recent years, it has been reported that epoxy fatty acids, in which unsaturated bonds have been epoxidized via heating or oxidation, are present in lipid- or oil-containing foods. Monoepoxy and diepoxy fatty acids are detected in a wide variety of fat- or oil-containing processed foods, and their mean concentrations ranged from 13 ppm in cooked meat to 687 ppm in dry nuts (Mubiru *et al*., 2017; Fankhauser-Noti *et al*., 2006). It has been also reported that the concentrations of epoxides are higher than those of peroxides during the initial lipid oxidation in canola oil and margarine (Grüneis *et al*., 2019). Dietary epoxy fatty acids can be orally absorbed in healthy humans (Wilson *et al*., 2002).

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In general, compounds with an epoxide structure have high reactivity with biomolecules such as DNA and protein, and there is increasing concern about their toxic effects such as genotoxicity and cellular damage. For example, various epoxide-containing compounds in foods such as glycidol (produced in the oil and fat refining process, mainly in the deodorization process), glycidamide (acrylamide is produced by the Maillard reaction of reducing sugar and aspartic acid and is epoxidized *in vivo*), and polycyclic aromatic hydrocarbons (polycyclic aromatic hydrocarbons are produced in baking, drying, and heating foods and are epoxidized *in vivo*) are known to induce genotoxicity and carcinogenicity *in vitro* or *in vivo* in experimental animals (Bakhiya *et al*., 2011; Carere, 2006; Jägerstad and Skog, 2005). Recently, it was reported that epoxyoleic acid induced stronger cytotoxicity, oxidative stress, and apoptotic effects than oleic acid at high concentrations in HepG2 human hepatocellular carcinoma cells (Liu *et al*., 2018). However, the toxicity of epoxy fatty acids is largely unknown, and new studies are warranted. Therefore, in this study, we conducted *in vitro* genotoxicity and cytotoxicity studies of epoxy fatty acids to evaluate their possible effects on human health.

MATERIALS AND METHODS

Reagents

Epoxyoleic acid (EOA) was obtained from Toronto Research Chemicals (Ontario, Canada). Diepoxylinoleic acid (DELA) was synthesized in our laboratory via the epoxidation of linoleic acid with m-chloroperoxybenzoic acid (mCPBA) as follows. In total, 500 mg of linoleic acid (Tokyo Chemical Industry, Tokyo, Japan) and 767.9 mg of mCPBA (Fujifilm Wako, Osaka, Japan) were mixed at room temperature for 4 hr in a round-bottom flask containing 20 mL of chloroform (Kanto Chemical, Tokyo, Japan). Sodium carbonate (Fujifilm Wako) and sodium thiosulfate (Fujifilm Wako) were subsequently added to stop the reaction. Then, the reaction mixture was extracted using chloroform, and the organic layer was washed with water and brine, dried over sodium sulfate (Fujifilm Wako), and then evaporated to obtain the crude product. The crude product was purified via flash chromatography over silica gel (Silica gel 60, Merck Millipore, Burlington, MA, USA) eluted with hexane/ethyl acetate = 4/1 (v/v) and, DELA was obtained (427 mg, yield = 85.4%). The structure of DELA was confirmed using UHR-Q-TOF/MS (impact II, Bruker, Billerica, MA, USA), and the purity was confirmed as 93.3% using HPLC-UV (Agilent 1260 Infinity II, Agilent Technologies, Santa Clara, CA, USA).

EOA and DELA were dissolved in sterile analytical grade dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) immediately before compound treatment of the cells. Other reagents were of analytical grade and used as supplied.

Bacterial reverse mutation assay

Mutagenicity assays of EOA and DELA, with and without metabolic activation, were conducted as described previously (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). The *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and the *Escherichia coli* strain WP2 *uvrA* were obtained from the National Institute of Health Sciences. All strains were characterized prior to the study. Metabolic activation was performed using phenobarbital- and 5,6-benzoflavone-induced rat liver S9 mix (Oriental Yeast, Tokyo, Japan) with added cofactors. EOA and DELA were tested at a highest concentration of 5000 μg/plate, as specified in OECD TG471, in all tester strains in the presence and absence of metabolic activation (±S9). EOA and DELA were dissolved in DMSO. The positive controls, which were tested without metabolic activation, were 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (TA98, TA100, and *E. coli* WP2 *uvrA*), sodium azide (TA1535) and 9-aminoacridine hydrochloride (TA1537). 2-Aminoanthracene was tested in all strains with metabolic activation. All tester strains were pre-cultured for 8-10 hr, and 100 μL of the bacterial suspensions was mixed with 500 μL of phosphate buffer (pH 7.4) or rat liver S9 mix and 100 μL of test compound solution in the assay tubes. The assay tubes were pre-incubated at 37°C for 20 min, mixed with 2 mL of top agar containing 0.5 mM biotin and 0.5 mM histidine (for *Salmonella* strains) or 0.5 mM tryptophan (for *E. coli* WP2 *uvrA* strain), and plated onto minimum glucose agar. Two test plates per concentration were incubated at 37°C between 48 and 72 hr and then analyzed using an automated colony analyzer (aCOLyte3, Synbiosys, Cambridge, UK).

In vitro micronucleus (MN) test

MN assays were conducted using CHL/IU Chinese hamster lung cells (JCRB, Osaka, Japan) according to a previous report (Shibai-Ogata *et al.*, 2011) with some modifications as follows.

The cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% calf serum, 100 units/mL penicillin, and 75 units/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a 5% CO_2 humidified incubator at 37°C. Two thousand cells were seeded with 180 μL of medium into each well of clear bottom black 96-well

microplates (CELLBIND surface, Corning, Corning, NY, USA) and then pre-cultured for 24 hr before testing the chemicals. The highest concentration for EOA and DELA was 500 µg/mL based on the toxicity in preliminary range finding study. EOA and DELA were dissolved in DMSO at 100× the final highest concentration and then diluted by 50% in a stepwise manner with medium containing 10% DMSO alone or 10% DMSO and 20% S9 containing medium to obtain 10× the desired final concentrations. After pre-culture for 24 hr, 20 µL of the prepared medium containing test compounds were transferred to the cell culture microplates. The treatment medium included 1% DMSO in addition to the test chemicals. The cells were continuously treated with test chemicals for 30 hr in the absence of S9 mix or for 6 hr with or without S9 mix followed by a 24 hr recovery period in triplicate wells. For short-term treatment, cells were incubated with chemicals for 6 hr, after which the medium was replaced with fresh medium and cells were incubated for 24 hr to allow recovery. Negative controls were included in the test system in all experiments under each treatment condition. Mitomycin C was used as a positive control in the absence of rat liver S9. Cyclophosphamide was used as a positive control in the presence of rat liver S9. All incubations were performed out in a 5% CO_2 humidified atmosphere at 37°C. After the prescribed incubation time, cells were fixed with 20 µL of acetic acid/ethanol (1/3, v/v), incubated with 50 µL of 1% acetic acid/methanol solution for at least 30 min and then covered with PBS until the staining procedures.

Fixed cells were washed and double-stained with 1 µg/mL Hoechst 33342 (Dojindo, Kumamoto, Japan) and 2 µg/mL HCS CellMask Red (Thermo Fisher Scientific) in 50 µL of PBS for 30 min to stain the nucleus, MN, and cytoplasm. Then, cells were washed with PBS once and covered with 100 µL of PBS to acquire fluorescence images. Fluorescence images of cells stained with Hoechst 33342 (Ex: 390 nm, Em: 430 nm) and CellMask Red (Ex: 544 nm, Em: 588 nm) were acquired with a Cytell Cell Imaging System (GE Healthcare, Chicago, IL, USA) using a 10× objective lens. Next, the stored images were analyzed using IN Cell Developer Toolbox software (GE Healthcare) as described previously (Shibai-Ogata *et al.*, 2011). Twenty-five fields per well were acquired and analyzed, and the MN appearance ratio was calculated as the number of cells with micronucleation versus the total number of analyzable cells. Analyzable cells were used to calculate the relative cell count in treated cultures.

p53R2-dependent luciferase reporter gene assay

TK6/p53BS-*luc2P*/R*luc* cells, which stably express three-tandem repeat sequences for the p53R2-derived p53 binding site gene and downstream firefly luciferase and internal control Renilla luciferase, were developed in our laboratory as previously reported (Mizota *et al*., 2011; Ohno *et al*., 2013). The characteristics of the cells were confirmed according to their responses to the chemicals recommended for the evaluation of modified or new mammalian cell genotoxicity tests by the European Centre for the Validation of Alternative Methods as follows (Kirkland *et al*., 2008; Ohno *et al*., 2013).

The cells were maintained in RPMI1640 medium (Nissui Pharmaceuticals) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 75 units/mL streptomycin (Thermo Fisher Scientific) in a 5% CO_2 humidified incubator at 37°C. Ninety microliters of p53BS-*luc2P*/R*luc* stably expressing TK6 cells were plated at an initial concentration of 1×10^5 cells/well in white, clear-bottomed 96-well plates (Corning) and kept in a 5% CO_2 humidified atmosphere at 37°C until immediately before test compound treatment. The highest concentrations for EOA and DELA were set at 100 and 300 µg/mL, respectively, based on the toxicity in a preliminary range finding study. EOA and DELA dissolved in DMSO were serially diluted by threefold in a stepwise manner with medium containing 1% DMSO alone or 1% DMSO and 5% S9 to obtain 10× the target concentration. Then, 10 µL of the prepared solutions were added to 96-well plates and then incubated for 6.5 hr in a 5% CO_2 humidified atmosphere at 37°C. The Dual-Glo assay system (Promega) was used to detect p53R2-dependent firefly luciferase and internal control Renilla luciferase activity using a luminometer (TriStar LB942, Berthold Technologies, Bad Wildbad, Germany). Each assay was triplicated. Benzo[a]pyrene and Adriamycin were used as positive controls for assays with and without S9 metabolic activation, respectively.

The criteria for positive responses were described previously (Mizota *et al*., 2011), namely, relative p53R2-dependent firefly luciferase activity was 150% or greater and responded in a concentration-dependent manner (Ohno *et al*., 2013). The internal Renilla luciferase activity is under a constitutively active promoter, and it correlates with the ability of the cells to express protein. We identified cytotoxicity when the Renilla luciferase activity decreased to less than 50% of the negative control level.

Cytotoxicity assay

HepG2 human hepatocellular carcinoma cells (ATCC, Manassas, VA, USA) were cultured in Eagle's minimum

essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 100 units/mL penicillin, and 75 units/mL streptomycin in a 5% CO_2 humidified incubator at 37°C. One hundred microliters of cells were plated at an initial concentration of 2×10^4 cells/well in clear 96-well plates (Corning). Oleic acid, EOA, linoleic acid, and DELA were dissolved in DMSO at 333 × the final highest concentration and then diluted by 50% in a stepwise manner using 0.6% DMSO-containing medium to obtain dilutions of 2 × the desired final concentrations. The highest concentration for these compounds was set at 1000 µM based on a previous report (Liu *et al*., 2018). After pre-culture for 24 hr, 100 µL of the prepared medium containing the test compounds were transferred to cell culture microplates. The treatment medium included 0.3% DMSO in addition to the test chemicals. The cells were continuously treated with the test chemicals for 24 hr in triplicate wells. The viability of cells was determined using the WST-8 assay (Cell counting kit-8, Dojindo) and LDH assay (Cytotoxicity Detection Kit PLUS, Roche Diagnostics, Rotkreuz, Switzerland) with a microplate reader (iMark, Bio-Rad, Hercules, CA, USA)

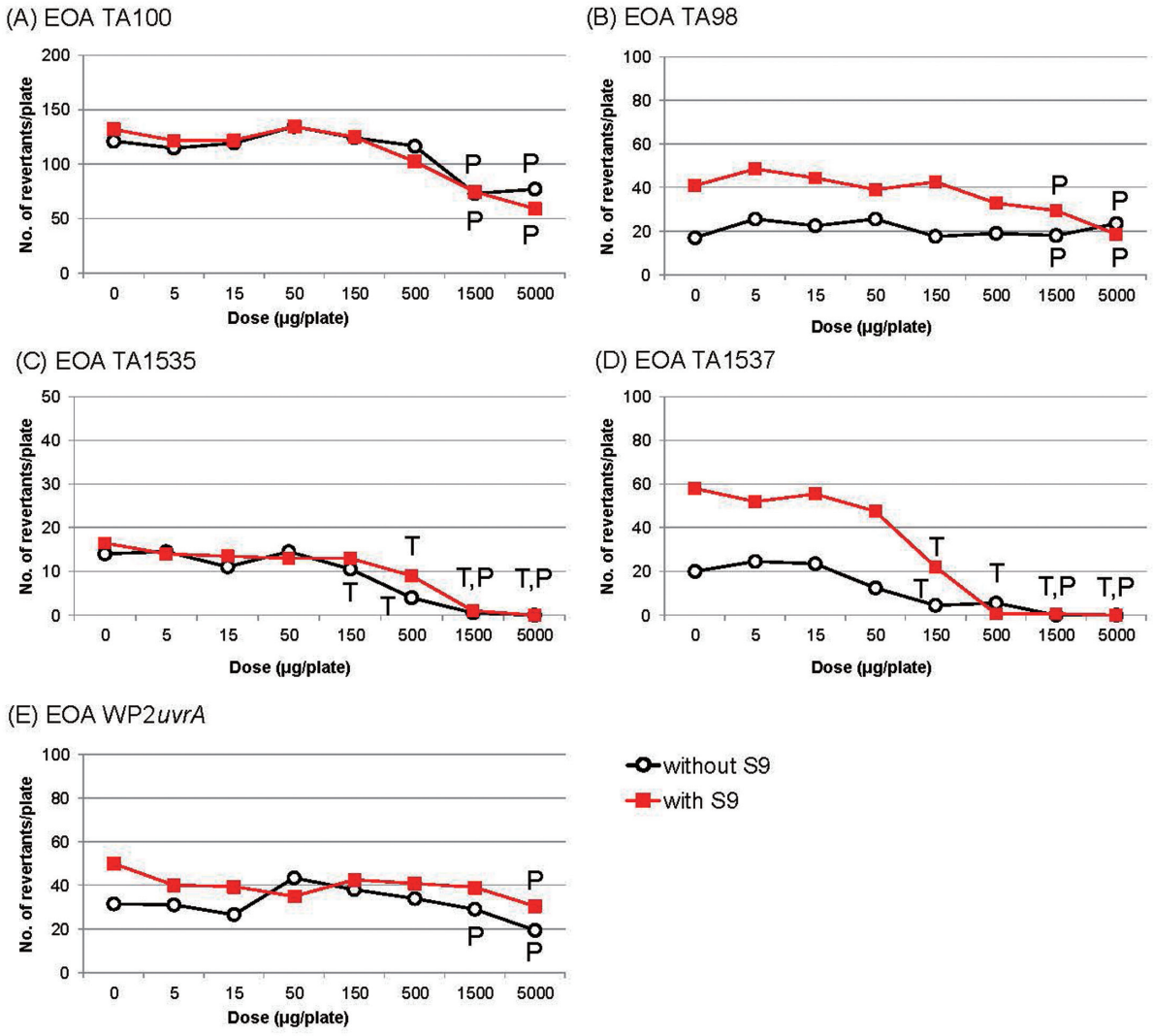

Fig. 1. Bacterial reverse mutation assays of epoxyoleic acid (EOA) and diepoxylinoleic acid (DELA). Each black open circle plot indicates the number of revertant colonies in the absence of rat liver S9 metabolic activation, and red closed square plots indicate that in the presence of rat liver S9 metabolic activation. Each value represents the mean of two replicates. "P" and "T" in each figure represent the precipitation of test compounds and growth inhibition of the tester strain, respectively.

following the manufacturer's instruction.

Statistical analysis

All statistical analyses were conducted using JMP version 15 (SAS Institute, Cary, NC, USA). For *in vitro* MN test, Dunnett's test was used to compare the effects of compound treatment between the control and treatment groups and Student's *t*-test was used to compare differences between the control and positive control groups. $p < 0.05$ was considered statistically significant.

RESULTS

Bacterial reverse mutation assay (Ames test)

The results of the bacterial reverse mutation assay are shown in Fig. 1. Neither EOA nor DELA increased the number of revertant colonies by more than twofold compared with the findings for the negative control in any of the tester strains in either the presence or absence of S9 activation. For EOA, precipitation was observed at 1500 µg/plate, excluding WP2 *uvrA* with S9 activation, for which precipitation was only observed at 5000 µg/plate. Growth inhibition was observed beginning at 150 or 500 µg/plate in TA1535 and TA1537, but it was not observed in TA98, TA100, and WP2 *uvrA*. For DELA, growth inhibition was observed at 1500 µg/plate in TA1535 and TA1537 and at 5000 µg/plate with TA98 and TA100, whereas no growth inhibition was observed in WP2 *uvrA*. The numbers of revertant colony for all positive controls exceeded twofold compared with those

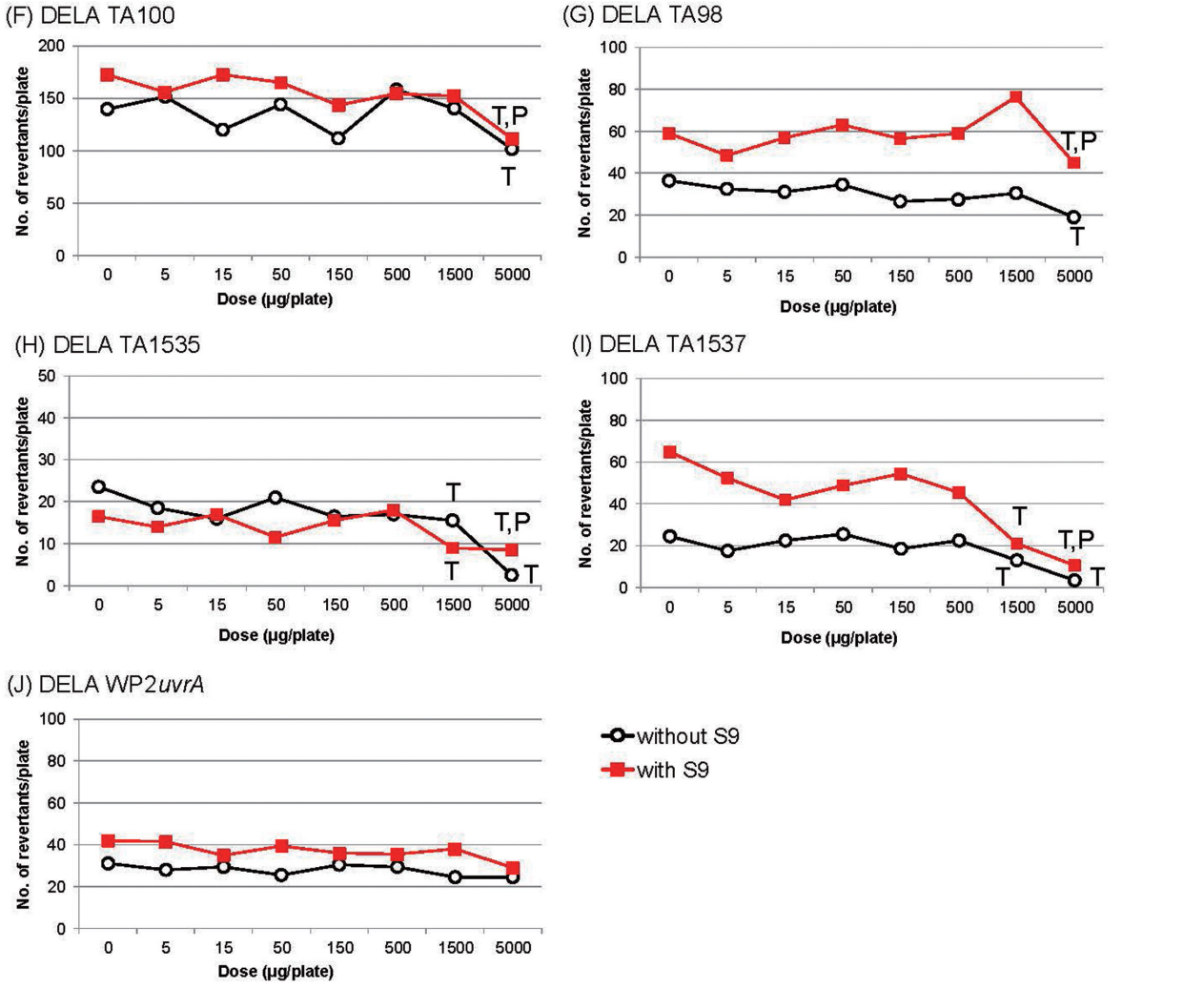

Fig. 1. (Continued).

for the negative control (data not shown). Based on these results, we considered that EOA and DELA were not mutagenic in the bacterial reverse mutation assay.

In vitro MN test

A summary of the MN test results is presented in Fig. 2. EOA and DELA, in the absence and presence of S9 activation, did not significantly increase the frequen-

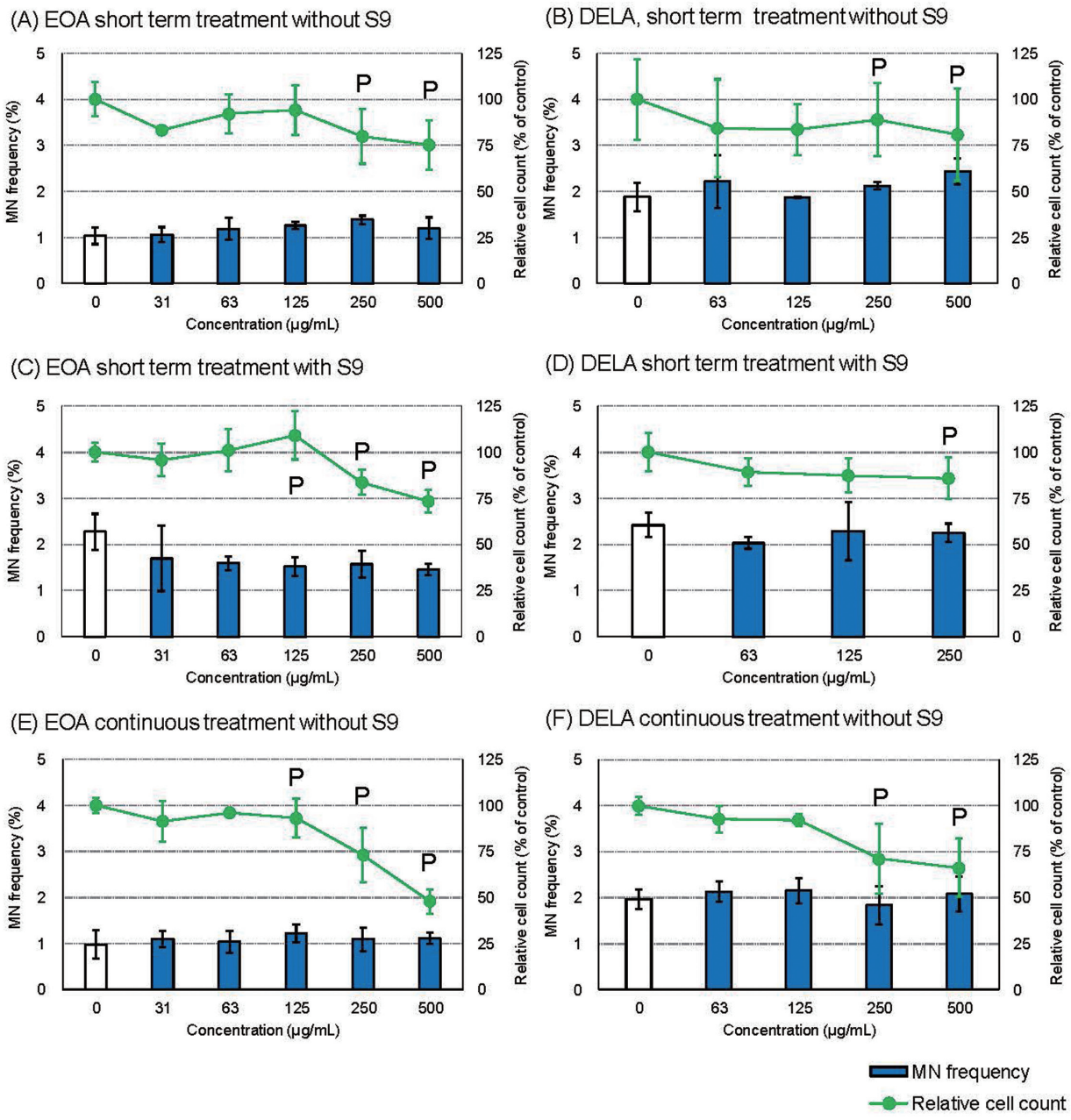

Fig. 2. The summary results of *in vitro* micronucleus assays of epoxyoleic acid (EOA) and diepoxylinoleic acid (DELA). Each white or blue bar indicates the frequency of micronucleated (MN) cells, and each green solid line indicates the relative cell counts in comparison with the negative control cell counts. "P" in each figure represents the precipitation of test compounds. Each value represents the mean ± S.D. of triplicate assays. $p > 0.05$ for all treatment conditions (vs. vehicle control, Dunnett's test).

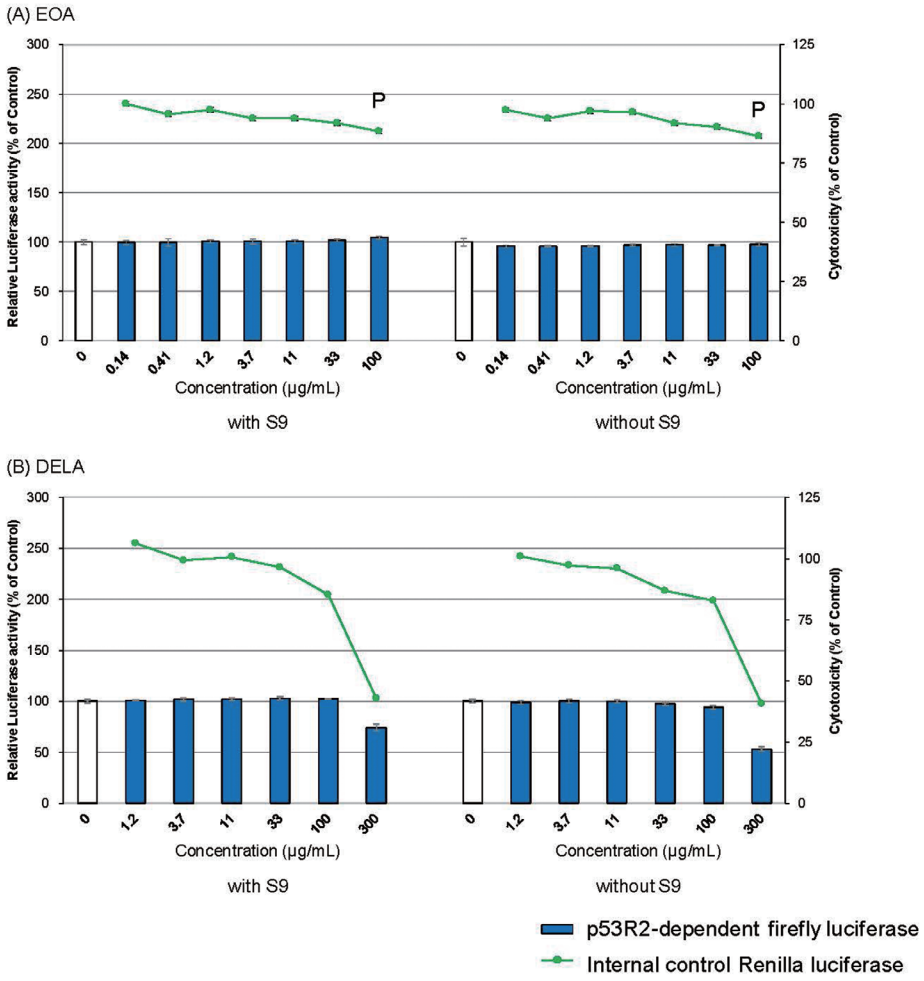

Fig. 3. Effects of epoxyoleic acid (EOA) and diepoxylinoleic acid (DELA) on p53R2-dependent luciferase activity in TK6/p53BS-*luc2P*/R*luc* cells. Each white or blue bar indicates the relative p53R2-dependent luciferase activity, and each green solid line indicates the relative Renilla luciferase activity in comparison with the negative control cells. "P" in each figure represents the precipitation of test compounds. Each value represents the mean ± S.D. of triplicate assays.

cies of micronucleated cells ($p > 0.05$) compared with the findings in the concurrent negative controls for all concentrations analyzed. For EOA, precipitation was observed beginning at 250 μg/mL without S9 activation and 125 μg/mL with S9 activation and cytotoxicity was not observed up to 500 μg/mL under all treatment conditions. For DELA, precipitation was observed beginning at 250 μg/mL under all treatment conditions and cytotoxicity was observed at 500 μg/mL during short-term treatment with S9 activation. Positive control compounds induced statistically significant increases in the frequencies of MN-containing cells compared with the findings in the negative controls with and without metabolic activation ($p < 0.05$). For short-term treatment without S9, 0.25 μM mitomycin C increased the frequencies of micronucleation by up to 21.3% ± 1.9%. In short-term treatment with S9, 20 μM cyclophosphamide increased the frequencies of micronucleation by up to 8.6 ± 0.8%, and for continuous treatment without S9, 0.125 μM mitomycin C increased the frequencies of micronucleation by up to 21.0% ± 1.3%.

Based on these results, we considered that EOA and DELA did not induce micronuclei in CHL/IU cells.

p53R2-dependent luciferase reporter gene assay

The results of the p53R2-dependent luciferase reporter gene assay are shown in Fig. 3. For EOA, neither p53R2-dependent nor Renilla luciferase activity was changed

Table 1. Summary results of the cell viability of oleic acid, epoxyoleic acid (EOA), linoleic acid, and diepoxylinoleic acid (DELA) in HepG2 cells as assessed using WST-8 assay.

Concentrations (μM)	Cell viability (% of control)			
	Oleic acid	EOA	Linoleic acid	DELA
0	100.0 ± 2.6	100.0 ± 6.9	100.0 ± 10.7	100.0 ± 2.7
31	98.4 ± 4.1	107.6 ± 1.6	110.1 ± 0.7	104.4 ± 3.9
63	95.2 ± 5.2	105.8 ± 1.0	111.0 ± 1.3	101.6 ± 5.0
125	89.0 ± 9.2	105.8 ± 1.1	108.5 ± 3.6	99.9 ± 7.3
250	89.8 ± 2.3#	101.1 ± 6.2	102.7 ± 3.8#	100.9 ± 4.7
500	87.4 ± 8.5#	91.3 ± 5.0#	96.9 ± 3.6#	86.9 ± 5.3
1000	77.4 ± 1.3#	68.7 ± 2.3#	92.3 ± 3.2#	59.8 ± 6.3#

Each value represents mean ± S.D. of triplicate assays. "#" represents the precipitation of test compounds.

Table 2. Summary results of the cytotoxicity of oleic acid, epoxyoleic acid (EOA), linoleic acid, and diepoxylinoleic acid (DELA) in HepG2 cells as assessed using LDH leakage assay.

Concentrations (μM)	Cytotoxicity (% of control)			
	Oleic acid	EOA	Linoleic acid	DELA
0	0.0 ± 2.5	0.0 ± 3.3	0.0 ± 2.0	0.0 ± 2.5
31	4.3 ± 1.0	7.7 ± 0.8	2.8 ± 3.2	2.3 ± 3.3
63	14.2 ± 1.3	14.0 ± 1.4	4.5 ± 1.1	3.6 ± 0.5
125	17.1 ± 3.4	14.9 ± 3.5	6.9 ± 3.6	2.9 ± 4.4
250	9.4 ± 3.0#	15.8 ± 3.6	9.9 ± 3.4#	3.2 ± 2.8
500	17.2 ± 7.0#	20.2 ± 3.1#	11.2 ± 4.0#	10.0 ± 1.5
1000	19.1 ± 3.2#	28.3 ± 1.9#	13.0 ± 1.7#	30.6 ± 0.9#

Each value represents mean ± S.D. of triplicate assays. "#" represents the precipitation of test compounds.

regardless of S9 activation and precipitation was observed at 100 μg/mL. For DELA, p53R2-dependent and Renilla luciferase activity was not changed with or without S9 activation up to 100 μg/mL, whereas their activity was decreased at 300 μg/mL. Positive controls for benzo[a]pyrene with S9 activation and Adriamycin without metabolic activation increased p53R2-dependent firefly luciferase activities by 421 ± 11 and 427 ± 18%, respectively. Therefore, we judged that EOA and DELA did not induce p53R2 activation in TK6/p53BS-*luc2P*/R*luc* cells.

Cytotoxicity assay using HepG2

The results of cytotoxicity assays in HepG2 cells are presented in Tables 1 and 2. There was no obvious decrease in WST-8 reducing potential at up to 250 μM for oleic acid, EOA, linoleic acid, and DELA, whereas a slight decrease tendency was observed for DELA at 500 μM. In correlation with the WST-8 assay results, these fatty acids tended to decrease in LDH extracellular leakage assay. Because both these assays did not reach the 50% inhibitory concentration compared to the controls, the IC_{50} values were not calculated. Precipitation was observed at 250 μM for oleic acid, 500 μM for EOA and linoleic acid, and 1000 μM for DELA. At the highest concentration at which precipitation was observed, weak cytotoxicity was observed. Although a previous study reported that EOA was more cytotoxic than oleic acid at 1000 μM, in our study, precipitation was observed at 1000 μM. Although the cellular toxicity of oleic acid and linoleic acid was weak at 1000 μM, these fatty acids induced precipitation at 250 or 500 μM, and they appeared less soluble than EOA and DELA, suggesting a possible difference in cellular exposure to EOA and DELA.

DISCUSSION

We tested EOA and DELA as the representative epoxy fatty acids because monoepoxy and diepoxy fatty acids have been detected in various types of foods in previous reports (Mubiru *et al*., 2017; Fankhauser-Noti *et al*., 2006). In this study, we focused on evaluating genotoxicity and cytotoxicity because if the genotoxic result is positive, the non-threshold mechanism of toxicity would be expected, and a previous report described the cytotoxic risk of epoxyoleic acid (Liu *et al*., 2018).

The bacterial reverse mutation assay, also known as the Ames test, sensitively detects mutagenic compounds by counting reverse mutant colonies that can synthesis

histidine (Maron and Ames, 1983). In particular, TA100 and TA1535, which carry base pair mutations, were reported to be sensitive to epoxide-containing mutagens (El-Tantawy and Hammock, 1980; Canter *et al*., 1986). *In vitro* MN assays can detect clastogenic compounds by counting the erratic nuclei formed during the anaphase of mitosis or meiosis and identify epoxide-containing genotoxic chemicals such as glycidamide (Koyama *et al.*, 2006). p53R2-dependent luciferase reporter gene assays detect both mutagenic and clastogenic compounds with high accuracy because *p53R2*, which encodes a subunit of ribonucleotide reductase, is activated by DNA damage induced by γ-ray and ultraviolet irradiation, as well as genotoxic chemicals (Ohno *et al.*, 2005). We previously developed a p53R2-dependent luciferase reporter gene assay using TK6 cells and demonstrated its utility for detecting a wide variety of genotoxic compounds (Mizota *et al.*, 2011; Ohno *et al.*, 2013). All of these assays are reported to detect epoxide-containing genotoxic compounds with high sensitivity, making them appropriate for evaluating *in vitro* genotoxic potential.

In our studies, EOA and DELA did not induce genotoxic response in all three *in vitro* genotoxic assays irrespective of the metabolic activation of rat liver S9, suggesting that both compounds have low reactivity with DNA molecules *in vitro*. The possible reason might be that these epoxy fatty acids were structurally different from other genotoxic epoxides. The electron density of aliphatic epoxides is low; thus, their reactivity with other biomolecules such as DNA and proteins might be weak. A previous report supports our explanation (Voogd *et al*., 1981). They conducted mutagenicity tests of various types of aliphatic epoxides including EOA using *Klebsiella pneumoniae*, and the result of terminal epoxides was positive and that of EOA was negative, indicating that the mutagenic potential of non-terminal and aliphatic epoxides was low. The fact that the cytotoxic concentrations without S9 were not stronger than those with S9 in our *in vitro* MN and p53R2-dependent luciferase reporter gene assays supports their low reactivity to biomolecules. From another viewpoint, the contents of epoxy fatty acids, which are generated from unstable peroxidized fatty acids, were reported to be much higher than those of peroxidized fatty acids, suggesting that epoxy fatty acids themselves are stable end-products with low reactivity with other biomolecules. It has also been reported that epoxidized fatty acid esters were more resistant to oxidation than their unepoxidized counterparts (Gan *et al.*, 1995).

In the cytotoxicity test, although cytotoxicity tended to be strong at the highest concentration at which precipitation was observed, there was no clear difference compared with the non-epoxidized fatty acid over the concentration range at which precipitation was not observed. Although we could not clearly explain the reason for the difference in findings between our study and that of Liu *et al.*, one possible explanation might be the precipitation of test compounds. Liu *et al.* did not mention the concentration at which precipitation occurred, but in our study, the solubilities of EOA and DELA were twofold to 4 fold higher than those of corresponding fatty acids such as oleic acid and linoleic acid. This difference might lead to differences in exposure in HepG2 cells and cytotoxicity. Further research is needed to confirm these differences. Nonetheless, in this study, the highest concentrations at which no precipitation occurred (approximately 250 μM) far exceeded the previously reported clinical C_{max} (0.18 and 0.08 μM, respectively) after ingesting 20 mg of monoepoxy fatty acid-containing triglycerides and 25 mg of diepoxy fatty acid-containing triglycerides. When considering the daily amount of epoxy fatty acids consumed in foods under the worst-case scenario (687 mg of intake based on the highest concentration of dietary epoxy fatty acid of 687 ppm ever reported in food × 1 kg daily food intake [EC, 2001] and C_{max} of 6.2 and 2.2 μM based on the linear exposure relationships), our *in vitro* tested concentration greatly exceeds the worst-case exposure scenario.

There are some limitations in this study because we did not conduct *in vivo* or long-term toxicity studies. Therefore, we searched for similar compounds and identified toxicity studies of epoxidized soybean oil (ESBO). ESBO, which is produced via the peroxidation of soybean oil, contains high levels of epoxy fatty acid-containing triglycerides, and it is used as stabilizer and plasticizer of polyvinyl chlorides. The toxicity test results for ESBO were reviewed by the European Food Safety Authority (EFSA, 2004). This report indicated that ESBO has extremely low acute toxicity in rats (LD_{50} > 5 g/kg), and carcinogenicity studies of ESBO in rats and mice were negative up to 2500 mg/kg/day. Meanwhile, 2-year chronic toxicity studies in rats observed slight changes in the uterine, liver, and kidney weight and no alteration of blood parameters at 1400 mg/kg/day and no effect on fertility or offspring development were observed in rats up to 1000 mg/kg/day. In addition, genotoxicity tests, including the Ames test, forward mutation assays in mouse lymphoma L5178Y cells, and chromosomal aberration assays using human lymphocytes with and without S9, were negative. Therefore, epoxy fatty acids were also suggested to have weak chronic toxicity.

In conclusion, we conducted *in vitro* genotoxicity and cytotoxicity studies. EOA and DELA did not induce *in*

vitro genotoxic responses. Both fatty acids induced weak cytotoxicity, but the effect was similar to that of oleic and linoleic acids at low concentrations. The cytotoxicity at high concentrations cannot be rigorously evaluated, due to differences in solubility into culture media between EOA and OA or between DELA and LA. These results could be useful for evaluating the health effects of epoxy fatty acids with sparse toxicity information.

Conflict of interest---- The authors declare that there is no conflict of interest.

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