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Research Article

SUPPLEMENTATION WITH 2:1 RATIO OF N-6:N-3 POLYUNSATURATED FATTY ACID IMPROVES LIVER STEATOSIS AND SERUM CYTOKINE LEVELS IN YOUNG OBESE BALINESE WOMEN: A RANDOMIZED CLINICAL TRIAL

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ABSTRACT

Objectives: In addition to the rise in obesity prevalence globally, morbidity due to nonalcoholic fatty liver disease is increasing. Primary modalities for preventing and managing this problem include dietary modification and improved physical activities. A daily diet with a low n-6:n-3 polyunsaturated fatty acid (PUFA) ratio is suspected to contribute to ameliorating liver steatosis (LS). The present study was conducted to elucidate the effects of an n-6:n-3 PUFA ratio of 2:1 in alleviating LS.

Methods: Twenty-four young obese women with LS were recruited from Denpasar, Bali, Indonesia. They were randomly allocated to an intervention or control group. Both groups were given linoleic acid:α-linolenic acid at ratios of 2035:970 and 240:100 g, respectively, for 12 weeks. Baseline and end-line data were obtained. All patients were advised to maintain their daily energy intake no more than 1500 kcal and to perform structured physical exercises once a week.

Results: The intervention significantly decreased the body fat (body mass index, p=0.040; triglyceride, p=0.008) and serum tumor necrosis factor-α (TNF-α) levels (p=0.002) and increased serum interleukin-10 (IL-10) levels (p=0.004). The severity of LS was reduced through the intervention (odds ratio=0.064; 95% confidence interval=0.013-0.310; p=0.001).

Conclusion: An increased intake of 2:1 n-6:n-3 PUFA ratio alleviated LS, decreased body fat composition and serum TNF-α levels, and increased serum IL-10 levels.

Keywords: Young obese women, 2:1 n-6:n-3 Polyunsaturated fatty acid ratio, Cytokines, Liver steatosis.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), or liver steatosis (LS), is an emerging global health problem. It has been associated with an increase in morbidity in western populations and is currently also affecting Southeast Asian populations. NAFLD is an accumulation of fat in the liver of nonalcoholic individuals. This accumulation of fat, particularly intrahepatic triglycerides (TGs), occurs due to several mechanisms, i.e, increased influx of free fatty acids into the liver, increased fat synthesis, decreased TG export through very low-density lipoprotein (VLDL), and reduced β-oxidation [1-3]. Fat accumulation indicates that hepatic fatty acid uptake from the plasma and *de novo* fatty acid synthesis occur at a faster rate than do β-oxidation and export [3]. NAFLD occurs as two entities, i.e, non-alcoholic fatty liver (NAFL) and succeeded by non-alcoholic steatohepatitis (NASH) in the later stage. NAFL is defined as hepatic steatosis without inflammation, whereas NASH is steatosis accompanied by inflammation [3]. NAFLD is the most common liver disorder and is the leading cause of hepatocyte injury and fibrosis. NAFLD is also associated with chronic liver diseases such as cirrhosis [4-8].

The two-hit theory explains the development and progression of NAFLD. The first hit initiates LS, and the second hit stimulates inflammatory processes. These two conditions may result in hepatocyte injury and fibrosis [4,5]. NAFLD is asymptomatic, undetectable, and progressive. Except for individuals who consume more than 20 g of ethanol daily, imaging studies or liver biopsies can be used to diagnose NAFLD. Liver biopsy is the definitive method for diagnosing NAFLD, yet this method is invasive, traumatic, and expensive. Ultrasound (US) imaging is an alternative method for diagnosing suspected NAFLD cases. This method is relatively safe and inexpensive, with good sensitivity and specificity [9].

Approximately 20-35% of the global population has LS, and 10% of cases are more severe than NASH. The prevalence of LS is higher among patients with obesity and type 2 diabetes (70-80%), and as many as 25-70% of these patients eventually develop chronic liver diseases such as NASH and fibrosis [6-8]. Obesity, a high-fat diet, a genetic predisposition, and microbiota have been identified as risk factors for NAFL [4-8]. In obese patients associated chronic inflammatory states, the adipose tissue releases more pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, whereas the release of IL-10 is decreased. Moreover, the levels of pro-inflammatory cytokines, such as TNF-α and IL-16, produced by the liver and adipose tissue are elevated during the activation of nuclear factor kappa B. TNF-α involves in inflammatory and metabolic alterations nearly stage of liver injury, leading to increased cytokines synthesis, which in turn induces cell migration, initiate fibrotic processes, and influence the progression of NAFL to NASH [4-8]. By contrast, IL-10 inhibits the synthesis of pro-inflammatory cytokines such as interferon-γ, IL-2, IL-3, and TNF-α. These cytokines are mainly secreted by T regulatory cells. Obesity is associated with decreased IL-10 concentrations [10]. Research has shown that an imbalance between

pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokine levels are associated with a high n-6:n-3 ratio in the cell membranes of obese patients. The amounts of n-3 polyunsaturated fatty acid (PUFA) (α-linolenic acid [ALA]) and long-chain (LC)-PUFA (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) in obese patients were shown to be, respectively, 30% and 35% lower than those in normal-weight patients. By contrast, n-6 PUFA (linoleic acid [LA]) and LC-PUFA (arachidonic acid [AA]) levels were shown to be relatively higher in obese patients than in non-obese patients [11]. Another study demonstrated that an increased n-6:n-3 PUFA ratio induces steatosis and inflammation, which leads to NAFLD [12]. Nutritional modification, such as increasing the n-3 PUFA proportion in the diet, can reduce the risk of obesity, inflammation, and oxidative stress and prevent the development of LS [13]. Few types of oil, such as canola oil, contain high n-3 PUFA (ALA) concentration. ALA is desaturated and elongated to EPA and DHA. n-3 LC-PUFA reduces lipogenic processes in the liver by inhibiting the sterol regulatory element binding protein-1c (SREBP1c) and carbohydrate-responsive element-binding protein. Furthermore, it also stimulates paroxysmal proliferator-activated receptor (PPAR)-α and activates a protein kinase (AMPK) to increase the β-oxidation of hepatic lipids. It also increases lipid oxidation in adipose and skeletal tissues through AMPK [14]. The present study was conducted to elucidate the effects of n-6:n3 PUFA supplementation at a ratio of 2:1 for alleviating LS and improving serum TNF-α and IL-10 levels in young Balinese obese women.

METHODS

Research design, participants, and intervention

This study was a randomized trial with a double-blind control group-based pre- and post-test design [15]. It involved young obese women (age, 18-25 years) in Denpasar. They were recruited between May and September 2013 (clinical trial ID ACTRN12615000757516).

This study is part of a larger research project investigating the effects of low-ratio n-6:n-3 PUFA supplementation for alleviating obesity and its comorbidities in young obese women in Bali. Sixty-six young obese women (body mass index [BMI] ≥25 kg/m^2) were screened for LS through US imaging (Logiq 500, GE, Solingen, Germany); 26 (39.4%) of them were found to have LS and were randomly enrolled in this study. They were allocated to either intervention or control group (13 patients in each group). Informed consent was obtained from all patients before enrolment. The study protocol was approved by the Research Ethics Committee of Udayana University, Sanglah Hospital (Ethical Clearance No. 787/UN.14.2/Litbang/2012, 17 September 2012).

The intervention group received supplementation with 30 mL of emulsion containing 10 g of canola oil (952 mg of saturated fatty acid [SFA], 6072 mg of monounsaturated fatty acid [MUFA], 2035 mg of LA, and 970 mg of ALA; n-6:n-3 PUFA ratio of 2:1) [16,17], and the control group received supplementation with 30 mL of placebo containing 2 g of palm oil (660 mg of SFA, 1000 mg of MUFA, 240 mg of LA, and 100 mg of ALA; n-6:n-3 PUFA ratio of 2:1) [17,18]. Supplementation was provided daily for 12 weeks (June 2013-September 2013). Furthermore, the participants were advised to restrict their daily energy intake to <1500 kcal. To ensure compliance, weekly meetings (every Sunday) were organized. During these meetings, they were advised to participate in aerobic exercises for 1 hr under the supervision of a gym instructor. They were given 250 mL of emulsions per week, and any complaints or adverse effects related to the supplementation were monitored. To ensure that, the previous week's dose was consumed as instructed, and the volume of the remaining emulsion in the used bottles was recorded.

During this period, two participants from the intervention group were lost to follow-up. In total, 24 patients (11 in the intervention group and 13 in the control group) completed the study protocol and were included in the analysis.

Data collection

All research variables were assessed on the first day of week 1 (baseline data) and last day of week 12 (end-line data). Energy intake was assessed using a semi-quantitative food frequency questionnaire. Body weight (BW) was measured using a digital scale (Omron HBF-362 model, Kyoto, Japan), with a precision of 0.1 kg, and body height (BH) was measured using a stature meter, with an accuracy of 0.1 cm. BMI was calculated as BW (kg)/BH (m)^2. Serum TG and gamma glutamyl transferase (GGT) levels were measured using the colorimetric method (Cobas 6000-Roche Diagnostic, Mannheim, Germany), with precisions of 1 mg/dL and 1 μg/L, respectively.

Serum TNF-α and IL-10 levels were assessed using an ELISA kit (Boster Biological Technology Ltd, Pleasanton, CA, USA), according to the manufacturer's instructions. The results were examined with an ELISA reader (pg/mL).

LS was assessed through US imaging, the results of which were independently interpreted by three radiologists. The final interpretation was determined through majority decision. The defining criteria for LS are as follows: (1) Normal liver (absence of steatosis and other liver disorders), (2) mild steatosis (appearance of a slightly bright liver parenchymal or hepatorenal echo contrast without intrahepatic vascular disorder), (3) moderate steatosis (more colored liver parenchymal or hepatorenal appearance in more areas without intrahepatic vascular disease), and (4) severe steatosis (diffused and brighter liver appearance with blunting intrahepatic vascular disorder) [19,20].

Statistical analyses

Statistical analyses were performed using Stata 12.1 (Stata Corp, College Station, TX, USA). A generalized linear model was employed to explore the relationships of variables within and between the groups. Repeated ordered logistic regression was used to assess the ORs between severe and less severe LS. The significance level for all statistical analyses was $p<0.05$ (95% confidence intervals [CI]) [21] (Fig. 1).

RESULTS

All participants were Balinese, with the mean age of 20.8±1.7 years (range, 18-24 years). Most participants were students of Udayana University (87.5%). All of the baseline characteristics, namely, age, occupation, selected nutrients intake (except cholesterol), BMI, waist circumference, TG, and GGT were comparable between the intervention and the control group. However, two nutrient intakes, namely, cholesterol and fiber were in extreme values. There was a high cholesterol intake in the intervention group (354 mg) compared to the control group (240 mg) ($p=0.033$). In contrary for the fiber consumption, both the intervention and the control group had a low-fiber intake (7.86 g and 12.8 g; $p=0.135$ respectively), less than Indonesian RDA (32 g) (Table 1). The sources of fat intake were mainly palm oil, chicken, and red meat (Table 2).

The reduced total energy intake between the baseline and end-line did not differ in the intervention and control groups ($p=0.053$ and $p=0.551$, respectively) and remained at >1500 kcal in both the groups. Moreover, no significant difference was observed in the changes in total energy intake between the two groups ($p=0.257$; Table 3).

In the intervention group, a significant reduction in the BMI and TG levels between the baseline and end-line was noted ($p=0.040$ and $p=0.008$, respectively), but this finding was not observed in the control group. The BMI and TG levels did not differ between the two groups (BMI, $p=0.150$; TG, $p=0.433$). A greater reduction in serum GGT levels occurred in the intervention group than in the control group, but this difference was not statistically significant.

Serum TNF-α and IL-10 concentrations were not normally distributed; therefore, log transformation was performed. In the intervention group, the concentration of serum TNF-α decreased from baseline and end-line ($p=0.002$), whereas the IL-10 concentration increased ($p=0.004$). By contrast, only the IL-10 serum concentration increased ($p=0.0038$; Table 3) in the control group.

Table 1: Baseline characteristics of participants stratified by group

Parameters	Group (mean±SE)		p
	Intervention (n=11)	Control (n=13)	
Age (years)	21.2±1.8	20.5±1.7	0.338
Occupation			
F (%)			
Student	10 (90.9)	11 (84.6)	0.589
Officer staff	1 (9.1)	1 (7.7)	
Unemployment	-	1 (7.7)	
Specified nutrients intake			
Energy (kcal)	2187±203	1714±170	0.848
Fat (g)	82.0±9.11	67.9±13.5	0.976
PUFA (g)	19.1±3.09	15.8±3.36	0.978
Cholesterol (mg)	354±48.1	240±40.3	0.033
Fiber (g)	7.86±0.91	12.8±3.36	0.135
BMI (kg/m^2)	33.8±2.32	34.1±1.33	0.916
Triglyceride (mg/dl)	157±16.5	130±22.5	0.580
GGT (µg/l)	25.8±2.94	25.2±2.88	0.351

PUFA: Polyunsaturated fatty acids, BMI: Body mass index, GGT: Gamma glutamyl transferase, SE: Standard error

Compared with the control group, a significant improvement was noted in the LS condition of the intervention group after the 12-week intervention, with an OR of 0.064 (95% CI 0.013–0.310, p=0.001; Table 3).

DISCUSSION

Following the present findings, most studies - both in animals and humans -have documented the benefits of n-3 PUFA supplementation for treating NAFLD by improving the cytokine profiles and reducing inflammation and oxidative stress.

In a recent animal model study, Konuma *et al*. used plant oils as a source of n-3 PUFA demonstrated that a low n-6:n-3 PUFA ratio improved serum lipid metabolism, inflammatory cytokines levels, oxidative stress, and endothelial function. They found that EPA effectively prevented the development and progression of NASH and ameliorated hepatic steatosis in mice [22]. Heerwagen *et al*. revealed that a high-fat diet significantly increased the n-3:n-6 PUFA ratio and reduced maternal obesity-associated inflammation in Fat-1 transgenic mice. In that study, wild-type mice fed with a high-fat diet exhibited a high serum concentration of 12 pro-inflammatory cytokines ($p<0.05$), but no such increase was observed in the transgenic mice [23]. Depner *et al.* reported that among n-3 LC-PUFAs, DHA was more effective than

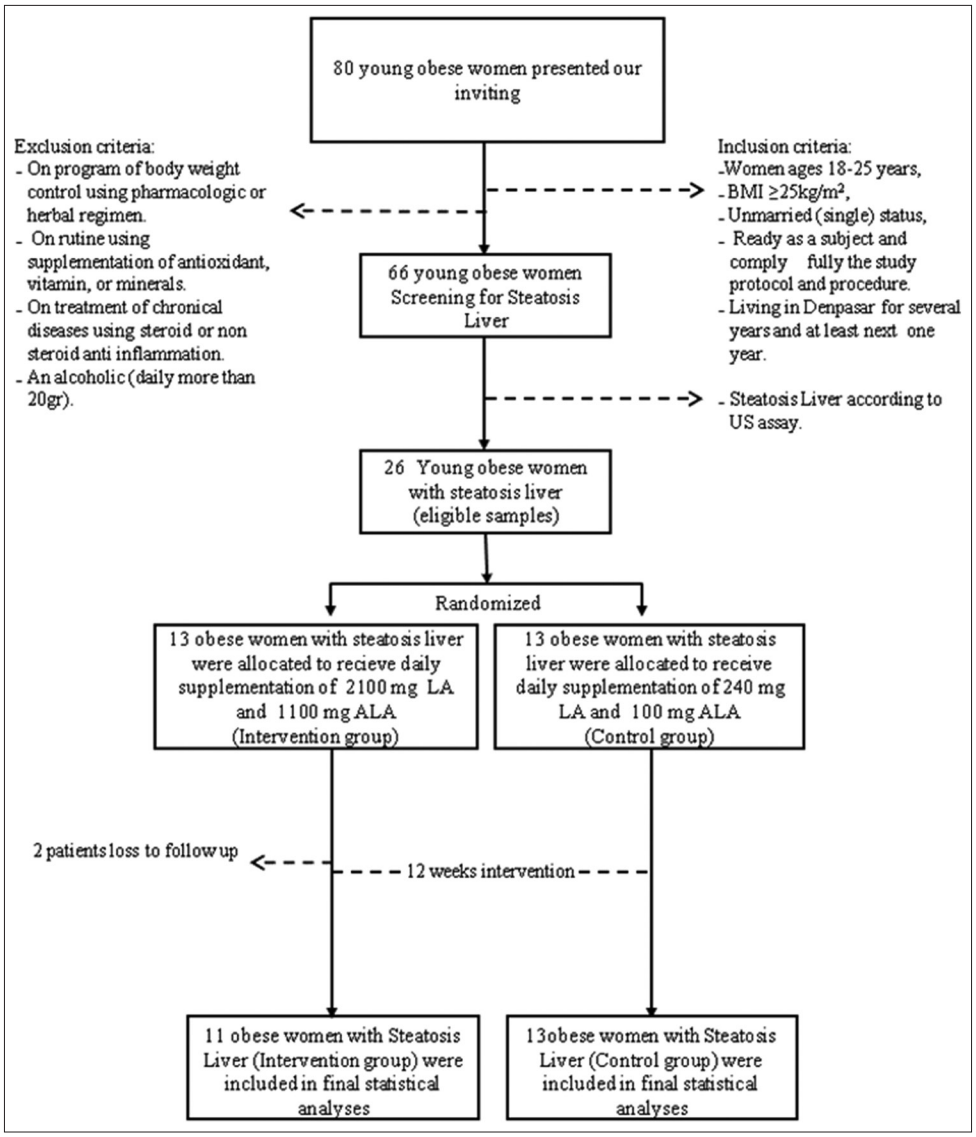

Fig. 1: Sequence of participants recruitment and pathways of research procedure

EPA in attenuating inflammation, oxidative stress, fibrosis, and hepatic damage [24]. Lionetti *et al.* described that EPA effectively minimized saturated fat-induced insulin resistance in mice and decreased leptin alleviated LS and improved secondary outcomes, such as BMI, TG and alanine transaminase levels, and homeostasis model assessment of insulin resistance values in both groups when compared to the control group [25]. A systematic review and meta-analysis of five randomized controlled trials (nine studies) in which n-3 LC-PUFA supplementation was provided for 8 weeks to 12 months to 355 adults with NAFLD demonstrated that n-3 LC-PUFA was beneficial in reducing liver fat ($p<0.001$) and aspartate aminotransferase ($p=0.02$) but not alanine aminotransferase activity [26]. Another study reported that a reduction in the n-6:n-3 PUFA ratio (3:1) in the diet resulted in decreased plasma TG levels in mRNA expression *in vivo* and *in vitro.* A decline in the circulating levels of leptin may reduce the white adipose tissue mass and eventually reduce the BW and degree of systemic inflammation in rats. In another study [27], marine n-3 PUFAs were found to have an anti adipogenic effect during obesity development in mice. The study showed that it inhibited the growth of fat cells through both hypertrophy and hyperplasia processes. In human studies, Nobili *et al*. have demonstrated that supplementation with two doses of 250 mg and 500 mg of DHA daily for 2 years in children with NAFLD patients aged 45-75 years [28-30].

Table 2: Baseline fatty food consumption patterns (time/day) of the participants (n=24)

Fat sources	F (%)	Mean±SE
Animal		
Beef	12 (50.0)	0.25±0.09
Pork	16 (66.7)	0.19±0.08
Lamb	8 (33.4)	0.27±0.09
Chicken	22 (91.7)	1.20±0.19
Full cream milk	10 (41.7)	0.25±0.72
Cheese	14 (58.3)	0.27±0.08
Butter/margarine	9 (37.5)	0.18±0.09
Fast food/street food		
Fried chicken	19 (79.2)	0.45±0.35
Meatball (bakso)	19 (79.2)	0.21±0.07
Plant		
Coconut oil	4 (16.7)	0.26±0.12
Palm oil	19 (79.2)	2.07±0.29
Olive oil	3 (12.5)	1.35±0.86
Fried food (camilan)	14 (58.3)	0.79±0.18

In the present study, the use of plant (canola) oil with an n-6 (LA):n-3 (ALA) PUFA ratio of 2:1 appeared to improve the composition of n-6 (AA) and n-3 (EPA and DHA) LC-PUFAs in the phospholipid cell membrane. ALA desaturated and elongated in animal and human bodies to form LC PUFAs (EPA, DPA, and DHA). By contrast, LA desaturated and elongated to form γ-linolenic acid and dihomo-γ–linolenic acid (DGLA), and DGLA desaturated to form n-6 LC-PUFA (AA) [31]. DGLA is a n-6 PUFA that produces anti-inflammatory eicosanoids, the prostaglandin 1 series (PG-1), which differ from the pro-inflammatory prostaglandin 2 series (PG-2) released by AA. In addition, ALA also desaturated and elongated to form EPA. Increased EPA levels potentially inhibit the δ-5 desaturase enzyme, which is required by DGLA to form AA [32]. Thus, this plant oil exerts its beneficial effects by increasing EPA levels and DLGA to produce PG-3 and PG-1 while inhibiting the AA formation that releases PG-2. Furthermore, a low n-6:n-3 PUFA ratio controls the inflammatory state by inducing the release of anti-inflammatory cytokines and inhibiting the release of pro-inflammatory cytokines.

In addition, Burdge *et al.* analyzed the EPA and DHA obtained from the desaturation and elongation of ALA. They observed that on average, as much as 36% of ALA was converted to n-3 LC-PUFA (21% EPA, 6% DPA, and 9% DHA) in young obese women. ALA conversion to n-3 LC-PUFA

Table 3: Relationship of intervention with energy intake, body lipid composition, cytokine levels, and liver steatosis (n=24)

Parameter group	Mean±SE			p
	Baseline	Endline	Difference	
Energy intake (kcal)				
Intervention (n=11)	2187±203	1691±209	−497±226	0.053
Control (n=13)	1714±170	1574±158	−140±206	0.511
p			0.257	
BMI (kg/m^2)				
Intervention (n=11)	33.8±2.32	33.0±2.31	−0.74±0.31	0.040
Control (n=13)	34.1±1.33	34.5±1.11	0.43±0.69	0.546
p			0.150	
Triglyceride (mg/dl)				
Intervention (n=11)	157.4±16.5	129.1±16.2	−28.3±8.5	0.008
Control (n=13)	129.9±22.5	117.9±11.5	−11.9±17.3	0.505
p			0.433	
GGT (µg/l)				
Intervention (n=11)	25.8±2.94	21.8±2.74	−3.97±2.15	0.074
Control (n=13)	25.2±2.88	24.7±3.24	−0.45±1.67	0.788
p			0.202	
Log. IL10				
Intervention (n=11)	0.82±0.24	1.55±0.07	0.73±0.23	0.004
Control (n=13)	1.31±0.11	1.54±0.05	0.23±0.10	0.038
p			0.053	
Log. TNF-α				
Intervention (n=11)	1.87±0.06	1.58±0.08	−0.29±0.08	0.002
Control (n=13)	1.89±0.08	1.71±0.13	−0.18±0.15	0.242
p			0.516	
Liver steatosis (0/1/2/3)†				
Intervention (n=11)	0/5/3/3	5/3/2/1	5/−2/−1/−2	0.064
Control (n=13)	0/6/1/6	3/4/3/3	3/−2/2/−3	(0.013-0.31)††
p				0.001

SE: Standard error of the mean, TNF: Tumor necrosis factor, IL: Interleukin, GGT: Gamma glutamyl transferase. p, analyzed using a general linear model. †Liver steatosis level: 0, none; 1, mild; 2, moderate; 3, severe. The data are presented as the frequency distribution and analyzed using the ordinal generalized model. ††OR (95% CI); OR of more severe to less severe liver steatosis

was also reported to be higher among young obese women than among young obese men, probably due to the lower level of lipid β-oxidation among women [33].

EPA and DHA regulate adiposity through two mechanisms. First, n-3 LC-PUFAs reduce endogenous lipid production by inhibiting the expression and processing of SREBP1c, which stimulates lipogenic gene transcriptions [14,34,35]. Second, n-3 LC-PUFAs are potent PPAR-α activators and PPAR-γ inhibitors that upregulate several gene expression pathways involved in the stimulation of fatty acid β-oxidation [14,35-38].

In the present study, reduced TNF-α and increased IL-10 levels were associated with reduced inflammation in the participants after the 12-week intervention. The release of GGT typically increases oxidative stress [39]; however, serum GGT levels were relatively decreased in this study, indicating that oxidative stress was reduced. Furthermore, GGT has been strongly associated with body lipids, particularly visceral fat, which contribute to the progression of NAFLD [23,24,40].

In animals study, other antioxidant nutrients also reported have a positive effect in reducing liver inflammatory and fibrosis. Abdel-Sttar *et al*. [41] studied that hesperidin (3,5,7-trihydroxy flavanone-7-rhamnoglucoside) and a flavanone glycoside present abundantly in citrus fruits, significantly ameliorated carbon tetrachloride (liver fibrosis inducer) in rats based on its antioxidant, anti-inflammatory, antilipidemic, and anti-fibrotic activities. Ahmed *et al*. [42] reported grape seeds (Vitis vinifera) extract contains flavonoids, polyphenols, anthocyanins, proanthocyanidins, procyanidins, and the stilbene derivative resveratrol bioactive phytochemicals that possess inhibitory activity on the fat-metabolizing enzymes, pancreatic lipase, and lipoprotein lipase. Vitis Vinifera seed extract has potent therapeutic implication in NASH accompanied with insulin resistance and severe inflammation. Human trials are clearly needed in the future in order to confirm whether or not the combination of those nutrients supplementation with n-6:n-3 PUFA on 2:1 ratio can strengthen the effects of the present study.

Many risk factors, such as unhealthy diet comprising high energy, high saturated fat, and high n-6:n3 PUFA ratio, along with sedentary lifestyle, are associated with obesity and insulin resistance. It facilitates the influx of free fatty acids from the adipose tissue into the liver, hepatic oxidative stress, cytokine production, reduced VLDL secretion, and the growth of the intestinal microbiome [43]. Unhealthy diet and a sedentary lifestyle can alter the stability of the intestinal microbiome composition. This leads to the development of chronic diseases such as obesity and metabolic dysfunction [44]. These factors are associated with the progression of NAFL to NASH.

NAFLD is asymptomatic and undetectable in its early stages, and a definitive pharmacologic regimen has yet to be identified. If NAFLD is neither identified in its early stages nor managed appropriately, the disease will naturally progress to the severe NASH stage, which entails inflammation, fibrosis, and cirrhosis [3]. Non-invasive US imaging is an appropriate first-line method for the early detection of asymptomatic NAFLD [40]. Schwenger *et al*. [3] recommended that an improvement in diet and increase in physical activity should be the first choice to treat NAFLD, followed by the management of insulin resistance, oxidative stress, and obesity-associated morbidity.

The results of this study suggest that the risk of NAFLD should be monitored among obese patients. Early identification and appropriate management of LS are crucial, particularly among obese patients. An improvement in the quality of life through the modification of daily life behaviors, such as by maintaining a healthy and balanced diet and increasing physical activities, is essential to effectively manage LS. Healthy diets and supplementation with a low n-6:n-3 PUFA ratio are beneficial in alleviating and treating NAFLD, improving the body lipid composition, and controlling inflammation.

We conclude that daily supplementation with an n-6:n-3 PUFA ratio of 2035:970 mg (LA:ALA) for 12 weeks alleviates LS reduces body fat composition and serum TNF-α levels and increases the serum concentration of IL-10 in young obese Balinese women.

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