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Original article

Oxidized dietary oils enhance immediate- and/or delayed-type allergic reactions in BALB/c mice

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ABSTRACT

Background: The consumption of cooking oils may exacerbate some allergic diseases. In the present study, the effects of naturally oxidized olive oil on immediate- and/or delayed-type allergic reactions were investigated in BALB/c mice.

Methods: Mouse models of 3 types of allergic reactions: contact hypersensitivity (CHS), active cutaneous anaphylaxis (ACA), and DNFB-induced hypersensitivity, were orally administered naturally oxidized olive oil that was obtained by keeping the oil at room temperature for more than 3 years. The effects of ultraviolet ray (UV)-irradiated olive oil and other dietary oils as well as their possible oxidation products on CHS were also investigated.

Results: Naturally oxidized olive oil had a high peroxide value (POV) and exacerbated CHS, ACA, and DNFB-induced hypersensitivity in a POV-dependent manner. UV-irradiated olive oil, corn oil, sesame oil and triolein had high POVs, but almost the same acid value (AV) and thiobarbituric acid-reactive substance (TBARS) level as fresh oils. Fresh olive oil and the representative oxidation product with a high AV or TBARS level had no effect on CHS, whereas all UV-irradiated oils and naturally oxidized olive oil exacerbated it.

Conclusions: Oxidized dietary oils that have high POVs exacerbated immediate- and/or delayed-type allergic reactions regardless of the different oil constituents or oxidation processes.

Introduction

The consumption of dietary fats has increased in industrialized countries in recent years, and this has mainly been attributed to an increase in the intake of fast foods, which include heated and processed dietary fats such as frying oil. The most frequently used cooking oils include olive oil, corn oil, and sesame oil, all of which contain the glycerol triesters of oleic acid and linoleic acid as their unsaturated fatty acids and palmitic acid and stearic acid as their saturated fatty acids.[1] Of these fatty acids, the oxidation of unsaturated fatty acids is slightly easier with heating, and radicals are more likely to be generated at the carbon–carbon double bond-neighboring methylene groups.[2,3] These radicals are very reactive and react easily with other lipid molecules. A series of chain reactions, including autoxidation, polymerization, cyclization, and decomposition are known to occur during the oxidation process,[3,4] and may be followed by the production of ketones, aldehydes, free organic acids, and hydroperoxides.[5,6] Measurements of the carbonyl value (COV),[7] the level of thiobarbituric acid-reactive substances (TBARS),[8] acid value (AV),[7] and peroxide value (POV)[7] revealed the accumulation of these oxidation products. Increased awareness of the adverse biological effects of some oxidation products has raised concern over the possible formation and presence of biologically active compounds in oxidized cooking oils. The ingestion of thermally-oxidized corn oil with a high POV by rats led to lipid peroxidation in the liver and kidney.[9,10] Oxidized soybean oil with a high POV has also been shown to induce oxidative stress in rats, resulting in peroxidative damage to muscle proteins and erythrocyte membranes.[11]

Evidence to show that enhanced oxidative stress is related to the exacerbation of allergic diseases in humans is increasing. Ambient levels of nitrogen dioxide (NO_2) have been shown to

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augment allergic inflammatory reactions in the airways of asthmatics.[12] The repeated exposure of patients with rhinitis to a peak ambient air level of ozone enhanced inflammatory airway responses.[13] An increase in ambient ozone concentrations has been correlated with an increased risk of childhood atopic dermatitis.[14] However, despite the importance to the adverse effects of oxidative stress on health, very little information is available on the relationship between the increased incidence of allergic diseases in humans and ingestion of oxidized frying oils or oxidation intermediates. Since skin is a primary site of the most prevalent human allergic diseases, animal models with allergic dermatitis including atopic dermatitis and contact hypersensitivity may be useful tools for investigating the adverse effects of oxidized cooking oil. Therefore, this study was designed to determine the effects of ingesting naturally oxidized olive oil, which is the most commonly used cooking oil with a high POV when preserved at room temperature for a long period of time, on mouse allergic reactions including contact hypersensitivity (CHS) as a delayed-type allergic reaction, active cutaneous anaphylaxis (ACA) as an immediate-type allergic reaction, and DNFB-induced hypersensitivity as the biphasic allergic reaction. We also investigated the effects of ultraviolet ray (UV)-irradiated olive oil, corn oil, sesame oil, and triolein as an oleate triester on CHS to elucidate whether the oxidation intermediates generated from other dietary oils with different constituents or chemical structures affect. Furthermore, we examined the effects of olive oil samples with high AVs or high TBARS levels on CHS to estimate whether the free organic acids and aldehydes produced from oxidized olive oil contributed to the allergic reactions. The results obtained revealed that peroxides including hydroperoxides expressed as POV that are ubiquitously generated by the oxidation of these cooking oils may accelerate skin allergic reactions.

Methods

Preparation of oxidized oils

Naturally oxidized olive oil was obtained by preserving it at room temperature for more than 3 years. The POV and AV of the oxidized olive oil were 36.6 ± 1.79–80.2 ± 2.99 mEq/kg and 0.42 ± 0.03–0.56 ± 0.29 mg/g respectively. The TBARS of the oxidized olive oil was not detected. UV-irradiated olive oil, corn oil, sesame oil, and triolein were prepared by irradiating 3.0-watt of UV light (major wavelength: 253.7 nm) at a distance of 50 cm from the opened glass dish (100 mmφ) for 48–72 h. Olive oil with a high AV or high TBARS level was adjusted to approximately 5 mg/g as the AV by adding oleic acid or 50 nmol/g as the TBARS level by adding the malondialdehyde (MDA) precursor, 1,1,3,3-tetramethoxypropan (TMP), respectively.

Animals

The protocol used here met with the Animal Experiment Guidelines of Setsunan University that were established by revising the guidelines of the Japanese Society for Pharmacology. This study was approved by the Committee for the Ethical Use of Experimental Animals at Setsunan University. All efforts were invariably made to minimize animal suffering, reduce the number of animals used, and utilize alternatives to *in vivo* techniques. Female BALB/c mice (5–6 weeks old) were purchased from Japan SLC, Inc., Shizuoka, Japan, and were acclimated in a specific pathogen-free room at 23 ± 1 °C and 47–67% humidity, under a 12 h light/darkness cycle (lights on at 7:00 a.m.) for at least a week before the start of the experiments.

CHS

Seven-week-old mice were sensitized by the topical application of 50 μl of 3% OXA (Sigma–Aldrich Inc., St. Louis, MO, U.S.A.) in a 3:1 (v/v) mixture of ethanol and acetone on the dorsal skin and were orally administered 100 μl of the test oil once a day or once every 2 days for 7 days. Mice were then challenged on the next day to elicit an allergic reaction by applying 7.5 μl of 0.1% OXA-ethanol solution to the right ears. The thicknesses of both ears were measured using a digital thickness gauge (Ozaki MFG Co. Ltd., Tokyo, Japan) while taking care to avoid compressing the edematous skin. The degree of ear swelling was calculated by subtracting the thickness of the left ear from that of the right ear.

ACA

Six-week-old mice were intraperitoneally injected with 1 μg of ovalbumin (OVA) mixed with 1 mg of aluminum hydroxide gel as the adjuvant and then orally administered 100 μl of the test oil once every 2 days for 2 weeks. Mice were intravenously injected with 0.25 ml of 0.5% Evans blue-saline solution on the next day, followed by the elicitation of ACA in the right ears by injecting 10 μl of 0.1 μg/μl OVA-saline solution. The left ears were sham-challenged by injecting 10 μl of saline solution. Thirty min after the challenge, mice were sacrificed under pentobarbital anesthesia and both ears were removed to measure extravasated dye. Non-immunized mice received the same antigenic challenge in order to determine the amount of dye detected non-specifically. The extraction and quantification of extravasated dye were performed as described by Inagaki et al.[15]

Blood samples were collected from the inferior vena cava after the OVA challenge. OVA-specific IgE plasma levels were determined using a commercial ELISA kit (SHIBAYAGI Co., Ltd. Gunma, Japan).

DNFB-induced dermatitis

Five-week-old mice were sensitized by the application of 7.5 μl of 0.15% DNFB in acetone on the right ears and orally administered 100 μl of the test oil 3 times per week for 4 weeks. Mice were also repeatedly challenged 3 times once a week to elicit an allergic reaction by applying 7.5 μl of 0.15% DNFB in acetone to the right ears. The thicknesses of both ears were measured using a digital thickness gauge (Ozaki MFG Co. Ltd., Tokyo, Japan) while taking care to avoid compressing the edematous skin. The degree of ear swelling was calculated by subtracting the thickness of the left ear from that of the right ear.

Chemical analyses

POV was determined by the American Oil Chemists' Society (AOCS) official method Ja 8-87[16] using 0.01 mol/l sodium thiosulfate after dissolving oils in acetic acid-chloroform (3:2) solution. POV (mEq/kg) was calculated with the added amount of sodium thiosulfate. AV was determined by the AOCS official method Cd 3d-63[17] after dissolving oils in diethyl ether-ethanol (1:1) solution. AV (mg/g) was calculated with the added amount of potassium hydroxide.

TBARS was determined by the method described by Ohkawa et al.[18] with slight modifications. To prepare the calibration curve, a TMP standard solution was prepared at more than 3 concentration levels without oil samples. After extracting the reaction solution in *n*-butanol –pyridine (15:1), the absorbance of the *n*-butanol phase was read at 532 nm using an absorption spectrometer. The amount of MDA was determined using a calibration curve and expressed as TBARS values.

Statistical analysis

Results were statistically analyzed using a one way analysis of variance (ANOVA) followed by Bonferroni's correction. Probability values ($p < 0.05$) were considered significant. Data in the figures were presented as the mean ± SD.

Results

Effects of oxidized olive oil on CHS

To investigate the effects of oxidized dietary oil on the delayed-type allergic reaction, mice that had been sensitization with OXA were orally administered oxidized or fresh olive oil for 7 days. CHS was elicited on the next day by the application of OXA to the right ears, and the time course of ear swelling was measured. No swelling was observed in the ears within the first 3 h regardless of the administration of oxidized or fresh olive oil, which indicated the absence of an immediate-type allergic reaction (Fig. 1). However, ear swelling was observed after 6–48 h of the elicitation, and peaked at 24 h. The extent of swelling in the oxidized olive oil-administered group was approximately 2-fold greater than that in the fresh olive oil-administered group.

To focus on the dose-dependent effects of oxidized olive oil on CHS, we prepared oxidized olive oils with different POVs by mixing with fresh oil in order to adjust to the same administration amount. Ear swelling due to CHS was enhanced in a POV-dependent manner (Fig. 2A). We also investigated the effects of the dosing interval of oxidized olive oil on CHS. Although ear swelling in the alternate day-administered mice was enhanced in a POV-dependent manner, the extent of swelling in alternate day-administered mice was less than that in the every day-administered mice (Fig. 2B). No marked difference was observed in ear swelling between the every day- and alternate day-administration in the fresh olive oil-administered group.

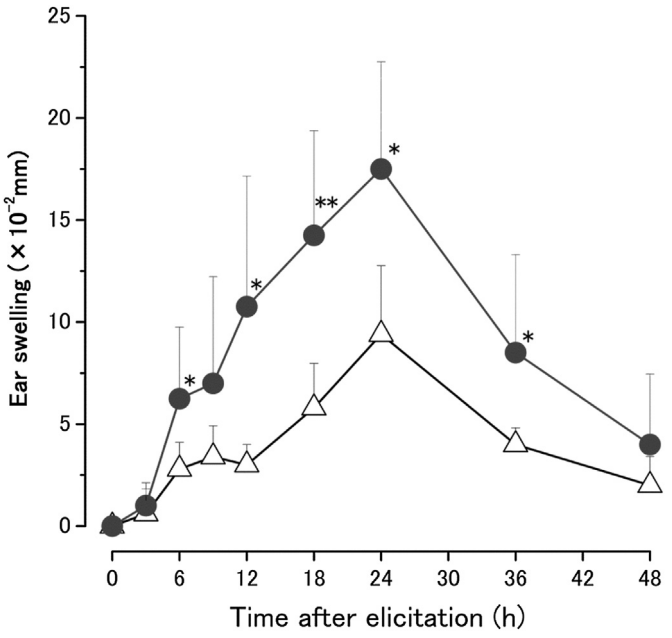

Fig. 1. Time-dependent effects of oxidized olive oil on ear swelling due to CHS. Mice were orally administered fresh olive oil (POV = 6.34 ± 1.50 mEq/kg) and oxidized olive oil (POV = 36.6 ± 1.79 mEq/kg) for 7 days and CHS was then elicited. Fresh olive oil (△) and oxidized olive oil (●). The values are the mean ± SD ($n = 4$). *$P < 0.05$, **$P < 0.01$ vs. fresh olive oil group.

Effects of oxidized olive oil on ACA

To investigate the effects of oxidized dietary oil on the immediate-type allergic reaction, mice were intraperitoneally administered OVA with aluminum hydroxide gel and orally administered oxidized or fresh olive oil 7 times on alternate days for 2 weeks. Extravasated dye was measured on the next day in the ear 30 min after the ACA elicitation. The amount of ear dye that leaked was greater in oxidized olive oil-administered mice than in fresh olive oil-administered mice (Fig. 3A). The titer of the OVA-specific IgE antibody in the plasma significantly increased in oxidized olive oil-administered mice (Fig. 3B).

Effects of oxidized olive oil on DNFB-induced dermatitis

The effects of oxidized dietary oil were investigated on biphasic allergic reactions (immediate- and delayed-type). Mice were orally administered oxidized or fresh olive oil 3 times a week while inducing allergic reactions by the application of 0.15% dinitrofluorobenzene (DNFB) 4 times to the right ears. The first DNFB application (sensitization) did not affect ear thickness (Fig. 4A). However, the ear showed a biphasic response which consisted of the immediate and delayed reaction after 1st challenge. The immediate reaction peaked at 1 h and the delayed reaction at 24 h after challenge, respectively. The 2nd (1st challenge), 3rd (2nd challenge), and 4th (3rd challenge) application of DNFB, ear swelling after 24 h, after 1–4 h, and after 1 h and 8–24 h, respectively, was significantly greater in oxidized olive oil-administered mice than in fresh olive oil-administered mice.

Effects of various UV-irradiated dietary oils and their possible reaction products on CHS

The effects of artificial oxidation and the oil constituents of several dietary oils on CHS were investigated. The oils with different fatty acid constitutions, olive oil, corn oil, sesame oil and the chemically-pure oleic triester, triolein were oxidized by UV-irradiation. All UV-irradiated oils had high POVs (Table 1). The AVs and TBARS levels of the UV-irradiated oils were similar to those of the fresh oils. Ear swelling was significantly greater in UV-irradiated oil-administered mice than in fresh oil-administered mice (Fig. 5).

We then investigated whether possible oxidation products due to UV-irradiation may have contributed to the exacerbation of CHS using fresh olive oil mixed with oleic acid or the malondialdehyde precursor as the representative product with a high AV or TBAS level (see Table 2). Although olive oil with a high POV significantly enhanced ear swelling, olive oil with a high AV or TBARS level induced the same extent of ear swelling as fresh olive oil (Fig. 6).

Discussion

In the present study, we demonstrated that naturally oxidized olive oil exacerbated 3 types of allergic reactions: CHS, ACA and DNFB-induced hypersensitivity. CHS and ACA represent a delayed-type allergic reaction that is caused by cell-mediated immunity[19] and an immediate-type allergic reaction that is caused by antibody-mediated immunity,[15] respectively. DNFB-induced hypersensitivity represents a biphasic allergic reaction that is caused by both cell-mediated and antibody-mediated immunity.[20] This contact dermatitis model shows mainly delayed-type allergic reaction mediated by Th1 type T cells and repeated application of DNFB also induces immediate-type allergic reaction with an increase in serum antigen-specific IgE.[21] Therefore, the results of the present study indicate that naturally oxidized olive oil may

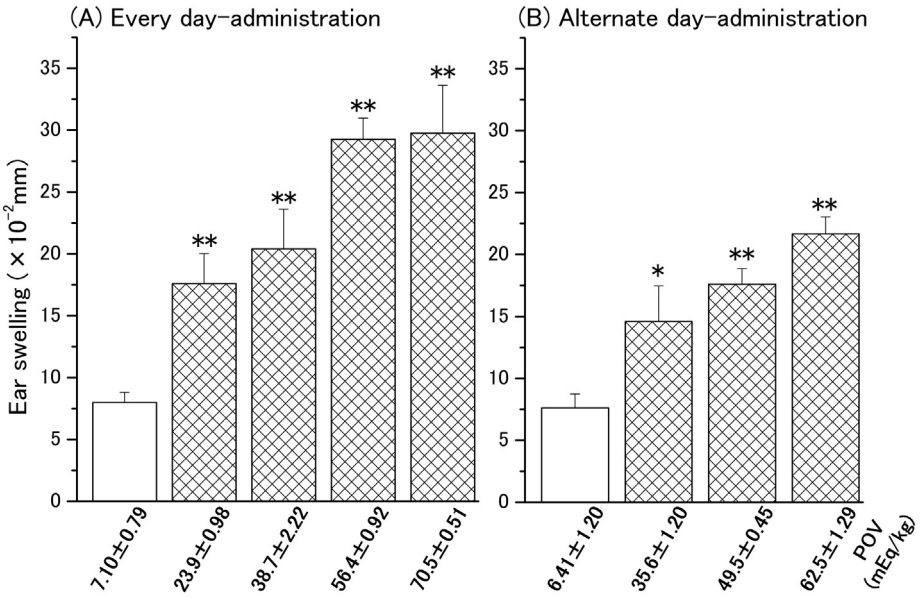

Fig. 2. Dose-dependent effects of oxidized olive oil on CHS. CHS was elicited 7 days after the OXA sensitization. Ear swelling was measured 24 h after the elicitation. Fresh olive oil (▭) and oxidized olive oil (▨). The values are the mean ± SD (n = 5–6). *P < 0.05, **P < 0.01 vs. the fresh olive oil group.

enhances both cell-mediated and antibody-mediated immunity due to promoting Th1 cell activity or IgE production.

As shown in Fig. 2, although fresh olive oil did not affect CHS regardless of the dosing interval, oxidized olive oil enhanced CHS in a POV- or dosing frequency-dependent manner. This result indicated that the exacerbation of CHS by oxidized olive oil may depend on the total amount of peroxides contained in the oxidized oil, which suggests that the ingestion of highly-oxidized dietary oil may be responsible for an increased risk of allergic diseases even though the incidence is low.

Most commercially available vegetable oils consist of oleic acid, linoleic acid, and/or linolenic acid as unsaturated fatty acids, and their compositions vary depending on the vegetable source.[22] When these oils are used as cooking oils, thermal oxidation, natural oxidation with aging, and/or photooxidation of the oil constituents can occur as possible oxidation processes. Thus, oxidation products may cause allergic reactions depending on the unsaturated fatty acid composition and oxidation processes. As shown in Fig. 2B and Fig. 5, we demonstrated that oxidized oil evaluated in terms of POV enhanced CHS regardless of oils with different fatty acid compositions or oxidation processes. The extent of CHS in the naturally oxidized olive oil group was similar to that in the UV-irradiated olive oil group. Taken together, the exacerbation of CHS by oxidation products generated from

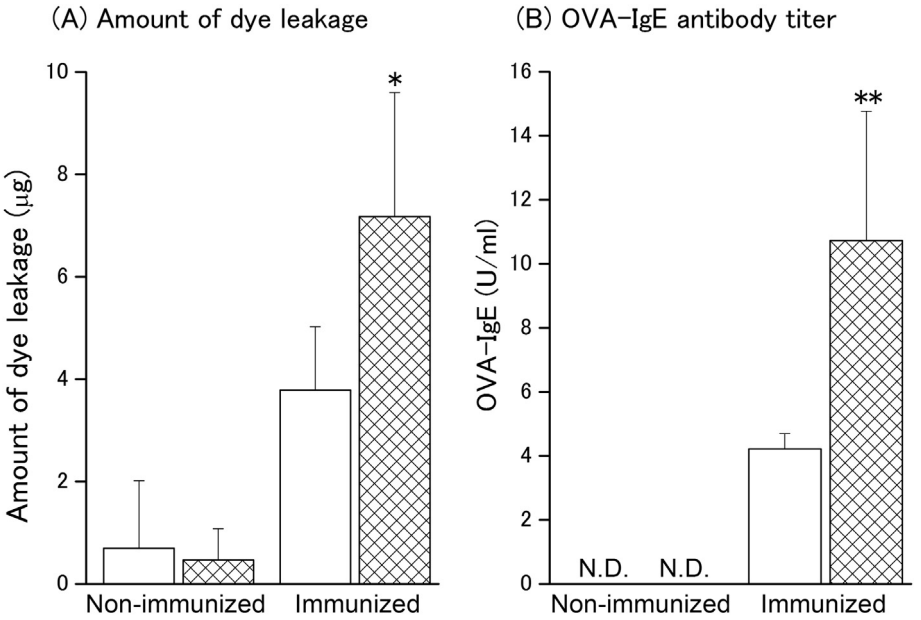

Fig. 3. Effects of oxidized olive oil on ACA. Mice were orally administered fresh olive oil (POV = 3.66 ± 1.75 mEq/kg) and oxidized olive oil (POV = 75.0 ± 3.03 mEq/kg) for 14 days and ACA was elicited. Fresh olive oil (▭), and oxidized olive oil (▨). The values are the mean ± SD (n = 5–6). *P < 0.05, **P < 0.01 vs. the fresh olive oil group. N.D., Not detected.

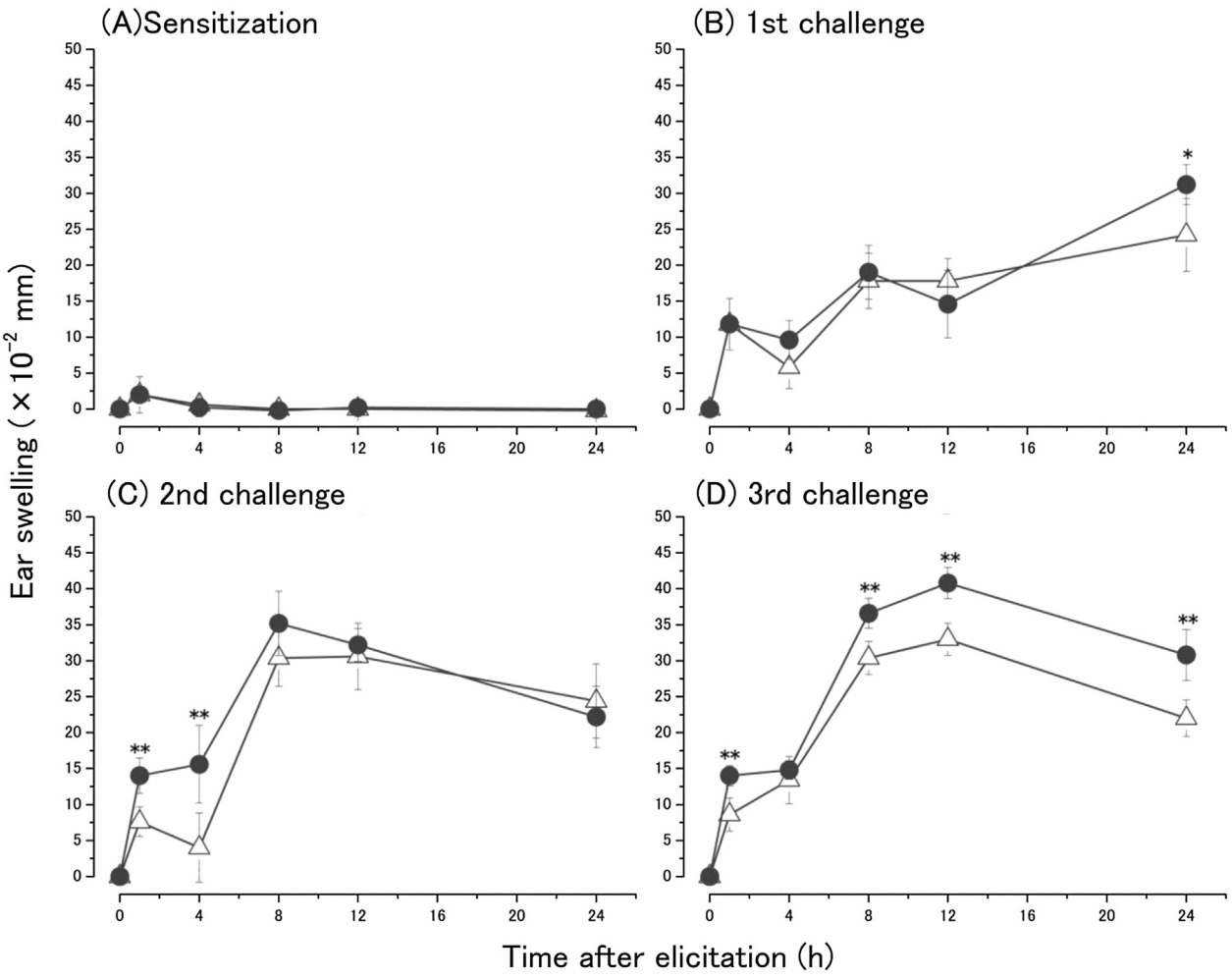

Fig. 4. Effects of oxidized olive oil on DNFB-induced dermatitis. Mice were orally administered fresh olive oil (POV = 3.95 ± 0.81 mEq/kg) and oxidized olive oil (POV = 80.2 ± 2.99 mEq/kg) 3 times a week for 4 weeks and allergic dermatitis was elicited repeatedly at 1-week intervals. Fresh olive oil (△) and oxidized olive oil (●). The values are the mean ± SD ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the fresh olive oil group.

unsaturated fatty acids may merely be dependent on the formation of hydroperoxides.

Since olive oil contains high amounts of oleic acid, hydroperoxides can be easily produced by oxidation[3] We also demonstrated that the hepatic oxidized glutathione content was increased in naturally oxidized olive oil, which indicated oxidative stress (data not shown). Many studies have demonstrated that peroxides in thermally oxidized oil induce oxidative stress.[22,23] The exacerbation of allergic diseases such as allergic asthma has been associated with an increase in oxidative stress, as indicated by the elevated levels of oxidative products in asthma patients.[24] Oxidative stress

Table 1
Properties of UV-irradiated oil.

Variety	UV-irradiation	POV (mEq/kg)	AV (mg/g)	TBA (μmol/g)
Olive oil	−	5.85 ± 1.22	0.34 ± 0.02	N.D.
	+	57.2 ± 0.76	0.40 ± 0.02	N.D.
Corn oil	−	5.90 ± 2.85	0.16 ± 0.03	N.D.
	+	68.2 ± 0.73	0.19 ± 0.06	N.D.
Sesame oil	−	5.11 ± 1.72	0.14 ± 0.02	N.D.
	+	63.5 ± 0.47	0.37 ± 0.10	N.D.
Triolein	−	3.94 ± 0.54	0.34 ± 0.03	N.D.
	+	52.3 ± 0.16	0.39 ± 0.06	N.D.

N.D., Not detected.

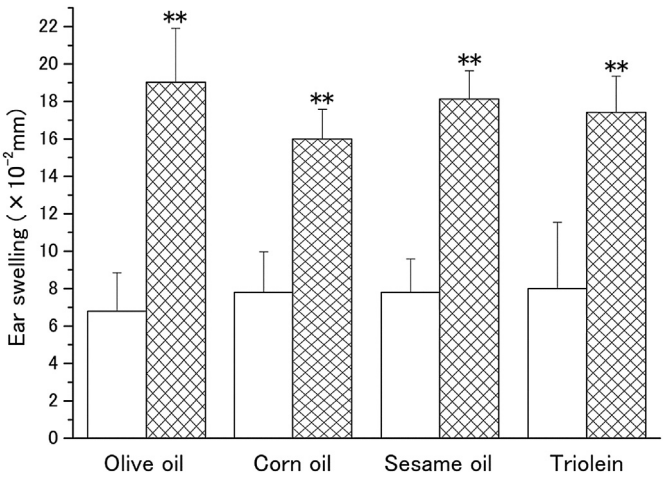

Fig. 5. Effects of various UV-irradiated frying oils on CHS. CHS was elicited 7 days after the OXA sensitization. Ear swelling was measured 24 h after the elicitation. The values are the mean ± SD ($n = 5–6$). Fresh oil (□) and oxidized oil (▨). $^{**}P < 0.01$ vs. the fresh oil group.

Table 2
Properties of olive oil with a high POV, high AV, and high TBARS level.

Index	Fresh oil	High POV	High AV	High TBARS
POV (mEq/kg)	9.40 ± 0.56	55.5 ± 0.95	9.20 ± 0.27	9.23 ± 0.24
AV (mg/g)	0.41 ± 0.02	0.51 ± 0.01	5.34 ± 0.02	0.42 ± 0.03
TBARS (nmol/g)	N.D.	N.D.	N.D.	52.4 ± 0.14

N.D., Not detected.

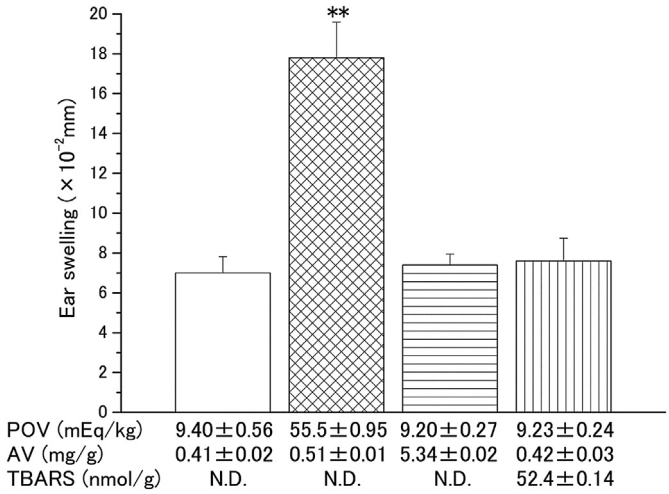

Fig. 6. Effects of possible oxygenated products in oxidized olive oil on CHS. CHS was elicited 7 days after the OXA sensitization. Ear swelling was measured 24 h after the elicitation. The values are the mean ± SD ($n = 5–6$). Fresh oil (▭), high POV oil (▨), high AV oil (▤), and high TBARS oil (▥). $^{**}P < 0.01$ vs. the fresh oil group.

and altered antioxidant defenses are involved in the pathophysiology of acute exacerbations in atopic dermatitis.[25] Polyunsaturated fatty acids also mediate degranulation from rat basophilic leukemia.[26] Recently, the saturated fatty acids with 7–12 carbons have been shown to induce expression of thymic stromal lymphopoietin, a Th2-inducer cytokine, which suggests exacerbation of allergic inflammation.[27] However, oleic acid or the malondialdehyde precursor did not affect CHS in the present study. Therefore hydroperoxides generated from unsaturated fatty acids are principally involved in the exacerbation of allergic reactions.

Although naturally oxidized olive oil are likely to affect both Th1 and Th2 responses, the underlying mechanisms are unclear. The oxidative stress by hydrogen peroxide treatment reduces the interferon-γ production of activated Th1 clones and potentiates interleukin-4 secretion of activated Th2 clones.[28] Oxidative stress may be related not only to lymphocytes function but also antigen-presenting cells. Csillag et al. showed that pollen exposure-induced oxidative stress could enhance dendritic cell function including development of naive T lymphocytes toward effector T cells with a mixed profile of cytokine production.[29] Another study also showed that activated dendritic cells enhanced Th1 and Th2 responses *in vitro* and *in vivo*.[30] Taken together, a biphasic response by naturally oxidized olive oil may be due to functional changes of dendritic cells.

In conclusion, oxidized dietary oils that contain high levels of hydroperoxides exacerbate both immediate- and delayed-type allergic reactions regardless of differences in oil constituents or oxidation processes. The present study has provided information that will support additional investigations on the enhancing effects of oxidized dietary oils on allergic reactions. Further studies also will be needed to clarify the enhancing mechanisms of the unsaturated fatty acid hydroperoxides in cell-mediated and antibody-mediated immunities including an involvement of inflammatory cytokines.

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Conflict of interest
The authors have no conflict of interest to declare.

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