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Original article

Ozonated ethyl ester of α-linolenic acid alleviates hepatic de novo lipogenesis in LXR agonist–treated HepG2 cells

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Summary

Ozonated ethyl esters of unsaturated fatty acids (zFEEs) are derived from the double bonds of oleic (zOEE), linoleic (zLEE), and α-linolenic acids (zLnEE). The *cis* double bonds of unsaturated fatty acids readily react with ozone, producing ozonated fatty acid ethyl esters (zFEEs) as reaction products. This study investigated the impact of ozonated ethyl esters of unsaturated fatty acids (zOEE, zLEE, and zLnEE) on lipogenic enzyme expression in HepG2 cells treated with an LXR agonist. Lipid accumulation was induced by LXR agonist, which activates genes involved in lipid synthesis and storage. The corresponding non-ozonated fatty acid ethyl esters (OEE, LEE, and LnEE) were also examined for comparison with ozonated fatty acid ethyl esters. HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were then co-treated with 10 μg/mL of each compound and 50 nM LXR agonist in DMEM containing 0.1% BSA for 24 h. The control group was treated with 0.1% ethanol and 50 nM T0901317 (an LXR agonist). Intracellular triglyceride (TG) levels were assessed using Oil Red O staining and absorbance measurements, whereas the mRNA expression of lipogenic enzymes (SREBP-1, FASN, SCD, CD36, and ABCA1) was quantified *via* RT-qPCR. No cytotoxicity was observed in HepG2 cells following treatment. Notably, zOEE and zLnEE significantly altered TG levels compared with the control group in LXR agonist-stimulated cells, as evidenced by Oil Red O staining. Furthermore, zLnEE significantly downregulated CD36, ABCA1, and SREBP-1 mRNA expression compared to the control, although FASN and SCD expression remained unchanged. These findings suggest that ozonated ethyl esters of unsaturated fatty acids may inhibit fatty acid synthesis and reduce TG accumulation in HepG2 cells.

Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a growing global health concern, characterized by excessive hepatic lipid accumulation during high-fat diet consumption[1)]. The shift from non-alcoholic fatty liver disease (NAFLD) to MAFLD was introduced to diagnose the disease based on the presence of metabolic risk factors (e.g., obesity, type 2 diabetes, or metabolic syndrome), regardless of other coexisting liver diseases. This highlights the central role of metabolic dysfunction in disease progression aligning with its strong association with insulin resistance and systemic inflammation[1)]. The pathogenesis of NAFLD involves complex metabolic disturbances, including increased free fatty acid flux from adipose tissue, enhanced *de novo* lipogenesis, and impaired lipid oxidation[2)]. Hepatic steatosis, a hallmark of NAFLD, arises from excessive triglyceride (TG) deposition because of dysregulated lipid metabolism[3)]. The liver X receptor (LXR) pathway plays a crucial role in lipid homeostasis by modulating the expression of genes involved in cholesterol efflux (*ABCA1*) and lipogenesis (*SREBP-1c* and *FASN*)[4)]. However, excessive LXR activation can exacerbate hepatic steatosis by promoting *de novo* lipogenesis, making it a potential therapeutic target for MAFLD intervention[5)].

Emerging evidence suggests that ozonated fatty acid derivatives mitigate MAFLD by modulating lipid metabolism. Ozonated olive oil, for instance, has been shown to suppress hepatic steatosis in obese *db/db* mice by altering lipogenic pathways rather than activating lipolysis[6)].

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Reportedly, ozonated olive oil intake reduces hepatic fat accumulation and liver injury in a rat model of obesity by suppressing pro-inflammatory cytokines[7]. These findings suggest that ozonated lipids may exert beneficial effects by inhibiting lipid synthesis and inflammation, which are key drivers of MAFLD progression[8,9].

The *cis* double bonds of unsaturated fatty acids readily react with ozone to produce ozonated fatty acid ethyl esters (zFEEs) as reaction products, which form bioactive compounds with potential metabolic regulatory effects[10]. Unlike their nonozonated counterparts, zFEEs may influence lipid metabolism by reducing TG synthesis and fatty acid uptake in hepatocytes. Prior research indicates that oxidized fatty acids can suppress LXR agonist-induced lipid accumulation in HepG2 cells, suggesting a possible mechanism involving the downregulation of lipogenic genes (*SREBP-1c* and *FASN*) and lipid transporters (*CD36*)[11]. T0901317 (an LXR agonist) robustly upregulates *ABCA1* to promote cholesterol export[23]. However, excessive *ABCA1* activation may paradoxically exacerbate hepatic lipid accumulation (simple steatosis) by diverting fatty acids toward esterification and VLDL secretion[24]. Given that MAFLD progression is closely linked to excessive *de novo* lipogenesis and impaired lipid export[12], zFEEs could offer a novel therapeutic approach by targeting these pathways.

Recent studies have emphasized the role of ozonated lipids in metabolic regulation. Ozonated olive oil modifies hepatic lipogenic gene expression, leading to reduced TG storage[6]. Additionally, Guo et al. reported that modulating fatty acid oxidation and lipogenesis can alleviate NAFLD in preclinical models[3]. Building on these findings, this study investigated whether zFEEs (zOEE, zLEE, and zLnEE) can inhibit TG accumulation and suppress fatty acid synthesis in LXR agonist-treated HepG2 cells. We hypothesized that zFEEs, particularly zLnEE, may attenuate hepatic steatosis by downregulating key lipogenic enzymes (SREBP-1c, FASN, and SCD) and fatty acid transporters (CD36). In addition, we evaluated the mRNA expression of ABCA1, a well-known LXR target gene, to examine its potential modulation of LXR signaling.

Understanding the mechanisms by which zFEEs influence hepatic lipid metabolism may provide new insights into MAFLD treatment strategies. Given the limitations of current pharmacological interventions, which often focus on weight loss and insulin sensitization[13], alternative approaches targeting lipid metabolic pathways are needed. The aim of this study was to elucidate the potential of zFEEs as therapeutic agents by evaluating their effects on TG accumulation and lipogenic gene expression in an *in vitro* MAFLD model. These findings may contribute to the development of novel nutraceutical or pharmaceutical strategies for managing metabolic disorders associated with fatty liver disease.

Materials and methods

Study design

The experimental compounds in this study consisted of ozonated fatty acid ethyl esters (zFEEs), which included three main derivatives: ozonated ethyl ester of oleic acid (zOEE), ozonated ethyl ester of linoleic acid (zLEE), and ozonated ethyl ester of α-linolenic acid (zLnEE) (Fig. 1). For comparison, the corresponding non-ozonated ethyl esters were also evaluated: ethyl ester of oleic acid (OEE), ethyl ester of linoleic acid (LEE), and ethyl ester of linoleic acid (LnEE). The synthetic LXR agonist T0901317 (50 nM) was employed throughout the study to activate the liver X receptor pathway and induce a steatotic phenotype in HepG2 cells. This experimental design allowed us to systematically investigate the effects of both ozonated and non-ozonated fatty acid derivatives on LXR-mediated lipid accumulation. This study employed eight distinct treatment groups to comprehensively evaluate the therapeutic potential of these compounds. The normal group received only the vehicle control (0.2% ethanol) without an LXR agonist. The control group was treated with 0.1% ethanol and 50 nM T0901317 (an LXR agonist). The groups of OEE, LEE, LnEE, zOEE, zLEE, and zLnEE groups were treated with 50 nM LXR agonist and administered 10 μg/mL of each

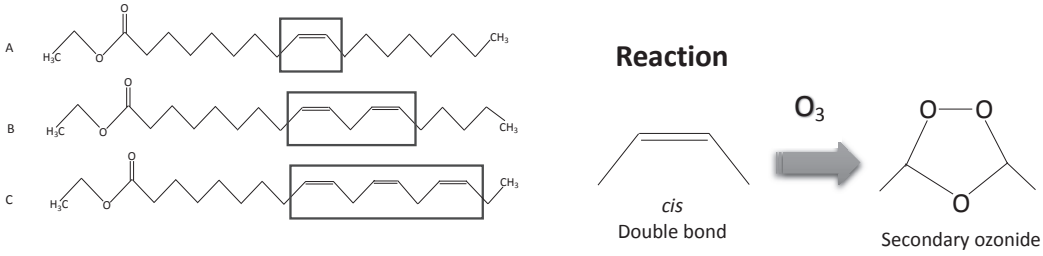

Fig. 1 Chemical structures of ethyl esters of unsaturated fatty acids: (A) oleic acid; (B) linoleic acid; (C) α-linolenic acid. Formation of secondary ozonides from cis double bonds.

compound.

Materials

OEE (> 95.0% oleic acid ethyl ester) and LEE (> 97.0% linoleic acid ethyl ester) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and LnEE (≥99% α-linolenic acid ethyl ester) was purchased from Merck KGaA (Darmstadt, Germany). Ozonated ethyl esters of unsaturated fatty acids (zOEE, zLEE, and zLnEE) were prepared according to the experimental conditions described below[22]. Each fatty acid ethyl ester (150 mg) was dissolved in 15 mL of dichloromethane (super dehydrated) and cooled to –20℃ using a mixture of acetone and liquid nitrogen. Ozone gas, generated by an ozone gas generator (ED-OG-R6; EcoDesign Co., Ltd., Saitama, Japan), was bubbled into each fatty acid ethyl ester solution at a rate of 1.0 L/min for 5 min. The ozone generation rate was set to 4 g/h. After bubbling, the mixture was stirred at –20℃ for 2 h to stabilize the product. Dichloromethane was removed using a rotary evaporator, and 15 mL of Infinity Pure ethanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to prepare an ethanol solution of ozonated fatty acid ethyl esters. The reaction was confirmed by ^{1}H and ^{13}C NMR spectroscopy, as described in our previous study[25]. Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan), containing 10% fetal bovine serum (FBS), penicillin, and streptomycin (100 U/mL), was used for cell culture. All other chemicals and solvents used in the experiment were of commercial grade.

Cell culture

The HepG2 cells were incubated under a humidified atmosphere of 5% CO_2 at 37 ℃. Cells were cultured at a density of 5 × 10^6 cells/mL in DMEM supplemented with 10% FBS and antibiotics (penicillin/streptomycin). HepG2 cells were obtained from JCRB 1054; Health Science Resources Bank (Osaka, Japan).

*MTT (*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*) assay*

HepG2 cell suspensions were prepared at a concentration of 5 × 10^6 cells/mL. Cells were seeded in 96-well culture plates at a density of 100 μL/well. After 24 h of incubation, HepG2 cells were co-treated with 10 μg/mL of each compound and 50 nM LXR agonist in 0.1% BSA-containing DMEM for 24 h at 5% CO_2 and 37℃. Then, 25 μL of MTT solution (Nacalai Tesque, Kyoto, Japan; final concentration 1 mg/mL) was added to each well, followed by incubation for 30 min at 37℃. The supernatant was then discarded and 2-propanol was added 120 μL per well before shaking for 1 h at 24℃. After that, take 80 per well into new 96-well. Finally, absorbance was measured using a microplate reader (Molecular Devices, CA, USA) at 570 nm with 650 nm as the reference wavelength.

Oil Red O staining

HepG2 cells at a concentration of 5 × 10^6 cells/mL were seeded in 24-well culture plates, at 400 μL/well. After 24 h of incubation, HepG2 cells were co-treated with 10 μg/mL of each compound and 50 nM LXR agonist in 0.1% BSA-containing DMEM for 24 h at 5% CO_2 and 37℃. For cell staining, the cells were washed twice with 400 μL of PBS, fixed with 400 μL of 4% PFA in PBS for 10 min at 24℃, and then washed again with PBS. Next, 400 μL of 60% 2-propanol was added for 1 min. The cells were stained with 400 μL of filtered 60% Oil Red O solution for 20 min at 24℃, followed by two quick rinses with 60% 2-propanol. Finally, the cells were washed twice with 400 μL PBS, with the last PBS wash left in the wells for microscopic analysis. Then, lipid droplets of HepG2 cells were observed using BZ II observation software, with 20× magnification, 0.45 NA (numerical aperture), and a Keyence BZ-2 Analyzer microscope (Keyence Corporation,

Table 1 Primers of quantitative real time PCR

mRNA	Forward (from 5' to 3')	Reverse (from 5' to 3')
β -actin	*ATTGGCAATGAGCGGTTCC*	*GGTAGTTTCGTGGATGCCACA*
SREBP-1	*CGGAACCATCTTGGCAACAGT*	*CGCTTCTCAATGGCGTTGT*
FASN	*AAGGACCTGTCTAGGTTTGATGC*	*TGGCTTCATAGGTGACTTCCA*
SCD	*TTCCTACCTGCAAGTTCTACACC*	*CCGAGCTTTGTAAGAGCGGT*
CD36	*CTTTGGCTTAATGAGACTGGGAC*	*GCAACAAACATCACCACACCA*
ABCA1	*ACCCACCCTATGAACAACATGA*	*GAGTCGGGTAACGGAAACAGG*

Note:
SREBP-1, Sterol Regulatory Element-Binding Protein 1
FASN, Fatty Acid Synthase
SCD, Stearoyl-CoA Desaturase
CD36, Cluster of Differentiation 36
ABCA1, ATP-Binding Cassette Subfamily A Member 1

Japan).

RT-qPCR analysis

Cell suspensions of HepG2 cells were prepared at a concentration of 5×10^6 cells/mL and seeded in 48-well plates at a density of 250 μL/well. After 24 h of incubation, the cells were attached and treated with zFEEs (10 μg/mL) and T0901317 (50 nM). All cells were incubated for an additional 24 h. Total RNA was extracted using Sepasol Reagent (Nacalai Tesque) and the isolated RNA was reverse-transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan). The cDNA templates were then diluted and mixed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) containing PCR primers. The thermal cycling program consisted of 40 cycles. Primer sequences used in this study are listed in Table 1. The mRNA levels of target genes were normalized to β-actin and calculated using the 2^{-DDCt} method.

Statistical analysis

Data are presented as mean ± SD (n = 3). Statistical significance was analyzed using SPSS software (IBM, version 25). Different letters indicate significant differences among groups at $p < 0.05$, as determined by one-way ANOVA followed by Tukey's post hoc test. Significance was set at $p < 0.05$.

Results and Discussion

Cytotoxicity assessment

The MTT assay revealed no cytotoxic effects on HepG2 cells following 24-h treatment with 10 μg/mL of ozonated ethyl esters (zOEE, zLEE, zLnEE) in the presence of 50 nM LXR agonist (Fig. 2). These findings align with those of previous studies demonstrating the biocompatibility of ozonated fatty acid derivatives at therapeutic concentrations[7,14]. The absence of cytotoxicity suggests that zFEEs are safe for further investigation as potential antisteatotic agents, corroborating reports that ozonated lipids exhibit selective bioactivity without compromising cell viability[10]. However, Liu et al. found that ozone-derived products at doses of 20–50 μg/mL may reflect tissue-specific sensitivity thresholds[15]. Collectively, these studies confirm the non-cytotoxic nature of ozone-based treatments at therapeutic concentrations, although our hepatic model (10 μg/mL) employed lower doses than previous studies. The fatty acid derivatives used in this study were synthesized in the ethyl ester form. Those are generally more stable in storage and less prone to oxidation than free fatty acids, making them a practical choice for experimental workflows[26]. While fatty acid ethyl esters are not primary physiological form for circulation, those were selected for their enhanced lipophilicity and stability, which promote efficient and consistent cellular uptake in vitro compared to free fatty acids[27]. Furthermore, the ethyl ester group provides chemical stability during the ozonation process. It is important to note that fatty acid ethyl esters release the active free fatty acids within the hepatocyte[28]. This approach ensures targeted intracellular delivery and validated strategy for studying fatty acid-mediated effects in cell culture models.

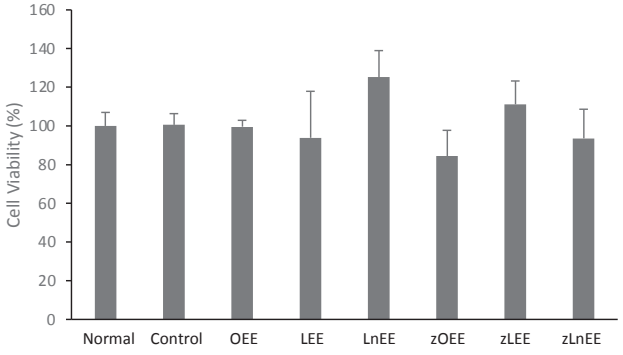

Fig. 2 Viability of HepG2 cells treated with LXR agonist (50 nM) and test compounds for 24 h. Values represent mean ± SD (n = 3). The normal group received only vehicle control (0.2% ethanol) without LXR agonist. The control group was treated with 0.1% ethanol and 50 nM T0901317 (LXR agonist). The experimental groups (OEE, LEE, LnEE, zOEE, zLEE, and zLnEE) were treated with LXR agonist 50 nM, and administered 10 μg/mL of each compound.

Inhibition of TG accumulation

zOEE and zLnEE significantly reduced intracellular TG levels compared to the control group in LXR agonist-stimulated HepG2 cells. Quantification *via* Oil Red O absorbance measurements showed a trend toward decreased lipid droplets relative to the control, the relative values showed normal as 100, control as 122, OEE as 116, LEE as 124, LnEE as 129, zOEE as 92, zLEE as 111, and zLnEE as 78. Those results consistent with earlier reports that ozonated lipids disrupt TG storage[6] (Fig. 3). These results mirror *in vivo* findings that ozonated olive oil attenuated hepatic steatosis in *db/db* mice by suppressing SREBP-1c-mediated lipogenesis[6]. Mechanistically, ozonated compounds may inhibit diacylglycerol acyltransferase (DGAT), a key enzyme in TG synthesis[11], and enhance β-oxidation, thereby promoting fatty acid catabolism[16].

Modulation of lipogenic gene expression

zLnEE significantly downregulated the mRNA expression of CD36, ABCA1, and SREBP-1 by 50–60% compared

to the control, but had no significant impact on FASN or SCD (Fig. 4). Previous studies have demonstrated that oxidized fatty acids may inhibit LXR agonist-mediated lipid build-up in HepG2 cells, suggesting a potential mechanism through the suppression of lipogenic gene expression (particularly *SREBP-1*)[11]. The slight reduction in FASN and SCD levels implies that zFEEs may inhibit lipogenesis, although not significantly. This selective gene suppression suggests that zFEEs target specific pathways involved in lipid metabolism. SREBP-1 reduction blunts LXR-induced lipogenesis, a mechanism also observed with omega-3 fatty acids[16]. While our study indicated that zLnEE downregulates LXR-induced SREBP-1 expression, Schulman's review emphasized that LXRs typically activate SREBP-1c to promote lipogenesis[17], suggesting that ozonated fatty acids may override canonical LXR signaling through com-

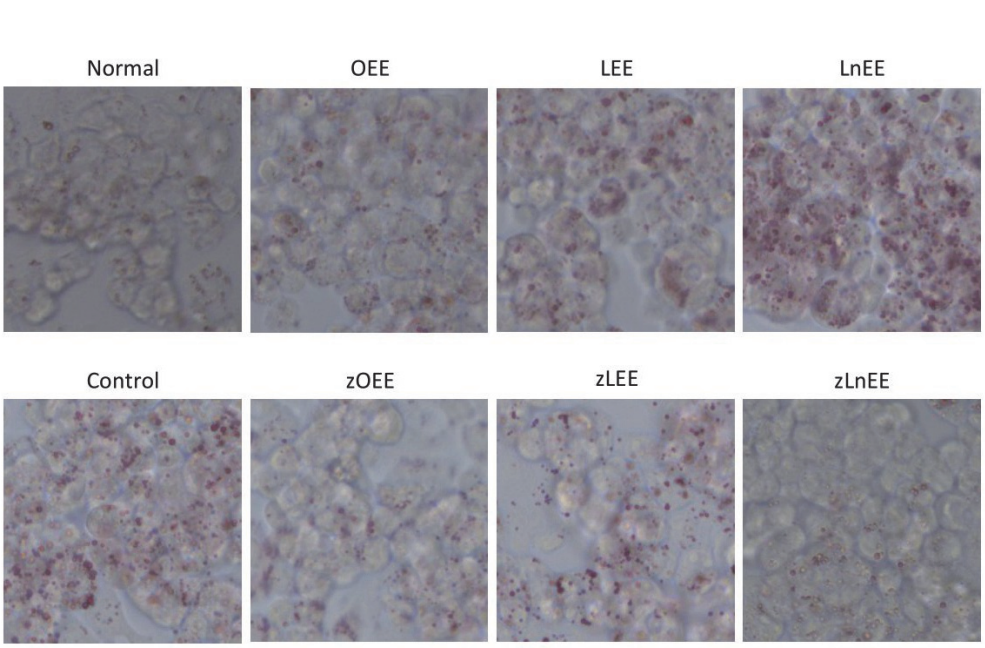

Fig. 3 Oil Red O staining of HepG2 cells treated with LXR agonist (50 nM) and samples for 24 h (20× magnification, 0.45 NA). The normal group received only vehicle control (0.2% ethanol) without LXR agonist. The control group was treated with 0.1% ethanol and 50 nM T0901317 (LXR agonist). The experimental groups (OEE, LEE, LnEE, zOEE, zLEE, and zLnEE) were treated with LXR agonist 50 nM, and administered 10 μg/mL of each compound.

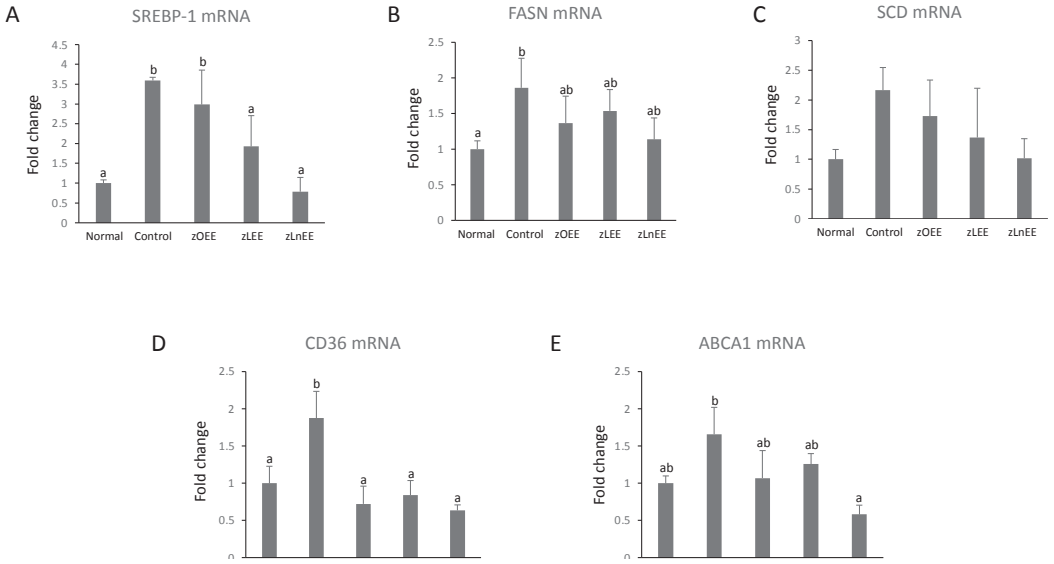

Fig. 4 mRNA expression of lipogenic enzymes. The normal group received only vehicle control (0.2% ethanol) without LXR agonist. The control group was treated with 0.1% ethanol and 50 nM T0901317 (LXR agonist). The experimental groups (OEE, LEE, LnEE, zOEE, zLEE, and zLnEE) were treated with LXR agonist 50 nM, and administered 10 μg/mL of each compound. The values represent mean ± SD (n = 3). Significance was determined by Tukey's test; bars with different letters are considered significantly different from each other ($p < 0.05$).

petitive receptor modulation. CD36 suppression reduces fatty acid uptake, a critical driver of NAFLD progression[18]. This inhibition is consistent with findings that ozonated oils decrease lipid transport in hepatocytes (9). Our results showed that zLnEE significantly reduced hepatic TG accumulation and CD36 expression. Cho et al. reported that ozonated sunflower oil decreased hepatic lipid levels by 35% in hyperlipidemic zebrafish by suppressing fatty acid uptake pathways[19]. As described in the Introduction, we evaluated ABCA1 mRNA expression, a well-known LXR target gene, to examine the potential modulation of LXR signaling by zFEEs. In LXR agonist-treated HepG2 cells, zLnEE significantly downregulated *ABCA1* expression. *ABCA1* is generally considered a protective gene that promotes cholesterol efflux, and its suppression may therefore seem counterintuitive. However, *ABCA1* downregulation can limit cholesterol export and potentially redirect lipids toward oxidation[4,20]. While Lee and Tontonoz emphasized the atheroprotective effects of *ABCA1* upregulation, the current results paradoxically show improved hepatic lipid homeostasis despite *ABCA1* suppression. This suggests that ozonated fatty acids may selectively modulate LXR target genes or bypass canonical LXR signaling pathways[21], highlighting a potential mechanism by which zFEEs attenuate TG accumulation without broadly activating LXR-mediated lipogenesis.

Conclusion

Unlike single-target drugs, zFEEs offer distinct advantages, such as dual-action modulation that simultaneously reduces lipid uptake (*via* CD36) and fatty acid synthesis (*via* SREBP-1). However, challenges remain in optimizing bioavailability and tissue-specific delivery. This study has limitations, including the fact that the stability of zLnEE was lower than that of zLEE, depending on the number of ozonides. Thus, unless supported by additional evidence, further studies are required to clarify the minor effects of zLEE. In this study, we found that zLnEE, an ozonated α-linolenic acid derivative, reduces hepatic TG accumulation by selectively suppressing CD36, ABCA1, and SREBP-1 in LXR-activated HepG2 cells. The absence of cytotoxicity and the multitarget effects of zFEEs make them novel candidates for MAFLD therapy.

Conflict of interest

Tsukishima Foods Industry Co., Ltd. provided research funding to TS and the samples to the Laboratory of Marine Bioproducts Technology at Kyoto University. YK is an employee of Tsukishima Foods Industry Co. Ltd. The other authors declare no conflicts of interest.

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