ABSTRACT
Adjuvant therapy is often used to optimize the antihyperlipidemic effect of simvastatin. Omega-3 and vitamin D supplementation are among recommended adjuvant therapies to low-intensity statins. 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 type-1 dyslipidemic rats. 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 a low and a high dose. Dyslipidemia was induced with high-fat diets for four weeks, followed by treatments for the next two weeks. Blood samples were withdrawn before and after simvastatin treatments. Additionally, aspartate transaminase (AST) and alanine transaminase (ALT) levels were analyzed to assess liver function. The administration of a high-fat diet induced type-1 dyslipidemia and increased ALT levels (p<0.05). Treatment with low-dose simvastatin did not result in significantly improved triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLc), or non-HDLc levels. When combined with high-dose vitamin D, simvastatin significantly reduced TG and increased HDLc levels (p<0.05), hence improving AIP levels. This improvement was not shown in rats treated with omega-3 or vitamin D at a lower dose. 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-1 dyslipidemic rats. This result may be translated in clinics to reduce the risk of coronary syndrome in type-1 dyslipidemia patients.

KEYWORDS: Vitamin D, Omega-3, Atherogenic Index, Simvastatin, Adjuvant Therapy

1. 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 (LDL) receptors at the surface and thus increases LDL uptake, decreases LDL plasma and other apolipoprotein-B, and lowers triglyceride (TG) levels. Other effects of simvastatin include reducing oxidative stress and inflammation of the vesel walls, and thereby it may provide protection against atherosclerotic lesions. 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 HDL compared to other statin agents. Various studies show that up to 65–75% of the incidence of atherosclerosis cannot be prevented by lowering LDL-cholesterol with statin therapy. 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 even when treated with high doses of statins. Therefore, the combination therapy of statins with other lipid-lowering agents has become one of the most used strategies that can be applied in the clinic.
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. 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. 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. 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 compared with statin monotherapy in primary and secondary prevention. One meta-analysis found that omega-3 supplementation for 4.4 years did not have a significant effect on reducing the incidence of coronary heart disease or other major vascular events. Based on this inconsistent data, another candidate must be considered for adjuvant therapy to statin treatment for dyslipidemia patients.

Vitamin D (known as calciferol) has long been associated with bone growth and bone strength. However, substantial evidence suggests that vitamin D deficiency is also associated with an increased risk of cardiovascular disease. 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 (RAA) system (anti-hypertensive effect), and improved glycemic control.

Various data regarding the relationship between vitamin D and blood lipid levels are still 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 triglyceride and LDLc levels and positively associated with TC. 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. In many studies, vitamin D deficiency has been strongly associated with liver disease. Vitamin D’s role in the liver has been greatly understood since the development of the vitamin D receptor. Vitamin D correlates inversely to non-alcoholic fatty liver disease in a dose-dependent manner. 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. 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.

2. Materials and methods
2.1 Drugs and chemicals
2.1.1 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 EPA and 120 mg DHA (Blackmores Co., Australia). Vitamin D was obtained as tablets containing cholecalciferol 400 IU (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 low-intensity simvastatin at a dose of 20 mg/day in humans. The dose of omega-3 was 206 mg/kg of rats’ b.w., which is equivalent to 2,000 mg/day in humans. Vitamin D was given 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.

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

2.2 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 with 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).

2.3 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 with 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 given for two weeks along with the high-fat diet. The rats’ b.w. was measured daily during the experimental period.

2.4 Blood collection and biochemical analysis

Blood samples were collected following the four-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 lateral veins using a Vacutainer tube (BD Vacutainers), then centrifuged at 4,000 rpm for 10 min at room temperature. The clear supernatant serum was then frozen and stored at 20 ºC for biochemical analysis.

2.5.1 Lipid profile measurement

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

\[ \text{Non−HDLc} = \text{TC} - \text{HDLc} \]

2.5.2 Atherogenic index of plasma

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

\[ \text{AIP} = \log \left( \frac{\text{TG}}{\text{HDLc}} \right) \]

AIP can be used to portray the size of atherogenic lipoprotein particles. If AIP increases, the atherogenic lipoprotein particles are smaller, making it easier to move and undergo oxidation, thereby accelerating the process of atherosclerosis.

2.5.3 Serum liver marker function enzyme measurement

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

2.6 Statistical Analysis

Data were analyzed using SPSS version 25. The normality of the collected data was tested using the Shapiro–Wilk test, 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.

3. Results

3.1. 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 significant weight gain compared to rats that did not receive a high-fat diet. Hence, treatments with simvastatin only or with adjuvant therapies did not cause any significant change in rats’ b.w. at any time point.

3.2. Anti-dyslipidemia activity of simvastatin, omega-3, and Vitamin D

The results of lipid profile measurement following four weeks of high-fat diet administration are shown in Table 1. In all dyslipidemia groups, the serum TG levels were significantly higher than in the healthy controls (p<0.05), but the TC, HDLc, and non-HDLc levels did not significantly rise. This indicates that the administration of a high-fat diet and 15% fructose in water for six weeks only induced Frederickson phenotype-1 dyslipidemia. The 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, with mean reductions of 13.6% and 12.4%, respectively. The HDLc and non-HDLc levels were also not significantly changed with 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 to simvastatin treatment alone. In contrast, the administration of vitamin D, especially in a high dose, led to a significant reduction in TG by 36.4% compared to the placebo group (112.6±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 to the administration of simvastatin alone (64.94±10.59 vs. 39.13±6.89, p=0.016). This group even had higher HDLc levels than the control group (64.46±7.01, p=0.042). Interestingly, we found that the HDLc levels of the simvastatin plus omega-3 group were lower than all of the dyslipidemia groups, including the untreated dyslipidemia group (25.06±2.10 vs. 46.71±5.20, p=0.040).

3.3. Anti-atherogenic potential of omega-3 and vitamin D as adjuvant therapy

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
Several studies have shown that consuming a high-fat diet and fructose can induce lipid abnormalities, obesity, metabolic diseases, and cardiovascular diseases. In general, experimental animals fed with a high-fat diet and fructose solution experienced Frederickson phenotype-1 dyslipidemia, characterized by triglyceride 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 triglycerides.

The addition of omega-3 as an adjuvant to simvastatin therapy had a superior effect on lowering TG level, with a mean value of 127.38±13.36 mg/dL compared to 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 triglyceride levels, so its use is more recommended in hypertriglyceridemia conditions. Omega-3 can reduce serum triglyceride concentrations by about 30%. 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.

The group receiving combination therapy with simvastatin and high-dose vitamin D showed the greatest outcome in reducing TG levels, even resembling the TG levels of the normal group. However, the TC and non-HDLc levels were not significantly improved. These results are consistent with the study of Paloma et al. (2014) who found that vitamin D supplementation (4,000 IU/day) could reduce serum triglycerides without affecting the other lipid profile parameters. Another study examining the effect of vitamin D on serum showed that vitamin D could lower triglyceride levels by 30.5% every time the dose was doubled. In vitro studies showed that incubation with calcitriol (1,25(OH)2D3) may increase the expression and activity of lipoprotein lipase in cultured adipocytes, resulting in enhanced triglyceride hydrolysis in chylomicrons and VLDL hydrolysis in blood vessels.

This study also showed that the combination therapy of simvastatin and high-dose vitamin D could increase the HDL levels in the dyslipidemia group high enough to exceed HDL levels in the normal group. This is in accordance with the research of Elm et al. (2021) who reported that vitamin D3 levels were positively related to HDL2-C levels. Research by Skjødt et al. (2013) showed an increase in HDL 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 show the relationship between them. The observational study of Williams et al. (2014) 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. (2016) who provided vitamin D supplementation to children aged 10–14 years and found a substantial rise in serum HDL levels compared to 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 to simvastatin treatment alone. These results are in accordance with a study conducted by Wang et al. (2012) who found that vitamin D supplementation lowered LDLc levels but had no significant effect on TC. Although the mechanism is not clear, various studies have shown the association between vitamin D deficiency and dyslipidemia. Vitamin D is hypothesized to increase lipolysis by suppressing parathyroid hormone secretion. 25-hydroxy vitamin D is also known to inhibit sterol regulatory element-binding protein as a major regulator of lipogenesis.

According to the 2019 ESC/EAS Guidelines for the management of dyslipidemia, omega-3 does not reduce TC levels but lowers TG. However, Ibrahim et al. (2020) 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: 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.\textsuperscript{1} Its level also correlates with apolipoprotein-B levels and is a good predictor of cardiovascular risk.\textsuperscript{35} In this study, the combination of simvastatin therapy 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, so rats did not experience an increased level of TC. Since 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 on non-HDLc levels was difficult in this study. However, another study reported a similar result in a clinical setting, where vitamin D supplementation in type 2 diabetes mellitus patients had no significant effect on non-HDLc levels.\textsuperscript{35}

The administration of the combination therapy with simvastatin and high doses of vitamin D in this study showed 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 the normal rats. This result is consistent with another study by Wang et al. (2016)\textsuperscript{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 the synthesis and secretion of TG by increasing the absorption of calcium in the intestine. Increased intestinal calcium levels may decrease fatty acid absorption due to the formation of insoluble calcium–fat complexes. In addition, calcium can increase the conversion of cholesterol into bile salts so that cholesterol levels will decrease.\textsuperscript{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.

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 to simvastatin alone. Vitamin D in high doses could also lower the ALT level, with a mean value of 66.0±1.39 µ/L. The hepatoprotective effects of omega-3 and vitamin D have been studied previously. Omega-3 PUFA in low doses has a hepatoprotective effect if given daily.\textsuperscript{37} Omega-3 prevented hepatic damage by improving hepatic function and normalizing lipid profiles in the serum and liver.\textsuperscript{38} Similarly, vitamin D deficiency has been found to correlate with increased hepatic inflammation by increasing toll-like receptors.\textsuperscript{39} Hardi et al. (2016)\textsuperscript{40} reported that a combination of vitamin D and calcium had a superior effect on lowering serum ALT and could improve the stage of non-alcoholic fatty liver disease on ultrasonography. This means that vitamin D benefits on liver function may or may not be related to its effect on lipid profiles. This hepatoprotective effect of vitamin D can be beneficial to improve clinical outcomes in dyslipidemic patients.

5. Conclusion

The 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 the AIP in the rat model of type-I dyslipidemia. Vitamin D in high doses (6,000 IU/day) as adjuvant therapy to simvastatin may not just alleviate dyslipidemia but also be beneficial to decrease the risk of atherosclerosis. Furthermore, the administration of high doses of vitamin D or omega-3 as an adjuvant to simvastatin therapy offered a hepatoprotective effect. Further work is needed to clarify the molecular mechanism underlying the cardioprotective, atheroprotective, and hepatoprotective potentials of vitamin D.

6. Acknowledgement

The authors would like to thank Ms. Mutmainnah and Ms. Fika who have helped us preparing the blood samples during the experiment.

References


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

<table>
<thead>
<tr>
<th>Group</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>Non-HDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control (HC)</td>
<td>102.18</td>
<td>6.63</td>
<td>101.16</td>
<td>7.91</td>
</tr>
<tr>
<td>Placebo control (PC)</td>
<td>103.58</td>
<td>14.67</td>
<td>255.70</td>
<td>42.66*</td>
</tr>
<tr>
<td>Simvastatin (S)</td>
<td>115.60</td>
<td>6.14</td>
<td>192.64</td>
<td>15.96*</td>
</tr>
<tr>
<td>Simvastatin+ Omega-3 (S+O)</td>
<td>113.83</td>
<td>13.10</td>
<td>224.22</td>
<td>19.50*</td>
</tr>
<tr>
<td>Simvastatin+ MVD (S+D Low)</td>
<td>119.58</td>
<td>11.32</td>
<td>197.40</td>
<td>34.87*</td>
</tr>
<tr>
<td>Simvastatin+ HVD (S+D High)</td>
<td>100.58</td>
<td>14.95</td>
<td>170.00</td>
<td>36.66</td>
</tr>
</tbody>
</table>

P value | 0.791 | 0.022 | 0.177 | 0.771 |

Data are presented as mean ± standard deviation. *p≤ 0.05 compared to the healthy control group.
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.
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). HC=healthy control, PC=placebo control, S=simvastatin, S + O=simvastatin and Omega-3, S + D Low=simvastatin and vitamin D low dose, S + D High=simvastatin and vitamin D high dose. Data are presented as mean ± standard deviation. *p<0.05 compared to the healthy control group.
Figure 3. Results of Atherogenic Index of Plasma (AIP) calculation after administration of therapies for 2 weeks in each group. Data are presented as mean ± standard deviation. *p≤ 0.05 compared to the healthy control (HC) group. "p≤ 0.05 compared to the simvastatin (S) group.
Figure 4. The effect of adjuvant treatments on the levels of serum liver enzymes in rats. Aspartate transaminase (AST) (A) and alanine transaminase (ALT) (B). HC=healthy control, PC=placebo control, S=simvastatin, S + O=simvastatin and Omega-3, S + D Low=simvastatin and vitamin D low dose, S + D High=simvastatin and vitamin D high dose. Data are presented as mean ± standard deviation. *p ≤ 0.05 compared to the healthy control (HC) group. †p ≤ 0.05 compared to the simvastatin (S) group.