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ORIGINAL ARTICLE

Vitamin D was Superior to Omega-3 as a Simvastatin Adjuvant in Improving Blood Lipids and Atherogenic Index in Type-I Dyslipidemic Rats

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ABSTRACT

Objectives: Adjuvant therapy is often used to optimize the antihyperlipidemic effect of simvastatin. Omega-3 and vitamin D supplementation are recommended as adjuvant therapies to low-intensity statins. This study aimed to compare the effects of vitamin D and omega-3 as adjuvant therapy to simvastatin to improve the lipid profiles and atherogenic index of plasma (AIP) in type-I dyslipidemic rats.

Materials and Methods: Thirty-six male rats were randomized and divided into six groups: healthy control, dyslipidemic rats with no treatment, and dyslipidemic rats treated with either low-dose simvastatin only or omega-3 or vitamin D at low and high doses. Dyslipidemia was induced with high-fat diets for four weeks, followed by treatment for the next two weeks. Blood samples were withdrawn before and after simvastatin treatment. In addition, aspartate transaminase (AST) and alanine transaminase (ALT) levels were analyzed to assess liver function.

Results: Administration of a high-fat diet-induced type 1 dyslipidemia and increased ALT levels ($p < 0.05$). Treatment with low-dose simvastatin did not significantly improve triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLc) or non-HDLc levels. When combined with a high-dose vitamin D, simvastatin significantly reduced TG and increased HDLc levels ($p < 0.05$), thereby improving AIP levels. This improvement was not observed in rats treated with omega-3 or vitamin D at a lower dose.

Conclusion: We concluded that high-dose vitamin D as an adjuvant to simvastatin therapy was superior to omega-3 in improving TG, HDL, and AIP levels. High-dose vitamin D also improved ALT levels in type-I dyslipidemic rats. This result may be translated in clinics to reduce the risk of coronary syndrome in patients with type-I dyslipidemia.

Key words: Vitamin D, omega-3, atherogenic index, simvastatin, adjuvant therapy

INTRODUCTION

Simvastatin is one of the most widely used antihyperlipidemic drugs. It competitively inhibits the action of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase). The reduction in hepatocellular cholesterol promotes increased lipoprotein [low-density lipoprotein (LDL)] receptors at the surface and thus increases LDL uptake, decreases LDL plasma and other apolipoprotein-B, and lowers triglyceride (TG) levels.[1] Other effects of simvastatin include reducing oxidative stress and inflammation of the vesicle walls, thereby providing protection against atherosclerotic lesions.[2]

The use of simvastatin is associated with cheaper and more affordable treatment for most patients, but it is still inferior in decreasing LDL and increasing high-density lipoprotein (HDL) compared with other statin agents.[3] Various studies show that up to 65-75% of the incidence of atherosclerosis cannot be prevented by lowering LDL-cholesterol with statin therapy.[4] One of the reasons is that statins are often not titrated to their optimal dose in practice, mainly due to concerns about their side effects, including myopathy and impaired liver function. Another reason is that a small proportion of patients have refractory hypercholesterolemia and cannot reach targets

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even, when treated with high doses of statins.[5] Therefore, combination therapy of statins with other lipid-lowering agents has become one of the most commonly used strategies in the clinic.[6]

The combination of a statin with polyunsaturated fat (PUFA) omega-3, based on the guidelines for the management of dyslipidemia, is a recommended option for the treatment of dyslipidemia, especially hypertriglyceridemia.[2] Fish oil, which is rich in omega-3 fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), is one form of safe and well-tolerated adjuvant therapy.[7] Growing evidence both from epidemiological data and case-control studies have indicated that routine consumption of fatty fish as well as long-chain n-3 PUFAs may lower the risk of cardiovascular death.[8]

However, the effect of omega-3 PUFAs on cardiovascular mortality, based on various studies, has been inconsistent. Studies in Japan reported that EPA therapy was associated with a 19% reduction in cardiovascular events and that combination therapy of EPA plus a statin would be more cost-effective than statin monotherapy in primary and secondary prevention.[9,10] One meta-analysis found that omega-3 supplementation for 4.4 years did not significantly reduce the incidence of coronary heart disease or other major vascular events.[11] Based on these inconsistent data, another candidate must be considered for adjuvant therapy to statin treatment for patients with dyslipidemia.

Vitamin D (known as calciferol) has long been associated with bone growth and strength.[12] However, substantial evidence suggests that vitamin D deficiency is also associated with an increased risk of cardiovascular disease.[13] The mechanisms by which vitamin D may improve cardiovascular disease are still unclear, but several hypotheses have been proposed, including direct effects on the myocardium, downregulation of the renin-angiotensin-aldosterone system (anti hypertensive effect), and improved glycemic control.[14-16]

Various data regarding the relationship between vitamin D and blood lipid levels remain inconsistent. Another study evaluating the association of vitamin D deficiency with the risk of dyslipidemia and heart disease found serum 25(OH)D levels to be inversely related to TG and LDLc levels and positively associated with total cholesterol (TC).[17] A meta-analysis found that vitamin D supplementation at various doses (from 300 IU *per* day to a 200,000-IU single dose) had an effect on LDLc levels but no significant effect on TC, HDL or TG levels.[18]

In many studies, vitamin D deficiency has been strongly associated with liver disease. The role of vitamin D in the liver has been greatly understood since the development of the vitamin D receptor.[19] Vitamin D correlates inversely with nonalcoholic fatty liver disease in a dose-dependent manner.[20] One study with 6,800 patients found that lower serum levels of vitamin D in patients correlated with an elevation of alanine transaminase (ALT) levels.[21]

Accordingly, this study aimed to compare the effect of vitamin D and omega-3 as adjuvant therapy to simvastatin to improve the lipid profiles and atherogenic index of plasma (AIP) in dyslipidemic rats. In addition, serum liver enzyme levels were measured to assess liver function following a high-fat diet and therapy. The results of this study may be important to determine, whether vitamin D and its recommended dose can be used as adjuvant therapy to simvastatin to improve the lipid profile and reduce the risk of atherosclerotic disease and liver dysfunction in dyslipidemic patients or not.

MATERIALS AND METHODS

Drugs and chemicals

Drugs of choice

Simvastatin was obtained from 10 mg simvastatin tablets (Kimia Farma Co., Indonesia). Omega-3 was obtained as soft capsules containing 180 mg of EPA and 120 mg of DHA (Blackmores Co., Australia). Vitamin D was obtained as tablets containing 400 IU cholecalciferol (Novapharin Co., Indonesia). All drugs were purchased from local pharmacies. The dose of simvastatin was 2 mg/kg body weight (*b.w.*) of rats, which is equivalent to a low-intensity simvastatin dose of 20 mg/day in humans. The dose of omega-3 was 206 mg/kg of rat *b.w.*, which is equivalent to 2,000 mg/day in humans. Vitamin D was administered in two different doses: 62 IU/kg rats' *b.w.*, equivalent to 600 IU/day in humans; and 620 IU/kg rats' *b.w.*, equivalent to the dose recommended for vitamin D insufficiency and deficiency treatment of 6,000 IU/day in humans.

Chemicals

Serum TC, TG, HDL levels, aspartate transaminase (AST), and ALT levels were determined using reagent kits (Human Diagnostic Worldwide, Germany).

Animals

Thirty-six male Wistar rats (150-200 g) were obtained from a certified animal house (UD, Wistar Bantul, Yogyakarta). The rats were then transported and cared for in the Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Hasanuddin University. They were housed under a controlled room temperature of 25 °C and humidity with 12 h light and dark cycles. The animals were fed standard pellets and water *ad libitum* during the adaptation period. All animal protocols complied with the Institutional Standard of Animal Care, and ethical clearance was obtained (409/UN 4.6.4.5.31/PP36/2022).

Experimental design

After a week of acclimatization, the rats were randomized and assigned to one of the six groups (n: 6 *per* group). The healthy control rats were fed standard pellets, whereas the dyslipidemia groups received a high-fat diet containing 5% duck egg yolk and 18% beef lard mixed with standard pellets and fructose in water (15%) for four consecutive weeks to induce dyslipidemia. After four weeks, the dyslipidemia groups were randomly assigned to receive either no treatment, simvastatin treatment only, omega-3 with simvastatin, low-dose vitamin D (62 IU/kg), or high-dose vitamin D (620 IU/kg) with simvastatin. These treatments were administered for 2 weeks along with the high-

fat diet. The rats' body weight was measured daily during the experimental period.

Blood collection and biochemical analysis

Blood samples were collected following the 4-week high-fat diet administration (at the end of week 4) and after all treatments were administered for two weeks (at the end of week 6). All rats were fasted for 12 h and anesthetized with ether before blood sampling. A 3 mL blood sample was collected from the lateral veins using a Vacutainer tube (BD Vacutainers) and then centrifuged at 4,000 rpm for 10 min at room temperature. The clear supernatant serum was then frozen and stored at 20 °C for biochemical analysis.

Lipid profile measurement

TC, TG, and HDLc levels were analyzed using a blood chemical analyzer (Humalyzer 3500, Human Diagnostic Worldwide, Germany). The serum non-HDLc level was calculated using the equation:

Non-HDLc= TC-HDLc [1]

Atherogenic index of plasma

AIP, an indicator of small dense LDLc and a predictor of coronary atherogenicity, was calculated using the following formula:

AIP=Log (TG/HDLc)

AIP can be used to determine the size of atherogenic lipoprotein particles. If AIP increases, the atherogenic lipoprotein particles become smaller, making it easier to move and undergo oxidation, thereby accelerating the process of atherosclerosis.[22]

Serum liver marker function enzyme measurement

AST and ALT levels were analyzed using a blood chemical analyzer (Humalyzer 3500, Human Diagnostic Worldwide, Germany).

Statistical analysis

Data were analyzed using SPSS version 25. The normality of the collected data was tested using the Shapiro-Wilk test and then further analyzed using one-way ANOVA (95% confidence interval), if the data were normally distributed or Kruskal-Wallis analysis, if they were not normally distributed. Statistical significance was defined as a p value of < 0.05.

RESULTS

Effects of simvastatin, omega-3, and vitamin D on body weight

Figure 1 shows the pattern of body weight gained by the rats after exposure to a high-fat diet and different treatments. Regardless of the high-fat diet treatment, the dyslipidemic rats did not show a significant weight gain compared with rats that did not receive a high-fat diet. Hence, treatments with simvastatin only or adjuvant therapies did not cause any significant change in the *b.w.* at any time point.

Anti-dyslipidemia activity of simvastatin, omega-3, and vitamin D

The results of the lipid profile measurements following four weeks of high-fat diet administration are shown in Table 1.

In all dyslipidemia groups, serum TG levels were significantly higher than in the healthy controls ($p < 0.05$), but TC, HDLc, and non-HDLc levels did not significantly increase. This indicates that administration of a high-fat diet and 15% fructose in water for six weeks only induced Frederickson phenotype-1 dyslipidemia.

Serum lipid levels after two weeks of treatment are depicted in Figure 2. Treatment with low-dose simvastatin in dyslipidemic rats only caused an insignificant decrease in TC and TG levels, with mean reductions of 13.6% and 12.4%, respectively. HDLc and non-HDLc levels were also not significantly changed by simvastatin treatment. In the groups treated with omega-3 as an adjuvant to simvastatin, the TC level was slightly increased, and the TG and HDL levels were slightly reduced compared with simvastatin treatment alone. In contrast, the administration of vitamin D, especially at a high dose, led to a significant reduction in TG by 36.4% compared with the placebo group (112.66 ± 19.36 *vs.* 177.20 ± 17.93, $p = 0.019$). Moreover, the combination therapy of simvastatin and high-dose vitamin D significantly increased HDLc levels compared with the administration of simvastatin alone (64.94 ± 10.59 *vs.* 39.13 ± 6.89, $p = 0.016$). This group had higher HDLc levels than the control group (64.94 ± 10.59 *vs.* 43.46 ± 7.01, $p = 0.042$). Interestingly, we found that the HDLc levels of the simvastatin plus omega-3 group were lower than those of all the dyslipidemia groups, including the untreated dyslipidemia group (25.06 ± 2.10 *vs.* 46.71 ± 5.20, $p = 0.040$).

Anti-atherogenic potential of omega-3 and vitamin D as adjuvant therapies

Figure 3 shows the AIP of rats after two weeks of treatment. The normal rats had an average AIP of 0.155 ± 0.082. Meanwhile, the groups that received a high-fat diet (dyslipidemia groups) predominantly had higher AIPs. The highest mean AIP was found in rats receiving simvastatin plus omega-3 therapy (0.702 ± 0.077), which was significantly higher than that of the controls. Unlikely, the AIP value of the rats treated with simvastatin plus a high-dose vitamin D therapy was near that of the normal controls and substantially lower than that of simvastatin alone ($p < 0.05$) or simvastatin plus omega-3 therapy groups ($p < 0.05$).

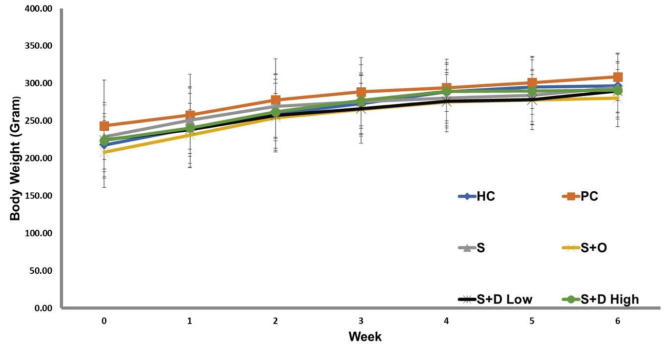

Figure 1. The effect of simvastatin, omega-3, and vitamin D on the bodyweights of rats of different therapeutic groups. Data are presented as mean ± standard deviation

Table 1. Lipid profile levels of rats after consuming a high-fat diet for 4 weeks before therapy administration

Group	TC	TG	HDL	Non-HDL
	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)
Healthy control (HC)	102.18 ± 6.63	101.16 ± 7.91	33.73 ± 3.18	74.53 ± 7.59
Placebo control (PC)	103.58 ± 14.67	255.70 ± 42.66*	47.07 ± 3.05	62.77 ± 21.23
Simvastatin (S)	115.60 ± 6.14	192.64 ± 15.96*	52.59 ± 10.07	70.01 ± 11.13
Simvastatin + Omega-3 (S + O)	113.83 ± 13.10	224.22 ± 19.50*	30.48 ± 3.42	92.76 ± 10.23
Simvastatin + MVD (S + D low)	119.58 ± 11.32	197.40 ± 34.87*	47.39 ± 6.78	77.1 ± 9.66
Simvastatin + HVD	100.58 ± 14.95	170.00 ± 36.66	43.86 ± 9.21	73.77 ± 18.84
(S + D high)				
***P* value**	**0.791**	**0.022**	**0.177**	**0.771**

Data are presented as mean ± standard deviation. *$p \leq 0.05$ compared with the healthy control group.
TC: Total cholesterol, TG: Triglyceride

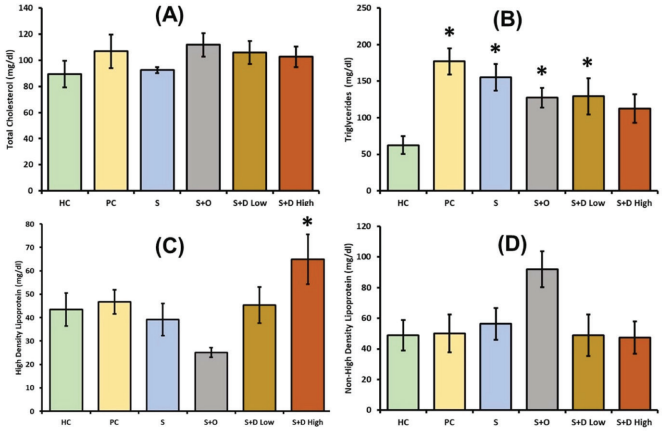

Figure 2. The effect of different treatments on serum lipid levels in rats. Total cholesterol (TC) (A), triglyceride (TG) (B), high-density lipoprotein cholesterol (HDLc) (C), and non-high-density lipoprotein cholesterol (non-HDLc) (D)

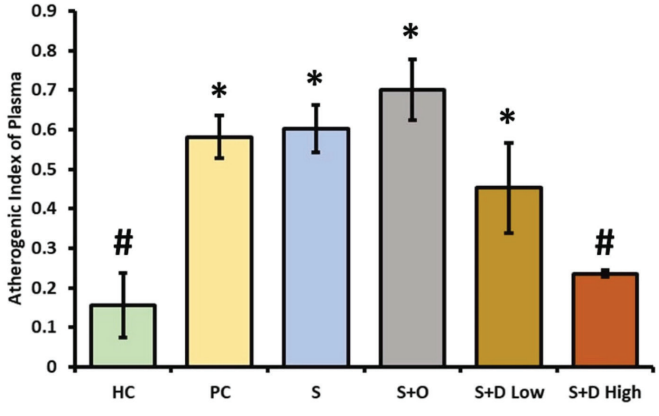

Figure 3. Results of atherogenic index of plasma calculation after administration of therapies for 2 weeks in each group

Liver function test after different treatments

The levels of serum liver enzymes after two weeks of treatment are depicted in Figure 4. In the placebo control group, rats only receiving a high-fat diet without antihyperlipidemic treatment

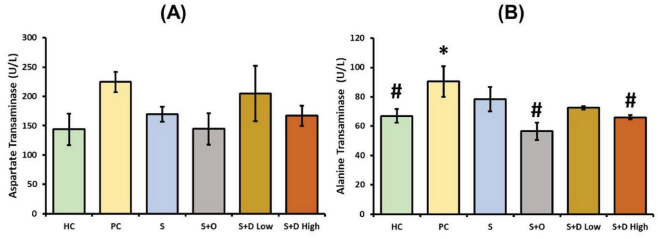

Figure 4. The effect of adjuvant treatments on the levels of serum liver enzymes in rats. Aspartate transaminase (A) and alanine transaminase (B)

experienced an increase in AST and ALT levels, although only the ALT level achieved statistical significance ($p < 0.05$). After the 2-week treatment with simvastatin, AST and ALT of the dyslipidemic rats were not significantly improved with mean reductions of 24.5% and 13.4%, respectively. Although the AST levels were not significantly changed with added adjuvant therapies, the administration of omega-3 or a high-dose vitamin D as an adjuvant to simvastatin led to a significant reduction in ALT by 37.60% and 27%, respectively, compared with the placebo group (56.5 ± 5.86 *vs.* 90.5 ± 10.45, $p = 0.003$ and 66.0 ± 1.39 *vs.* 90.5 ± 10.45, $p = 0.018$). The ALT value of rats treated with omega-3 and high-dose vitamin D as an adjuvant to simvastatin therapy was near that of normal controls.

DISCUSSION

Several studies have shown that consumption of a high-fat diet and fructose can induce lipid abnormalities, obesity, metabolic diseases, and cardiovascular diseases.[23,24] In general, experimental animals fed a high-fat diet and fructose solution experienced Frederickson phenotype-1 dyslipidemia, characterized by TG abnormalities > 99% due to lipoprotein lipase deficiency. Phenotype-1 dyslipidemia can occur due to the intake of high triacylglycerol content and enhanced lipogenesis activity that produces TGs.[12,24]

The addition of omega-3 as an adjuvant to simvastatin therapy had a superior effect on lowering TG levels with a mean value of 127.38 ± 13.36 mg/dL compared with 155.20 ± 18.05 mg/dL

with simvastatin alone. This is in accordance with the 2019 ESC/EAS Guidelines for the management of dyslipidemia, which state that omega-3 fatty acid therapy has the main effect of lowering TG levels, so its use is more recommended in hypertriglyceridemia conditions. Omega-3 can reduce serum TG concentrations by approximately 30%.[2] In the liver, omega-3 suppresses endogenous triacylglycerol production by decreasing levels of sterol receptor element-binding protein-1c (SREBP-1c) and selectively increasing the degradation of apo B-100, further reducing the production of triacylglycerol-rich VLDL. Another mechanism is the upregulation of fatty acid oxidation in the liver and skeletal muscle, thereby reducing the VLDL synthesized material.[25,26]

The group receiving combination therapy with simvastatin and a high-dose vitamin D showed the greatest reduction in TG levels, even resembling the TG levels of the normal group. However, the TC and non-HDLc levels did not significantly improve. These results are consistent with those of Paloma et al.,[27] who found that vitamin D supplementation (4,000 IU/day) could reduce serum TGs without affecting other lipid profile parameters. Another study examining the effect of vitamin D on serum showed that vitamin D could lower TG levels by 30.5% every time the dose was doubled.[28] *In vitro* studies have revealed that incubation with calcitriol ($1,25(OH)_2D_3$) may increase the expression and activity of lipoprotein lipase in cultured adipocytes, resulting in enhanced TG hydrolysis in chylomicrons and VLDL hydrolysis in blood vessels.[15,29]

This study also displayed that the combination therapy of simvastatin and high-dose vitamin D could increase HDL levels in the dyslipidemia group to a level high enough to exceed HDL levels in the normal group. This is in accordance with the research of Elmi et al.,[30] who reported that vitamin D3 levels were positively related to HDL2-C levels. Skaaby et al.[28] showed an increase in HDLc levels every time the dose of vitamin D was doubled. Although the molecular mechanism of the relationship between vitamin D and HDLc has not been fully elucidated, various observational studies have shown the relationship between them. The observational study of Williams et al.[31] of 4,274 children in the UK showed that high 25-OH-D levels were associated with cardioprotective levels of HDLc, apoprotein A1, and adiponectin. The results of this observational study are reinforced by the results of the experimental study by Tavakoli et al.,[32] who provided vitamin D supplementation to children aged 10-14 years and found a substantial rise in serum HDL levels compared with the control group.

In this study, adjuvant therapy with omega-3 or vitamin D did not have a beneficial effect in reducing TC levels compared with simvastatin treatment alone. These results are in accordance with those of a study conducted by Wang et al.,[18] where vitamin D supplementation lowered LDLc levels but had no significant effect on TC. Although the mechanism is not clear, various studies have shown an association between vitamin D deficiency and dyslipidemia. Vitamin D is hypothesized to increase lipolysis by suppressing parathyroid hormone secretion.[28] 25-Hydroxyvitamin D also inhibits sterol regulatory element-binding protein as a major regulator of lipogenesis.[33]

According to the 2019 ESC/EAS Guidelines for the management of dyslipidemia, omega-3 does not reduce TC levels but lowers TG.[2] However, Ibrahim Fouad[34] found that the administration of omega-3 monotherapy (500 mg/kg) in rats with hyperlipidemia induced by a high-fat diet for six weeks had a TC-lowering effect. Further research is required to evaluate the comparative effect of each therapy with simvastatin, vitamin D, and omega-3.

The non-HDLc level was calculated in this study to estimate the total number of atherogenic particles in plasma.[1] It also correlates with apolipoprotein-B levels and is a good predictor of cardiovascular risk.[35] In this study, the combination of simvastatin and adjuvant therapy (either omega-3 or vitamin D) had no significant impact on non-HDLc levels. A confounding factor is that the administration of the high-fat diet and fructose in rats only induced type-I dyslipidemia; therefore, rats did not experience an increased level of TC. Because the non-HDLc level was calculated from the TC level, the non-HDLc levels also remained unchanged after receiving a high-fat diet. Therefore, observing the role of either vitamin D or omega-3 in non-HDLc levels was difficult in this study. However, another study reported a similar result in a clinical setting, where vitamin D supplementation in patients with type 2 diabetes mellitus had no significant effect on non-HDLc levels.[35]

The administration of combination therapy with simvastatin and high doses of vitamin D in this study exhibited a superior effect in reducing AIP in the dyslipidemia group. The AIP of the group receiving combination therapy of simvastatin and high doses of vitamin D almost resembled the AIP of normal rats. This result is consistent with another study by Wang et al.,[17] which reported that serum 25(OH)D concentrations were negatively associated with AIP in men. Reduced TG and increased HDL will result in a smaller AIP calculation, indicating the normal diameter of LDL (atherogenic lipoprotein) and a lower risk of atherosclerosis. Vitamin D can inhibit TG synthesis and secretion by increasing calcium absorption in the intestine. Increased intestinal calcium levels may decrease fatty acid absorption because of the formation of insoluble calcium-fat complexes. In addition, calcium can increase the conversion of cholesterol into bile salts so that cholesterol levels decrease.[17,36] Further study is important to provide a complete measurement of atherogenic index parameters, including the cardiac risk ratio and atherogenic coefficient, as well as confirmation by histopathological examination of the heart and blood vessels.

Study limitations

In this study, we also found that the addition of omega-3 or high-dose vitamin D as an adjuvant to simvastatin therapy had a superior effect on lowering ALT levels compared with simvastatin alone. Vitamin D in high doses could also lower ALT levels with a mean value of 66.0 ± 1.39 µ/L. The hepatoprotective effects of omega-3 and vitamin D were previously studied. Omega-3 PUFA in low doses has a hepatoprotective effect, if administered daily.[37] Omega-3 prevents hepatic damage by improving hepatic function and normalizing lipid profiles in the serum and liver.[38] Similarly, vitamin D deficiency correlates with increased hepatic inflammation by increasing toll-like

receptors.[39] Lorvand Amiri et al.[40] reported that a combination of vitamin D and calcium had a superior effect on lowering serum ALT levels and could improve the stage of non-alcoholic fatty liver disease on ultrasonography. This means that vitamin D benefits on liver function may be related to its effect on lipid profiles. This hepatoprotective effect of vitamin D can improve clinical outcomes in patients with dyslipidemia.

CONCLUSION

Administration of high doses of vitamin D as an adjuvant to simvastatin therapy was more effective than omega 3 in improving TG and HDL levels. Vitamin D (both maintenance and high doses) as an adjuvant to simvastatin therapy was also superior to omega-3 in improving AIP in a rat model of type-1 dyslipidemia. Vitamin D in high doses (6,000 IU/day) as adjuvant therapy to simvastatin may not just alleviate dyslipidemia and decrease the risk of atherosclerosis. Furthermore, the administration of high doses of vitamin D or omega-3 as an adjuvant to simvastatin therapy had a hepatoprotective effect. Further work is needed to clarify the molecular mechanism underlying the cardioprotective, atheroprotective, and hepatoprotective potentials of vitamin D.

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Ethics

Ethics Committee Approval: All animal protocols complied with the Institutional Standard of Animal Care, and ethical clearance was obtained (409/UN 4.6.4.5.31/PP36/2022).

Peer-review: Externally peer reviewed.

Authorship Contributions

Surgical and Medical Practices: D.L, A.A., Concept: D.L, Y.Y.D, A.A., Design: D.L, Y.Y.D, A.A., Data Collection or Processing: D.L, Y.Y.D, B.O.M., Analysis or Interpretation: D.L, Y.Y.D, A.A., Literature Search: D.L, B.O.M., Writing: D.L, Y.Y.D.

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