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Genotoxicity evaluation of alpha-linolenic acid-diacylglycerol oil

Hiroshi Honda[a,*,1], Yurika Fujita[a,1], Aya Hayashi[b], Naohiro Ikeda[a], Yuichi Ito[a,*], Osamu Morita[a]

[a] R&D Safety Science Research, Kao Corporation, 2606 Akabane, Ichikai–Machi, Haga–Gun, Tochigi 321-3497, Japan
[b] Drug Safety Research Laboratories, Shin Nippon Biomedical Laboratories Ltd., 2438 Miyanoura, Yoshida, Kagoshima 891-1394, Japan

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ABSTRACT

The alpha-linolenic acid (ALA)-diacylglycerol (DAG) oil is an edible oil enriched with DAG (>80%) and ALA (>50%). Although DAG oil, which mainly consists of oleic and linoleic acids has no genotoxic concerns, the fatty acid composition could affect the chemical property of DAG. Therefore, the purpose of this study was to evaluate the genotoxicity of ALA-DAG oil using standard genotoxicity tests in accordance with the OECD guidelines. ALA-DAG oil showed negative results in the bacterial reverse mutation test (Ames test) and *in vitro* micronucleus test in cultured Chinese hamster lung cells with and without metabolic activation, and in the *in vivo* bone marrow micronucleus test in mice. Our results did not show any genotoxicity, suggesting that the fatty acid composition had no deleterious effects. We conclude that ALA-DAG oil had no genotoxicity concerns under the testing conditions.

1. Introduction

Diacylglycerol (DAG; Fig. 1a) is a natural component found in various edible oils and has been widely consumed for numerous years in the human diet. Recently, a high-purity DAG oil (DAG content >80%) was enzymatically prepared from plant oils for commercial use [1]. Several clinical studies focusing on the beneficial health effect of DAG oil in humans have revealed that it could have a preventive effect on body fat accumulation [2,3].

The fatty acid composition of dietary oils is considered to affect their physiological action. For example, dietary (n-3) polyunsaturated fatty acids showed beneficial effects on lipid metabolism and immune response in previous studies [4–8]. Among them, alpha-linolenic acid (ALA; Fig. 1b) has been reported to decrease the blood lipid concentration and reduce body and fat weight [1,6,9–12]. In fact, DAG oil, which mainly contains ALA as the constitutive fatty acid (ALA-DAG oil; Fig. 1), is beneficial for suppressing body weight gain and fatty liver [13–15]. Ando et al. [16] recently reported that ALA-DAG oil has a superior effect on the postprandial fat oxidation compared to oleic and linoleic acid-rich DAG. It suggests that the conjugation between ALA and DAG could affect the chemical properties of each conjugant and might increase their inherent physiological activity. Therefore, the toxicokinetics and dynamics of ALA-conjugated DAG might differ from those of DAG conjugated with other fatty acids.

The safety evaluations of DAG oil containing oleic and linoleic acids have mainly focused on the acute and repeated toxicity [17,18], developmental and reproductive toxicity [19], genotoxicity, carcinogenicity [20,21], and high-dose dietary DAG oil clinical studies [22], all of which have demonstrated no adverse effects. Although glycidol fatty acid esters (GEs) found in DAG oil as process-related contaminants led to safety concerns about the possible release of glycidol [23], an animal genotoxic-carcinogen, during the digestion, the internal exposure levels of glycidol in DAG-oil exposed subjects were considered negligible [24–26]. However, little is currently known about the toxicity including genotoxicity of ALA-DAG oil itself and the potential toxicological role of the fatty acid composition of DAG because the ALA content of the previously tested DAG oils tested was low (8.7%) [21].

Although ALA showed no genotoxicity in the bacterial reverse mutation test (Ames test) and mouse lymphoma test performed by the National Toxicology Program (NTP) [27,28], a slight increase in the revertant colonies was observed under previous experimental

Abbreviations: 2AA, 2-aminoanthracene; 9AA, 9-aminoacridine hydrochloride hydrate; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; ALA, alpha-linolenic acid; B(a)P, benzo(a)pyrene; CLC, Colchicine; CP, cyclophosphamide; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; LA, linolenic acid; MMC, mitomycin C; MNPCE, micronucleated polychromatic erythrocyte; NaN_3, sodium azide; NCE, normochromatic erythrocyte; TAG, triacylglycerol.

* Corresponding authors.
E-mail addresses: honda.hiroshi@kao.co.jp (H. Honda), ito.yuichi@kao.co.jp (Y. Ito).

[1] These authors contributed equally.

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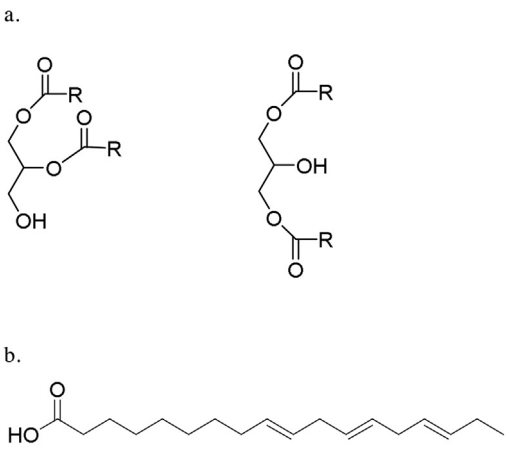

Fig. 1. Diacylglycerol (DAG), alpha-linolenic acid (ALA), and alpha-linolenic acid-diacylglycerol (ALA-DAG) oil. a. Diacylglycerol (DAG), R: One of the fatty acids in Table 1. b. Alpha-linolenic acid (ALA). Alpha-linolenic acid (ALA)-diacylglycerol (DAG) oil is an edible oil enriched with DAG (>80%), and ALA (>50%) as a fatty acid composition.

conditions. This effect was considered non-genotoxic because the response was either very weak or observed only at a dose range beyond that recommended by the Organisation for Economic Co-operation and Development (OECD) guideline [29]. However, as described earlier, the fatty acid composition (ALA content) could affect the chemical properties of DAG. Thus, the genotoxicity of ALA-DAG oil needs to be evaluated. Since it is difficult to compare the *in vitro* experimental doses used in genotoxicity tests with the ALA-DAG oil intake in human, an *in vivo* genotoxicity assay would be useful for quantitative risk evaluation.

The present study was specifically focused on the effect of the fatty acid composition of DAG oil on its potential genotoxicity. The ALA-DAG oil was investigated using *in vitro* and *in vivo* genotoxicity tests, which are accepted by the OECD guideline.

2. Materials and methods

2.1. Test chemicals and bacterial strains

The chemicals used as positive controls included: 2-(2-furyl)-3-(5-nitro-2furyl)acrylamide (AF-2), 2-aminoanthracene (2AA), and sodium azide (NaN_3), purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan); and 9-aminoacridine hydrochloride hydrate (9AA), purchased from Sigma-Aldrich Chemical Corp, Inc., (St. Louis, MO, USA). Mitomycin C (MMC), cyclophosphamide monohydrate (CP), and colchicine (CLC) were purchased from Kyowa Hakko Kirin Co., Ltd., (Tokyo, Japan), Sigma-Aldrich Chemical Corp, Inc., (St. Louis, MO, USA), and Wako Pure Chemical Industries, Ltd., (Osaka, Japan), respectively. The olive oil used as a negative control in the bone marrow micronucleus assay in mice was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The bacterial strains *Salmonella typhimurium* and *Escherichia coli* were obtained from the National Institute of Technology and Evaluation (Chiba, Japan).

2.2. Study design

All studies were performed in compliance with the Good Laboratory Practice (GLP) regulations and in accordance with the OECD Guidelines for the Testing of Chemicals No. 471, 474, and 487 [29–31]. There were no deviations from the guidelines.

2.3. Preparation of ALA-DAG oil

The ALA-DAG oil was synthesized by esterifying glycerol with fatty acids obtained from linseed oil using immobilized lipase as previously described [32]. The fatty acid and glycerol composition of the test oils are shown in Table 1. We used the Deutsche Gesellschaft für Fettwissenschaft (DGF) standard method [33] to confirm that the level of GEs in the ALA-DAG oil was comparable to that of the other edible oils (<0.3 μg/g) [34].

Table 1
Glycerol and fatty acid composition of alpha-linolenic acid-diacylglycerol (ALA-DAG) oil.

Components of ALA-DAG oil	wt%
Ester distribution	
Monoglyceride	0.7
Diglyceride (DAG)	83.1
Triglyceride and others[a]	16.2
Fatty acid composition	
Palmitic acid	2.2
Stearic acid	1.2
Oleic acid	21.4
Linoleic acid	16.5
Linolenic acid	57.6

[a] The level of GEs in the ALA-DAG oil was comparable to that in the other edible oils (<0.3 μg/g) [34].

2.4. Bacterial reverse mutation test (Ames test)

To evaluate the mutagenicity of the ALA-DAG oil, a bacterial reverse mutation test (Ames test) was performed with five bacterial test strains (*S. typhimurium*: TA100, TA1535, TA98, and TA1537; and *E. coli*: WP2*uvrA*) using a pre-incubation method with and without metabolic activation. Dimethyl sulfoxide (DMSO) was used as the solvent control, and the positive controls were AF-2, NaN_3, 9AA (without metabolic activation), and 2AA (with metabolic activation).

2.4.1. Dose selection

The study consisted of two independent tests, which were the dose-finding test and the main test. The highest dose was set at 5000 μg/plate for the dose-finding test in accordance with the OECD guidelines [29], and the lower doses were set at a common ratio of approximately 3. In the dose-finding test, we did not observe a ≥ 2-fold or dose-dependent increase in the number of revertant colonies for the test substances compared to that in the negative control group. Furthermore, there was no growth inhibition of any of the test strains at up to 5000 μg/plate with and without metabolic activation. Therefore, the highest dose was set at 5000 μg/plate for the main test while the lower doses were set at a common ratio of 2.

2.4.2. Experimental procedure

The genotoxicity test was performed using a pre-incubation method [35] with and without metabolic activation. Briefly, 0.5 mL of either 0.1 mol/L sodium phosphate buffer (pH 7.4) or the S9 mix (for treatment without or with metabolic activation, respectively), and 0.1 mL of the bacterial suspension were added to a test tube. Then, 0.1 mL of the test substance preparation was added, the mixture was incubation at 37 °C for 20 min, 2 mL of the top agar was added to the test tube, and the mixture was overlaid onto a minimal glucose agar plate. The plates were subsequently placed in an incubator at 37 °C for 48 h and the revertant colonies were counted.

The S9 fraction (Oriental Yeast Co. Ltd., Tokyo, Japan) for the metabolic activation was prepared from phenobarbital and 5,6-benzoflavone-induced Sprague-Dawley rats. Subsequently, the S9 mix was prepared by mixing 1 vol of the S9 fraction with 9 vols of Cofactor-I (Oriental Yeast Co. Ltd., Tokyo, Japan) dissolved in water (as the nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system).

2.4.3. Determination criteria

A response was determined as positive when the test chemical caused a $\geq$ 2-fold dose-dependent increase in the mean number of revertant colonies per plate compared to the negative control followed by confirmation that the two independent tests were reproducible.

2.5. In vitro *micronucleus test in cultured cells*

To evaluate the micronuclei-inducing potential of the ALA-DAG oil in *in vitro*, the micronucleus test was performed using CHL/IU cells with short-term treatment with and without metabolic activation and continuous treatment for 24 h.

2.5.1. Grouping and dose selection

For the treatment of cells, the ALA-DAG oil was suspended in 1% DMSO as final concentration. Based on the results of the dose-finding test, the highest dose level was set at 2000 μg/mL, while the lower levels of 1000 and 500 μg/mL were set at a common ratio of 2 because no marked cytotoxicity was observed at up to 2000 μg/mL regardless of the treatment condition. Additional doses of 25 and 50 μg/mL were set for the short-term and continuous treatments, respectively, at which no test substance precipitation was anticipated at the end of treatment. DMSO was used as the negative control, whereas CP and MMC were the positive controls for determining the clastogenicity of treatments with and without metabolic activation, respectively. CLC was used as the positive control for determining the aneugenicity of treatment without metabolic activation.

2.5.2. Experimental procedure

The CHL/IU cells were plated at a density of 1×10^4 cells/mL and incubated for approximately 72 h. For the short-term treatment, the cells were treated with the ALA-DAG oil or control chemicals for 3 h with or without metabolic activation. Subsequently, the cells were washed and incubated in fresh medium for 21 h (the total experimental period was 24 h, which was equivalent to 1.5 cell cycles). For the metabolic activation condition, the S9 mix (final S9 concentration of 5%) was used at a final medium concentration of 16.7%. The S9 mix for the metabolic activation was prepared in a similar manner to that used for the Ames test. For the continuous treatment, the cells were treated with each concentration for 24 h. The number of viable cells was determined for all the conditions when the treatment was started and when the micronuclei specimens were prepared, as an indication of the effects of treatment on cell proliferation. Furthermore, the cell numbers were used to calculate the relative increase in cell count (RICC), which is described in the OECD Test Guideline 487 [30]. After the treatment, the cells were harvested, suspended in 0.075 M potassium chloride (KCl) solution for 10 min, fixed with Carnoy's fixative solution (in a 3:1 mixture of methanol and acetic acid), and then mounted onto clean glass slides. The specimens were stained with acridine orange solution and 4000 mononuclear cells per group (2000 cells/dish) were examined. The number and incidence (%) of mononuclear cells with micronuclei were scored in each group.

2.5.3. Statistical analysis and determination criteria

Fisher's exact test with Bonferroni correction was performed to determine whether the number of cells with micronuclei differed significantly between the negative control and the ALA-DAG-treated groups using the statistical analysis software (SAS) System, Release 9.2 (SAS Institute Inc., NC, USA). A significance level of 5% (one-sided) was used in the present study.

2.6. Bone marrow micronucleus test in mice

To evaluate the micronuclei-inducing potential of the ALA-DAG oil in *in vivo*, a bone marrow micronucleus test was performed using male Crl:CD1(ICR) mice.

2.6.1. Grouping and administration

A previous *in vivo* micronucleus test conducted on DAG oil [21] showed no changes in clinical signs or body weight at a dose of 2000 mg kg^{-1} day^{-1}, which is the upper limit dose specified in the OECD Guidelines for the Testing of Chemicals [31]. Therefore, 2000 mg kg^{-1} day^{-1} was set as the highest dose in the micronucleus test in the present study, while the lower doses were 1000 and 500 mg kg^{-1} day^{-1} with a common ratio of 2. Furthermore, olive oil and MMC were used as the negative and positive controls, respectively.

2.6.2. Experimental procedure

The different doses of the ALA-DAG oil were resuspended in olive oil and the 8-week-old male ICR (Crl:CD1) mice were treated with two oral doses by gavage, which were administered 24 h apart. The olive oil (negative vehicle control) was administered in the same manner as the ALA-DAG oil, while the MMC solution (positive control) was intraperitoneally administered as a single dose at 2 mg kg^{-1} day^{-1}.

The bone marrow smears were prepared from all the surviving animals 24 h after the last administration and were stained with acridine orange. The specimens were examined by using fluorescent microscopy to observe 4000 polychromatic erythrocytes (PCEs) and the number of micronucleated PCEs (MNPCEs) was counted. Then, 500 total erythrocytes (PCEs + normochromatic erythrocytes) from each animal were observed and the proportion of the PCEs was determined as an indicator of the suppression of bone marrow cell proliferation.

2.6.3. Statistical analysis and determination criteria

The method of Kastenbaum and Bowman [36] was used to determine any significant difference in the number of MNPCEs between the negative control group and each ALA-DAG oil-treated or the positive control groups. To analyze the incidence of PCE formation, Dunnett's multiple comparison test was performed to compare the negative control group and each ALA-DAG oil-treated group, and Student's *t*-test was used to compare the negative and positive control groups. These statistical analyses were performed at a significance level of 5% for both the one-tailed tests performed using the method of Kastenbaum and Bowman [36] and the two-tailed tests performed using the SAS software.

Table 2
Bacterial reverse mutation test (Ames test) of alpha-linolenic acid-diacylglycerol (ALA-DAG) oil (main test).

S9 mix	Group	Dose (μg/plate)	Number of revertant colonies/plate				
			Base-pair substitution mutations			Frameshift mutations	
			TA100	TA1535	WP2uvrA	TA98	TA1537
−	Negative control DMSO	–	88 ± 2	10 ± 1	22 ± 1	17 ± 3	6 ± 1
	ALA-DAG oil	313	103 ± 5	8 ± 0	23 ± 3	15 ± 1	5 ± 1
		625	89 ± 5	7 ± 2	21 ± 4	15 ± 3	6 ± 1
		1250	85 ± 3	8 ± 2	20 ± 3	15 ± 2	6 ± 1
		2500[a]	95 ± 11	8 ± 2	20 ± 3	15 ± 2	5 ± 1
		5000[a]	87 ± 3	5 ± 1	22 ± 2	13 ± 1	5 ± 1
+	Negative control DMSO	–	103 ± 2	8 ± 1	25 ± 3	23 ± 3	8 ± 1
	ALA-DAG oil	313	100 ± 4	7 ± 1	21 ± 3	24 ± 4	8 ± 1
		625	104 ± 7	6 ± 0	25 ± 2	26 ± 3	6 ± 1
		1250	117 ± 5	9 ± 1	25 ± 4	21 ± 1	7 ± 1
		2500[a]	112 ± 4	8 ± 1	24 ± 4	24 ± 4	9 ± 2
		5000[a]	116 ± 5	10 ± 2	27 ± 3	25 ± 4	8 ± 2
−	Positive controls						
	AF-2	0.01	468 ± 18				
	NaN_3	0.5		279 ± 13			
	AF-2	0.02			361 ± 15		
	AF-2	0.1				456 ± 26	
	9AA	80					189 ± 6
+	Positive controls						
	2AA	1	615 ± 19				
	2AA	2		219 ± 12			118 ± 19
	2AA	10			312 ± 37		
	2AA	0.5				406 ± 15	

[a] Test substance precipitation (on plates after 48 h incubation), DMSO: dimethyl sulfoxide, AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, 9AA: 9-aminoacridine hydrochloride hydrate, NaN_3: sodium azide, 2AA: 2-aminoanthracene.

3. Results

3.1. Ames test

The results of the main Ames test of the ALA-DAG oil using the five bacterial strains are shown in Table 2. The dose range for the dose-finding test was determined in the presence or absence of metabolic activation at doses of 15–5000 μg/plate at a common ratio of approximately 3. Based on the results of the dose-finding test, the main test was conducted using doses of 313–5000 μg/plate at a common ratio of 2. No bacterial toxicity was observed at any of the tested doses. The positive controls were simultaneously evaluated under the standard Ames test conditions during all the experiments and showed marked positive responses, which validated the test results. The test chemical was precipitated at >1250 μg/plate with and without metabolic activation.

The ALA-DAG oil did not cause a > 2-fold increase in the number of colonies/plate compared to the negative control in both the dose-finding test and main test. The results suggested that ALA-DAG oil has no mutagenic effect under the conditions of the present study.

3.2. In vitro *micronucleus test*

The result of the *in vitro* micronucleus test to determine the effects of the ALA-DAG oil in the CHL/IU cells is summarized in Table 3. We determined that this experiment was performed satisfactorily because (1) the proportion of cells with micronuclei was significantly increased in the positive control group and (2) the micronucleus analysis was possible for the three dose levels. The ALA-DAG oil did not exhibit cytotoxicity under any of the treatment conditions. In the short-term treatment without metabolic activation, the proportions of cells with micronuclei in the 25, 500, 1000, and 2000 μg/mL-treated groups were 1.9, 1.8, 1.9, and 1.8%, respectively, and there was no significant difference compared with that in the negative control group (1.5%). The corresponding proportions of micronucleated cells with metabolic activation were 1.4, 1.5, 1.7, and 1.9%, respectively, and no significant difference was observed compared with the negative control group (1.3%). Similarly, following continuous treatment for 24 h, the proportions of the micronucleated cells in the 50, 500, 1000, and 2000 μg/mL-treated cells were 1.4, 1.5, 1.8, and 1.8%, respectively, which was comparable to that in the negative control group (1.8%). Taken together, these findings demonstrate that the ALA-DAG oil was not clastogenic and aneugenic in the CHL/IU cells.

3.3. Bone marrow micronucleus test

The results of the bone marrow micronucleus test to determine the effect of ALA-DAG oil in mice are presented in Table 4. There was no significant increase in the incidence of MNPCEs or PCEs in the test substance-treated groups compared with the vehicle control group. The positive control, MMC, remarkably increased the incidence of MNPCEs ($P < 0.05$). Therefore, under the experimental conditions of the present study, ALA-DAG oil did not show clastogenic or aneugenic effects in *in vivo*.

4. Discussion

The results of the present study indicate that the ALA-DAG oil did not induce any genotoxic effects, because it did not show mutagenicity in the Ames test, or clastogenicity and aneugenicity in the *in vitro* and *in vivo* micronucleus tests. In the series of genotoxicity tests, the highest doses used were the maximum levels recommended by the OECD guidelines. Furthermore, the negative and positive controls used in each test exhibited the expected responses, validating the methodologies used. As a mammalian genotoxicity test, the *in vitro* micronucleus test was used to detect the aneugenicity and clastogenicity in the present study, because the *in vitro* chromosomal aberration test might not detect the aneugenicity adequately [37]. The results revealed that the ALA-DAG oil was not aneugenic and clastogenic in both the *in vivo* and *in vitro* micronucleus tests.

The GEs found in the DAG oil as an impurity have raised safety concerns about the possible release of glycidol [23,38], an animal

Table 3
In vitro micronucleus test of alpha-linolenic acid-diacylglycerol (ALA-DAG) oil in CHL/IU cells.

Treatment time – recovery time (hour)	S9 mix	Group	Concentration (μg/mL)	RICC (%)[a]	Micro-mononucleated cells[b]	
					No.	Incidence(%)
3–21	–	Negative control DMSO		100	59	1.5
		ALA-DAG oil	25	108.0	77	1.9
			500#†	104.0	73	1.8
			1000#†	94.0	74	1.9
			2000#†	92.0	72	1.8
		Positive controls				
		MMC[c]	0.1	94.0	500	12.5
		CLC[d]	0.75	58.0	255	6.4
3–21	+	Negative control DMSO		100	51	1.3
		ALA-DAG oil	25	91.2	56	1.4
			500#†	98.5	60	1.5
			1000#†	98.5	66	1.7
			2000#†	104.4	77	1.9
		Positive control				
		CP[c]	10	66.2	946	23.7
24–0	–	Negative control DMSO		100	72	1.8
		ALA-DAG oil	50#	95.7	56	1.4
			500#†	97.8	60	1.5
			1000#†	82.6	73	1.8
			2000#†	91.3	70	1.8
		Positive controls				
		MMC[c]	0.025	89.1	324	8.1
		CLC[d]	0.1	47.8	537	13.4

-:Without metabolic activation, +: with metabolic activation, RICC: relative increase in cell count, DMSO: dimethyl sulfoxide, MMC: mitomycin C, CLC: colchicine, CP: cyclophosphamide monohydrate, precipitation in treatment medium was observed at #start and †end of treatments.
[a] Relative values when RICC in negative control was considered as 100%.
[b] total of 4000 mononucleated cells were scored in each group.
[c] For clastogenicity.
[d] For aneugenicity.

Table 4
Bone marrow micronucleus test of alpha-linolenic acid-diacylglycerol (ALA-DAG) oil in mice.

Group	Dose (mg kg^{-1} day^{-1})	PCEs[c]		MNPCEs[d]	
		No.	Incidence (%) Mean ± SD	No.	Incidence (%) Mean ± SD
Negative control Olive oil[a]	–	1362	54.48 ± 2.58	22	0.11 ± 0.03
ALA-DAG oil[a]	500	1416	56.64 ± 2.78	27	0.14 ± 0.05
ALA-DAG oil	1000	1417	56.68 ± 2.81	27	0.14 ± 0.05
ALA-DAG oil	2000	1395	55.80 ± 3.46	28	0.14 ± 0.05
Positive control MMC[b]	2	1207	48.28[$] ± 5.14	1219[#]	6.10 ± 1.15

MMC: mitomycin C, PCEs: polychromatic erythrocytes, MNPCEs: micronucleated polychromatic erythrocytes.
[a] treated twice by oral gavage administered 24 h apart.
[b] treated once by intraperitoneal injection.
[c] total of 2500 erythrocytes scored in each group.
[d] total of 20000 PCE scored in each group.
[$] ($p < 0.05$ compared with negative control, Student's *t*-test (two-tailed).
[#] $p < 0.05$ compared with negative control, Kastenbaum and Bowman's method (one-tailed).

genotoxic-carcinogen, during the digestion process. Accordingly, in a previous study, we demonstrated that the genotoxicity of GEs would depend on the release of glycidol [39]. Furthermore, the total levels of the GEs in ALA-DAG oil (<0.3 μg/g) were equivalent to the level of other edible oils [34], which are considered non-genotoxic, and other potential genotoxic contaminants have not been reported, indicating that it was not necessary to consider the potential genotoxicity of contaminants in ALA-DAG oil.

ALA and its related substances have been reported to increase revertant colonies in the Ames test with metabolic activation. For example, NTP [28] reported that a slight increase in the revertant colonies was observed in the experiments with the TA97 and TA100 strains at ≥666 and ≥6000 μg/plate at maximum fold ratios of 1.71 and approximately 2 (with metabolic activation), respectively. Yamaguchi and Yamashita [40] reported that autoxidized linolenic acid increased the number of revertant colonies in the Ames test using TA98 and TA100 with metabolic activation. Similarly, Hageman et al. [41] reported that linoleic acid (LIA) peroxide showed a mutagenic response in TA97 and TA100 strains in the Ames test with metabolic activation. They suggested that there was a relationship between the secondary autoxidation of LIA by the S9 mix and bacterial mutagenicity. These studies suggest that the peroxide produced by the secondary autoxidation induced by the S9 mix might have increased the revertant colonies in the Ames test to evaluate the ALA-related compounds. The disposition of ALA-DAG oil could change compared to that of ALA, which raises concerns that DNA-reactive peroxides could easily be produced by the liver enzymes in the DAG form of the oil. However, Katsuta et al. [42] reported that the glyceride structure did not affect the amount and composition of volatile aldehydes produced by oxidation of edible oils during

frying. Similarly, in the present study, an increase in the number of revertant colonies was not observed with the TA100 or TA98 strains up to at least 5000 μg/plate, which indicates no enhancement of the bacterial mutagenicity. Therefore, we considered that the concern over the potential production of reactive peroxides from the ALA-DAG oil was unfounded.

Although the fatty acid composition of oils has been known to affect their efficacy [5–8,13–16], the effect of a difference in the fatty acid composition of DAG on its associated adverse effects has not been previously investigated. Therefore, a concern was raised that the conjugation between ALA and DAG could affect the chemical properties of DAG and might induce different adverse effect from those of DAG with other fatty acid compositions. However, results of this study indicate that DAG would be non-genotoxic even if ALA contents in the fatty acid composition increased up to 60%. Moreover, we examined the possibility that genotoxic substances could be generated *via* metabolic activation of ALA-DAG oil in the *in vitro* studies in the presence and absence of a rat S9 mix as well as *in vivo* evaluations and identified no genotoxic effects under all the test conditions used.

Assuming a 50 kg human takes 15 g of ALA-DAG oil as an edible oil, the daily intake of ALA-DAG oil is estimated to be 300 mg/kg. This intake level is approximately 7 times lower than the highest dose, 2000 mg/kg, used in the *in vivo* micronucleus test using mice. In addition, since the intake of ALA-DAG oil may be divided into several times in a day, the concentration of glycerides originating from ALA-DAG oil in the blood would be lesser than those of the administered dose to mice in the *in vivo* micronucleus test. Therefore, it is unlikely to show genotoxicity in humans under the normal use of edible oil. However, considering the possible difference in toxicokinetics for different species, uncertainty of excess intake on genotoxicity should be considered for precise risk assessment of the ALA-DAG oil.

Triacylglycerol and DAG are essential nutritional components of foods that have been consumed by humans for many years. The cosmetic ingredient review panels concluded that triacylglycerol is essentially non-genotoxic based on safety assessments [43]. Moreover, a previous study demonstrated that DAG oil has a comparable genotoxicity profile to that of triacylglycerol [21].

Thus, from the results of this study, we concluded that the ALA-DAG oil showed no evidence of genotoxicity under the conditions investigated. The genotoxicity profile of ALA-DAG oil was comparable to those of triacylglycerol, DAG, and ALA, suggesting its relative safety.

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