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Research Article

The Effect of Oleoylethanolamide Supplementation on Pyroptotic Cell Death in Obese Patients with Non-Alcoholic Fatty Liver Disease: A Double-Blind Randomized Controlled Clinical Trial

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver diseases globally. Recent interest has been aroused in the role of pyroptosis, a form of cell death, that contributes to hepatocyte destruction in liver damage. This study aimed to investigate the effects of OEA supplementation on the pyroptosis pathway in NAFLD patients.

Methods: The current study was a double-blind, randomized controlled clinical trial conducted in 65 adults with obesity and newly-diagnosed NAFLD. For 12 weeks, participants received personalized calorie-restricted diets plus either two daily 125-mg oleoylethanolamide (OEA) capsules or matching placebo capsules. The main objective was to assess the impact of OEA on serum levels of lipopolysaccharide-binding protein (LBP) and expression levels of key genes in the pyroptosis pathway.

Results: The intervention impacted the expression levels of Toll-like receptor 4 (TLR4), Myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain containing 3 (NLRP3), Cysteine-dependent aspartate-specific protease (Caspase) 1, Caspase 8, Interleukin (IL)-1β, and IL-18 in PBMCs, presented as log fold changes with median values and 95% confidence intervals. TLR4 and Caspase 8 expression levels increased, while it was decreased for the rest. However, none of the changes between groups were statistically significant (TLR4, p=0.48; MyD88, p=0.47; TRIF, p=0.06; NLRP3, p=0.70; Caspase 1, p=0.81; Caspase 8, p=0.15; IL-1β, p=0.98; IL-18, p=0.65). Serum LBP levels also showed no significant differences between groups (p=0.16) or within groups.

Conclusion: OEA supplementation could not make any significant difference in the pyroptotic pathway in obese adults with NAFLD. Further studies are warranted to corroborate these findings.

Introduction

Non-alcoholic fatty liver disease (NAFLD), encompassing a spectrum of chronic liver conditions, is characterized by an excessive fat accumulation of at least 5% of liver weight in the absence of other etiologies such as excessive alcohol consumption.[1] This spectrum comprises a range from simple steatosis (non-alcoholic fatty liver), which lacks hepatocellular injury, to non-alcoholic steatohepatitis (NASH), which is concurrent to inflammation and hepatocyte injury with or without fibrosis.[2] NAFLD is the primary cause of chronic liver diseases globally.[3,4] A meta-analysis of studies from 1989 to 2015 estimated a global prevalence of NAFLD at 25.2%.[5]

NAFLD prevalence is highly correlated with obesity, metabolic syndrome, and type 2 diabetes (7). It is predicted that the growth patterns of NAFLD and obesity will parallel each other in the coming years, with a 2019 study estimating NAFLD prevalence at 15-30% among the general population and 50-90% among the obese.[6,7] NAFLD's intimate link to metabolic syndrome makes lifestyle modification a fundamental aspect of its management.[8,9]

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Few mechanisms are involved in excessive intrahepatic fat accumulation, including increased hepatic influx of fatty acids (FAs), elevated *de novo* lipogenesis, and/or decreased clearance of FAs through very-low-density lipoprotein cholesterol (VLDL-C) secretion or β-oxidation.[10] The exact pathophysiology of NAFLD remains elusive, particularly regarding why some individuals may progress towards inflammation, fibrosis, and cirrhosis.

Recently, attention has been drawn towards pyroptosis, an intensely inflammatory form of programmed cell death reliant on inflammatory Cysteine-dependent aspartate-specific proteases (Caspases).[11-13] Pyroptosis has been suggested to play a role in hepatocyte death under liver injuries. Blocking nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3)-inflammasome, a key player in the pyroptosis pathway, has been indicated to positively affect NAFLD pathophysiology.[14-16] NLRP3-Inflammasome activation is suggested to be undertaken by a primary signal (signal I) in advance of or simultaneous with a secondary signal (signal II). Signal I is carried out by nuclear factor-kappa B (NF-κB)-activating receptors, such as tumor necrosis factor (TNF) receptor, toll-like receptors (TLRs), interleukin (IL)-1 receptor, and IL-18 receptor.[17-19] Subsequently, signal II is accomplished by the agonists that induce activation of NLRP3 and assembling of the inflammasome complex, ultimately resulting in Caspase 1 activation, production of mature IL-1β and IL-18, and also drives cleavage of gasdermin D (GSDMD), which generates an N-terminal fragment that oligomerizes to develop pores on the host cell membrane and causes rupture, lytic cell death, and release of the intracellular pro-inflammatory contents.[20]

Oleoylethanolamide (OEA) is an endocannabinoid-like compound that has been shown to positively affect NAFLD and related factors, such as steatosis, glycemic profile, lipid profile, the level of liver enzymes, and redox status.[21-25] OEA has also been indicated to ameliorate body inflammatory status,[24-29] and suppress appetite.[30,31] As mentioned before, the pyroptosis pathway has recently been shown to play a role in hepatocyte death under liver injuries. These arise the question whether OEA could also influence the inflammatory pyroptosis pathway and subsequently improve NAFLD pathophysiology.

Given the growing epidemic and burden of NAFLD, the development of novel adjuvant therapies is urgently required. In this context, we set out to explore whether OEA could improve NAFLD status by influencing the inflammatory pyroptosis pathway in human subjects with NAFLD. Considering that peripheral blood mononuclear cells (PBMCs) reflect the metabolic responses of hepatocytes and can be used to investigate the response of dietary interventions in relation to inflammation,[32,33] we decided to study the effects of our intervention on PBMCs of obese adults with NAFLD. As far as we are aware, our research represents the first human randomized controlled clinical trial examining the effects of OEA supplementation on the transcription of pyroptosis pathway genes in PBMCs of obese adults with NAFLD.

This study forms part of a larger project in which we previously demonstrated that OEA significantly improves anthropometric measurements, glycemic and lipid profiles, liver enzyme levels, and induces the expression of peroxisome proliferator-activated receptor (PPAR)-α, uncoupling protein (UCP)-1, and UCP-2.[22] Here, we hypothesized that the administration of OEA might affect the pyroptosis pathway. Therefore, the present trial aimed to investigate the effects of OEA supplementation on serum levels of lipopolysaccharide-binding protein (LBP) and expression levels of key genes in the pyroptosis pathway in obese adult patients with NAFLD.

Methods

Participant selection and study design

The current double-blind randomized controlled clinical trial included newly-diagnosed adults with NAFLD aged between 20-50 years old with a body mass index (BMI) ranging from 30-40 kg/m^2. The diagnosis of NAFLD was confirmed through ultrasonography (SonoAce X4 ultrasound system, South Korea) while in a fasting state, and performed by an expert radiologist. The exclusion criteria were (1) routine use of non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, and corticosteroids, (2) use of herbal medicines, hormonal drugs, hepatotoxic drugs (such as Amiodarone, Phenytoin, and Tamoxifen), anti-hypertensive drugs, weight-loss drugs, and lipid-lowering agents, (3) use of prebiotic and probiotic supplements, vitamins, minerals, antioxidants, and ω-3 supplements, (4) diagnosed pathological conditions affecting liver (e.g., liver transplantation, viral hepatitis, cystic fibrosis, hemochromatosis, Wilson's disease, alpha-1 antitrypsin deficiency, and acute systemic disease), (5) pregnancy, lactation, and menopause, (6) diagnosed thyroid disorders, kidney diseases, gastrointestinal diseases (e.g., Celiac disease), diabetes, heart failure, autoimmune diseases, malignancies, and severe psychological disorders, (7) and alcohol overconsumption over the past year.

The research was conducted at Tabriz University of Medical Sciences, Iran from February to June of 2019. The study protocol was approved by the Ethics Committee of Tabriz University of Medical Sciences (Tabriz, Iran), based on the Declaration of Helsinki standards (ethics code: IR.TBZMED.REC.1398.1131). This work was listed in the Iranian Registry of Clinical Trials (IRCT) (registration number: IRCT20110530006652N2, registration date: 07/02/2019). Informed consent was obtained from subjects and legal guardian(s).

Sample size, randomization, and stratification

As this research was the first human trial to study the effects of OEA supplementation on the pyroptosis pathway genes, the sample size was estimated based on the expression of NF-κB,21 a closely relevant factor in the pyroptosis pathway. Seventy volunteers were included in the study considering a confidence interval (CI) of 95%,

power of 90%, and allowing for a 10% potential drop-out rate, according to the following formula:

$$n = \frac{(Z_\alpha - + Z_\beta)^2 + (S_1^2 + S_2^2)}{d^2}$$

where Z_α and Z_β represent the Z-scores corresponding to the chosen significance level and study power, S_1^2 and S_2^2 denote the variance of the two groups, and d is the expected effect size.

To ensure an unbiased assignment of the participants, a computer-generated randomization list was created using Random Allocation Software. This software was operated by a colleague who was not involved in the study, thereby ensuring that the allocation was independent of the study team. To maintain balance and minimize bias, stratification was implemented, based on gender and fatty liver grading. Each participant was given a unique code associated with their assigned group. This ensured that the allocation process was accurately randomized and independent of any preferences or influences.

Supplements

OEA supplements were produced by the Nutrition Research Center of Tabriz University of Medical Sciences (Tabriz, Iran) through gas chromatography-mass spectrometry (GC-MS) under patent number 101756. The detailed process is explained in a previously published article.[31] All the capsules were uniform in shape and color.

Intervention protocol

The participants were instructed to consume one 125 mg OEA capsule or an equivalent placebo (starch) capsule 30-60 minutes prior to lunch and dinner (two capsules daily) for 12 weeks. They were provided with 90 capsules twice during the study. The dosage was determined based on a similar trial conducted by Payahoo *et al.*,[31] which reported no side effects. The rest of the capsules were asked to be returned to assess compliance by the participants. Consumption of ≥ 90% of the supplements was considered a good compliance.

All participants were also administered a personalized calorie-restricted diet with approximately 500 kcal/day less than their estimated energy expenditure and based on their previous dietary habits. Indirect calorimetry (COSMED, Italy) was used to estimate daily energy expenditure. Personalization was performed based on the participants' dietary habits, which were assessed using a 3-day food record at the baseline. Participants were instructed to keep a detailed record of everything they consumed over a period of two weekdays and one weekend day. The distribution of nutrients in relation to total amount of calories was, as follows: fat, 25 ~ 30%; protein, 10 ~ 15%; and carbohydrates, 55 ~ 60% of total energy expenditure. Dietary adherence was evaluated using 3-day dietary records at the end of the study. Once completed, each food record was reviewed in an interview with a registered dietitian. Potential confounding variables, including baseline values, age, changes in physical activity, energy intake, and BMI, were statistically controlled.

Demographics questionnaires and anthropometric measurements

Baseline characteristics, including demographic information such as age, gender, marital status, occupation, and level of education, as well as medical history and current medication use, were collected from the participants. A digital scale and a stadiometer, both from Seca, Germany, were employed for the accurate recording of weight and height, respectively. The participants were weighed in minimal clothing and without shoes while in a fasted state. Measurements were rounded off to the nearest 0.1 kg for weight and 0.1 cm for height.

Serum assay and gene transcription study

Blood samples (10 mL) were obtained in a fasting condition (10-12 hours, water permitted) at the baseline and endpoint of the study using Ethylenediaminetetraacetic acid (EDTA)-containing vacuum collection tubes. Samples were transferred from the sampling site to the laboratory, while preserving the cold chain, without freezing.

High-speed centrifugation was used to separate the serums (3,000 rpm for 5 minute). Serum samples were kept at -80°C, until LBP assay. According to the manufacturer's instructions, serum LBP was measured using a sandwich enzyme linked immunosorbent assay (ELISA) (Bioassay Technology Laboratory, ShanghaiCrystal day Biotech-Co, China). The intra-assay and inter-assay coefficient of variations (CV) were <8% and <10%, respectively.

PBMCs were isolated using Lymphodex solution (Ficoll and sodium diatrizoate, Inno-train, Germany) and density gradient centrifugation. Total ribonucleic acid (RNA) was purified using TRIzol™ solution (Invitrogen™, Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions. The extracted RNA was evaluated for quality and quantity by a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). Later, total RNA was converted to complementary DNA (cDNA) using oligo (dT), random primer, and reverse transcriptase, following the protocol of the cDNA synthesis Kit (BioFact™ RTase, South Korea).

The real-time polymerase chain reaction (PCR) technique was implemented using a Roche LightCycler® to assess the mRNA expression levels of TLR4, myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), NLRP3, Caspase 1, Caspase 8, IL-1β, and IL-18, using SYBR® Green Master mix (BioFACT™ 2X Real-Time PCR Master Mix, South Korea). Primer Bank was the reference for designing primer sequences (Table 1). The design of primer sequences was based on Primer Bank references (Table 1). Threshold cycle (CT) values were established for each sample, and fold changes were computed using the $2^{-\Delta\Delta CT}$ formula. β-actin was used as the housekeeping gene.

Table 1. Primer sequences of the study genes.

TLR4	Forward: TATTAAATGCTGCCACATGTC Reverse: GTTGGTTGAAATGCCCAC
MyD88	Forward: TTGGTTCTGGACTCGCCTT Reverse: GCACAGATTCCTCCTACAACG
TRIF	Forward: CCCCTCCTCCCTGTTCCCT Reverse: CCTCCAGCTTCTCCCGAACCC
NLRP3	Forward: AGCATCGGGTGTTGTTGTCA Reverse: AAGATAGCGGGAATGATGATATGAG
Caspase 1	Forward: GTCAAGCCGCACACGTCT Reverse: TTTCACATCTACGCTGTACCCCA
Caspase 8	Forward: TGCTTCATCTGCTGTATCCTCT Reverse: AGGGCACTTCAAACCAGT
IL-1β	Forward: GGAGAATGACCTGAGCACCT Reverse: GGAGGTGGAGAGCTTTCAGT
IL-18	Forward: GACTGTAGAGATAATGCACCC Reverse: TTTCTCACACTTCACAGAGAT

TLR4, Toll-like receptor 4; MyD88, Myeloid differentiation primary response 88; TRIF, TIR-domain-containing adapter-inducing interferon-β; NLRP3, Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain containing 3; Caspase 1, Cysteine-dependent aspartate-specific protease1; Caspase 8, Cysteine-dependent aspartate-specific protease8; IL-1β, Interleukin-1β; IL-18, Interleukin-18.

Statistical analysis

The statistical analyses were conducted using the IBM Statistical Package for the Social Sciences (SPSS) version 23 (IBM, USA). To verify the normality of the values, the Shapiro-Wilk test was employed in combination with an analysis of skewness, kurtosis, and histograms. Data were presented as mean (standard deviation (SD)) for parametric numerical data, median (interquartile range) for non-parametric numerical data, and frequency (percent) for categorical variables.

Differences at baseline between the study groups were assessed using the independent samples T-test or the Mann-Whitney U test, as appropriate. For the intragroup comparison, Paired samples T-test and the Wilcoxon matched-pair signed-rank test were applied, according to the nature of the data. Fisher's exact test and the Sign test were used to evaluate the differences of qualitative variables between and within groups, respectively. To adjust for confounding factors (baseline values, age, and changes in physical activity, energy intake, and BMI), we performed an analysis of covariance (ANCOVA) test via three separate models. When dealing with values with an abnormal distribution, quantile regression was employed to compare intergroup changes. This was done using STATA software version 16 (StataCorp, USA), and took into account the aforementioned potential confounders. The mean difference (95% CI) for parametric values and the median difference (95% CI) for non-parametric values were calculated using SPSS and STATA, respectively.

Percent changes were determined by the formula: ((end value – baseline value) / baseline value) * 100. The transcription data of genes was visualized using GraphPad

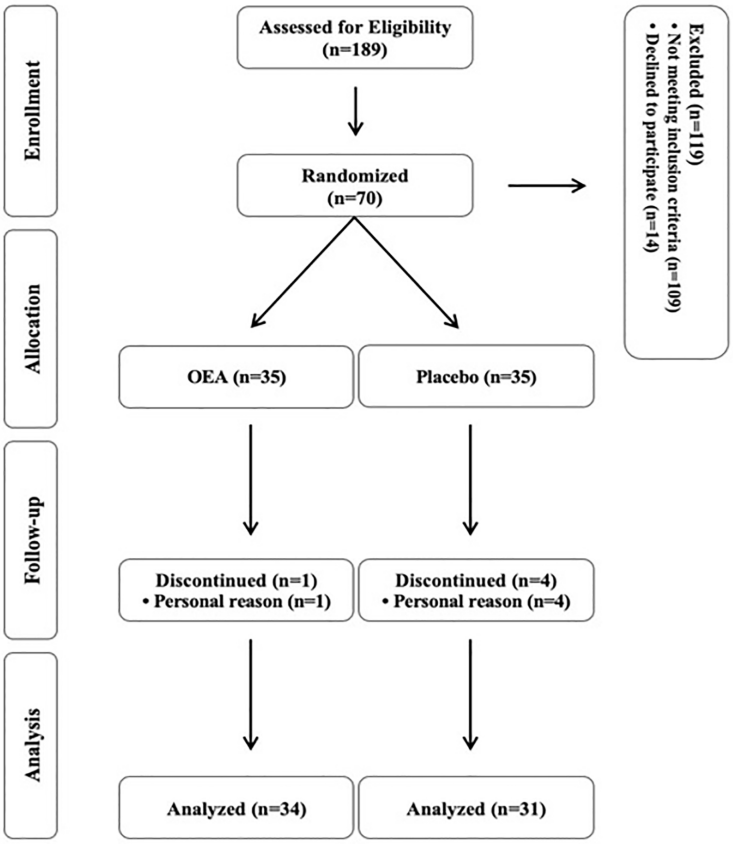

Figure 1. Study flow diagram. OEA, Oleoylethanolamide.

Table 2. Baseline characteristics of the study groups.

Parameters		OEA (n = 34)	Placebo (n = 31)	P
Age (years)		40.32 (7.4)	41.81 (7.7)	0.435[a]
Gender	Male	18 (52.9)	17 (54.8)	1.00[b]
	Female	16 (47.1)	14 (45.2)	
Marital status	Single	5 (14.7)	3 (9.7)	0.814[b]
	Married	26 (76.5)	26 (83.9)	
	Divorced or widow	3 (8.8)	2 (6.5)	
Education level	Illiterate	2 (5.9)	4 (12.9)	0.628[b]
	Diploma or lower	23 (67.6)	21 (67.7)	
	Bachelors or higher	9 (26.5)	6 (19.4)	
Occupation	Housewife	13 (38.2)	11 (35.5)	0.854[b]
	Employee	13 (38.2)	14 (45.2)	
	Self-employed	8 (23.5)	6 (19.4)	
Physical activity level	Low	25 (73.5)	26 (83.9)	0.668[b]
	Moderate	8 (23.5)	4 (12.9)	
	High	1 (2.9)	1 (3.2)	
Fatty liver severity	Mild	30 (88.2)	22 (71)	0.054[b]
	Moderate	3 (8.8)	9 (29)	
	Severe	1 (2.9)	0 (0)	

OEA, Oleoylethanolamide
Age is presented as mean (SD); other variables are presented as frequency (%).
[a] Independent Samples T-Test. [b] Fisher's exact test.

Prism 8 (GraphPad Software, USA). Statistical significance was assigned to p-values below 0.05.

Results

Figure 1 provides a depiction of the study flow, which concluded with a final analysis of 65 participants.

Baseline characteristics of the study groups

As outlined in Table 2, the baseline characteristics of the 65 participants, comprising 35 males (53.8%) and 30 females (46.2%), showed no significant disparity between the study groups. Variables such as age, gender, marital status, education level, occupation, physical activity level, and severity of fatty liver disease were evenly distributed between the two groups.

Effects of OEA on the anthropometric measures

Baseline anthropometric data, including weight, BMI, waist circumference (WC), hip circumference (HC), waist to hip ratio (WHR), and waist to height ratio (WHtR) did not show notable differences between the study groups. Over the course of the study, all these measures exhibited significant reductions in both groups, with the exception of weight in the placebo group which showed a non-significant decline. Details on the effects of OEA on the anthropometric measures could be found in our previously published article.[22]

Effects of OEA on the pyroptosis pathway

The impact of the intervention on the expression levels of TLR4, MyD88, TRIF, NLRP3, Caspase 1, Caspase 8, and IL-1β, and IL-18 in PBMCs is visualized in Figure 2. The data, displayed as log fold changes, are presented as median values with a 95% confidence interval. Upon comparison between the study groups, expression levels for TLR4 and Caspase 8 were increased, while the remaining genes had decreased expressions. However, none of the intergroup changes were statistically insignificant (TLR4 (p=0.48), MyD88 (p=0.47), TRIF (p=0.06), NLRP3 (p=0.70), Caspase 1 (p=0.81), Caspase 8 (p=0.15), and IL-1β (p=0.98), and IL-18 (p=0.65)).

The serum LBP levels did not differ significantly between the two groups, even after adjusting for potential

Table 3. Serum levels of LBP in the study groups.

		OEA (n = 34)	Placebo (n = 31)	MD (% Change), p
LBP (ng/ml)	Baseline	43.90 (16.0, 63.6)	38.80 (21.25, 65.3)	5.1 (13.14), 0.481[a]
	Endpoint	41.40 (14.9, 51.8)	32.82 (17.3, 53.6)	8.58 (26.14), 0.771[c1], 0.161[c2]
	MD (% Change), p	-2.50 (-5.69), 0864[a]	-5.89 (-15.41), 0336[a]	

OEA, Oleoylethanolamide; LBP, lipopolysaccharide binding protein: MD, median differences.
Data for baseline and end values are presented as median (25th, 75th).
a: Wilcoxon matched-pair signed-rank test.
b: Mann-Whitney U Test.
c1: Quantile regression, adjusted for baseline values (Model 1).
c2: Quantile regression, adjusted for baseline values, age and changes in physical activity and energy intake (Model 2).

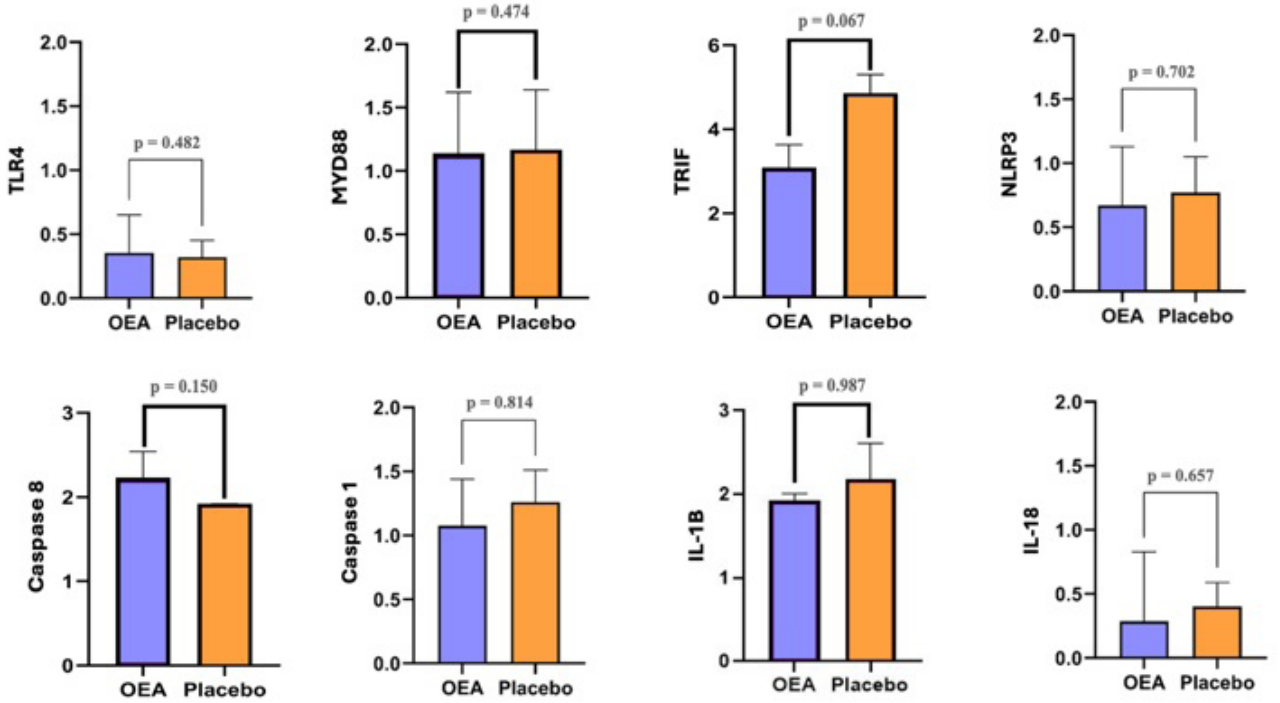

Figure 2. Effects of OEA on TLR4, MyD88, TRIF, NLRP3, Caspase 1, Caspase 8, and IL-1β, and IL-18 expression in the study groups. Values are presented as logarithms of fold changes (median (95% CI)). Data analysis was performed using quantile regression (adjusted based on model 3) and Wilcoxon signed-rank test (*P<0.05 vs. baseline). OEA, Oleoylethanolamide; TLR4, Toll-like receptor 4; MyD88, Myeloid differentiation primary response 88; TRIF, TIR-domain-containing adapter-inducing interferon-β; NLRP3, Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain containing 3; Caspase 1, Cysteine-dependent aspartate-specific protease1; Caspase 8, Cysteine-dependent aspartate-specific protease8; IL-1β, Interleukin-1β; IL-18, Interleukin-18.

confounders (p=0.16) (Table 3). Additionally, no significant differences were observed within either group.

Discussion

The present randomized controlled clinical trial was conducted to investigate the effects of OEA supplementation on the transcription of key pyroptosis pathway genes (TLR4, MyD88, TRIF, NLRP3, Caspase 1, Caspase 8, and IL-1β, and IL-18) and serum levels of LBP in obese adults with NAFLD. In our study, supplementation with 250mg/day of OEA over twelve weeks increased the expression of TLR4 and Caspase 8, while it was decreased for the rest. However, none of the changes between groups were statistically significant. Serum LBP levels also showed no significant differences between groups or within groups.

NAFLD has become the most prevalent form of chronic liver disease in recent times. Despite the recent approval of Resmetirom by the United States Food and Drug Administration for the treatment of non-alcoholic steatohepatitis,[34] lifestyle modifications remain the primary and most effective approach to managing this condition. Pyroptosis, a highly inflammatory form of programmed cell death, has recently been recognized as a potential pathway for hepatocyte death in response to liver injuries. Given that this is the pioneering human trial assessing the effects of OEA supplementation on the pyroptosis pathway, there were no direct comparative studies available. Nevertheless, several studies exploring the impact of OEA on key mediators of the pyroptosis pathway provide relevant insights. OEA has been found to inhibit the TLR4-mediated NF-κB signaling pathway and disrupt the extracellular signal-regulated kinase (ERK)1/2-dependent cascade (TLR4, ERK1/2, activator protein-1 (AP-1), and signal transducer and activator of transcription 3 (STAT3)).[27,29,35-40] Antón *et al.*[41] indicated that OEA pre-treatment (5 mg/kg, i.p.) in advance of intragastric alcohol gavage (3 times/day, 4 days) in rats attenuated the expression of TLR4 and high mobility group box 1 (HMGB1) danger signal, and prevented the NF-kB cascade. OEA diminished the levels of IL-1β, TNF-α, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and the monocyte chemoattractant protein-1 (MCP-1). It also blocked alcohol-induced lipid peroxidation, Caspase 8, and pro-apoptotic caspase-3 activation.

A study by Sayd *et al.*[29] in 2015 revealed that 10 mg/Kg i.p. OEA pretreatment (10 minutes in advance of LPS administration) led to a drop in plasma TNF-α levels, attenuated brain TNF-α, and inhibited the lipopolysaccharide (LPS)-induced NF-κB/IκBα (inhibitor of nuclear factor kappa B) upregulation in the nuclear and cytosolic extracts, respectively.OEA also decreased the expression of IL-1β, iNOS, COX-2, prostaglandin E2 (PGE2), and microsomal PGE2 synthase levels. Further, it diminished the LPS-induced oxidative/nitrosative stress. OEA also disrupted the LPS-induced anhedonia, as evidenced by the saccharine preference test. Consistently,

Yang *et al.*[28] utilized LPS (1 μg/ml) to stimulate THP-1 cells, a human monocytic cell line, with or without OEA (10, 20, and 40 μM). OEA acted as a potent anti-inflammatory agent and decreased TLR4 expression and the formation of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and raised PPAR-α expression. The protective role of OEA in the LPS-induced inflammation relied on PPAR-α and TLR4. Moreover, OEA inhibited the LPS-induced NF-κB activation, IκB-α degradation, and AP-1 expression. OEA also prevented the phosphorylation of ERK1/2 and STAT3. These effects suggest a potential role for OEA in inflammatory diseases. In this regard, Hu *et al.*[42] indicated that OEA administration (10 mg/Kg) could significantly inhibit the LPS/D-galactosamine (D-Gal)-induced hepatocytes injury, diminished the expression of B-cell lymphoma protein 2 (Bcl-2) and Bcl-2-associated X (Bax), and cleaved Caspase-3 to suppress hepatocyte apoptosis. Additionally, significant reductions were observed in the number of activated intrahepatic macrophages, as well as mRNA expression of TNF-α, IL-6, and MCP-1 after OEA administration. OEA obviously increased hepatic PPAR-α expression and reduced the expression of IL-1β in the liver and plasma through the inhibition of NLRP3 and Caspase 1, which indicated that OEA could suppress the NLRP3 inflammasome pathway.

In an RCT on obese NAFLD patients in 2018, Payahoo *et al.*[31] showed that 250 mg/day OEA supplementation for eight weeks could significantly reduce serum concentrations of IL-6 and TNF-α; nevertheless, reductions in malondialdehyde (MDA), high-sensitivity C-reactive protein (hs-CRP), and total antioxidant status (TAS) were not significant.[26] In a study in 2020, Tutunchi *et al.*[22] illustrated that hepatic fibrosis did not significantly improve in newly-diagnosed NAFLD patients intervened with 250 mg OEA and calorie restriction; however, it led to substantial reductions in NF-κB and IL-6 levels, while increasing IL-10. Also, the OEA group experienced a significant decline in fat mass and a rise in fat-free mass, compared to the placebo group.

The anti-inflammatory and cytoprotective effects of OEA have been shown to be, at least to an extent, due to PPAR-α, whose activation negatively modulates the transcription of inflammatory response genes by opposing the AP-1 and NF-κB signaling pathways. Moreover, OEA was found to disrupt the TLR4-mediated NF-κB signaling route and the ERK1/2-dependent cascade (TLR4/ERK1/2/AP-1/STAT3).[27,35-40]

Conversely, some studies suggest that OEA or its targets may intensify cell death. Chinetti *et al.*'s[43] *in vitro* study demonstrated that PPAR-α ligands could instigate apoptosis in macrophages activated with TNF-α/Interferon (IFN)-γ. In accordance, Tam *et al.*[44] highlighted that while OEA may inhibit pyroptosis, it can trigger necroptosis in mice subjected to post-influenza superinfection by Staphylococcus aureus. In their *in vitro* research in 2011, Lueneberg *et al.*[45] cultured cerebellar granule neurons with URB597 (25, 50, or 100 nM), a fatty acid amide hydrolase (FAAH) inhibitor, as well as OEA (25 nM) or PEA (100 nM). They indicated that URB597, OEA, or palmitoylethanolamide (PEA) all promote cellular death. While no mechanism of action was proposed, further investigations were recommended by the authors.

Discrepancies across these studies may arise due to variations in dosage, sample size, study duration, and specific methodologies. Our prior work,[22] along with the current work are components of the same overarching research project. We along with others have highlighted that OEA predominantly influences NAFLD pathophysiology through the PPAR-α pathway. This underscores the need for further focused research to elucidate more clearly the role OEA plays in hepatocyte pyroptotic death.

Our study presents several strengths, including its novelty as the first RCT to examine the effects of OEA supplementation on the pyroptosis pathway in NAFLD patients, the focus on newly-diagnosed NAFLD patients, recruiting merely the obese, and the incorporation of a personalized calorie-restricted diet for both study groups. It also had certain limitations. Serum concentration of OEA was not measured in the participants. We also utilized liver ultrasonography for diagnostic and fatty liver grading, but Fibroscan, which boasts greater accuracy, was not employed. Furthermore, while liver biopsy is the gold standard method, it was not deemed practical due to its invasive nature when working with human samples. It is of note that based on a meta-analysis, ultrasonography is considered the preferred imaging technique for screening fatty liver due to its low cost, safety, accessibility, and diagnostic accuracy, especially in the context of NAFLD.[46]

Conclusion

In summary, no notable differences were observed between the two study groups in the context of the pyroptosis pathway. We recommend future interventions to apply longer durations, increase dosage, and target patients with more advanced stages of NAFLD to garner a deeper understanding of the OEA's therapeutic potential.

Ethics Issues

The study protocol was approved by the Ethics Committee of Tabriz University of Medical Sciences (Tabriz, Iran), based on the Declaration of Helsinki standards (ethics code: IR.TBZMED.REC.1398.1131). This work is listed in the Iranian Registry of Clinical Trials (IRCT) (registration number: IRCT20110530006652N2, registration date: 07/02/2019). Informed consent was obtained from subjects and legal guardian(s).

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Data Sharing

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Milad Hasankhani: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing - Original Draft. **Maryam Saghafi-Asl:** Conceptualization, Methodology, Supervision, Writing - Review & Editing. **Mohammad Naemi Kermanshahi:** Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation. **Alireza Ostadrahimi:** Conceptualization, Supervision. Methodology. **Helda Tutunchi:** Investigation, Data Curation. **Neda Roshanravan:** Investigation, **Neda Gilani:** Formal Analysis, Validation.

Conflict of Interest

The authors report no conflicts of interest.

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