Research article
Effect of bortezomib on fatty liver in a rat model of atherosclerosis
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ABSTRACT

Introduction and Aim: Fatty liver is associated with atherosclerosis even though the exact mechanism remains unknown. Fatty liver and atherosclerosis correlate with inflammation. Interleukin 6 (IL-6) is recognized as an inflammatory marker. Bortezomib is a proteasome inhibitor that will inhibit the proteasome pathway and is expected to inhibit inflammation in atherosclerosis. The current research aimed to investigate the effect of bortezomib on the fatty liver of atherosclerosis rats and to analyze its correlation with serum IL-6 concentration.

Materials and Methods: Experimental subjects were 18 male Wistar rats (Rattus norvegicus) divided into three treatment groups, namely atherosclerosis group (I), atherosclerosis + bortezomib group (II), and control group (III). Bortezomib (50 μg/kg BW) was given twice intraperitoneally, on day 1 and day 3. The presence of fatty liver was evaluated using the percentage system. Serum IL-6 concentrations were measured using enzyme-linked immunosorbent assay kits.

Results: The highest amount of fatty liver was found in the atherosclerosis group (group I) (38.33%), while the lowest was in the control group (group III) (5.83%). There was a decreasing fatty liver percentage due to bortezomib administration (group II) (29.17%), and it was statistically significant. There is a significant correlation between the degree of fatty liver and serum IL-6 concentration.

Conclusion: The administration of bortezomib 50 μg/kg BW in atherosclerosis model rats can reduce the occurrence of fatty liver by reducing the inflammatory process.

Keywords: Atherosclerosis; bortezomib; fatty liver; interleukin 6; proteasome.

INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) is a world-wide problem and is the main cause of morbidity and mortality related to liver disease. NAFLD is a chronic liver disease characterized by steatosis and steatohepatitis in the liver parenchyma. This fatty liver can progress to cirrhosis, with or without hepatocellular carcinoma (HCC) (1,2). NAFLD is closely related to metabolic syndrome, abdominal obesity, dyslipidemia, and diabetes, thus increasing cardiovascular disease risk (2).

Inflammation is the underlying process of atherosclerosis. Low-density lipoprotein (LDL) cholesterol has been settled as the cause of atherosclerosis, so lowering the LDL cholesterol concentration is the main goal in reducing atherogenesis and limiting the occurring complications. Although statins have been used as therapy, there are some recurring cardiovascular attacks (3). Various studies have been conducted to obtain an ideal atherosclerosis therapy. Atherosclerosis treatment must focus on anti-inflammatory properties. Although statins and aspirin have been utilized as therapy for atherosclerosis, they are not specifically anti-inflammatory (4).

Proteasomes are protease complexes that play a role in inflammation in atherosclerosis, so they become the potential targets for atherosclerosis therapy (5). Several studies have analyzed the effect of proteasome inhibitor use on atherosclerosis. One of the proteasome inhibitors whose anti-atherosclerosis effect has been studied is bortezomib. Bortezomib was initially developed as an anti-cancer and has been used as a cancer therapy since 2003 (6). The effect of proteasome inhibitors as anti-atherosclerosis still needs further investigation because the effects of using proteasome inhibitors on atherosclerosis are varied. The given of Bortezomib 50 μg/kg for six weeks in LDLR -/- mice suppressed the early atherosclerotic lesion formation (7). However, the same dose of bortezomib administration did not provide a therapeutic effect in LDLR -/- mice with advanced atherosclerotic lesions (8). Some factors influencing the proteasome inhibitor effect are dose, administration duration, target proteasome subunit composition, and atherosclerosis stage. The inhibitor proteasome with the same dose can give different effects when used at different stages of...
atherosclerosis. The given low dose will have an advantageous effect, but this effect will be lost if the dose is increased (9).

The pathogenesis of NAFLD is still not clear. A multi-hit process is thought to be involved in its pathogenesis, where the first hit is the accumulation of fat in the liver associated with insulin resistance, and the second hit shows an increase in fatty acid oxidation, oxidative stress, and endotoxemia. Adipose tissue produces a number of cytokines called adipokines, which have local, peripheral, and central effects. Adipokines play a significant function in the pathogenesis of acute and chronic liver disease, further promote in liver cell death, inflammation, cholestasis, and fibrosis. The adipokines widely studied are tumor necrosis factor (TNF-α), interleukin-6 (IL-6), adiponectin, resistin, and leptin. Several studies have concluded that adipokine is involved in the pathogenesis of NAFLD and the progression to non-alcoholic steatohepatitis (NASH). Liver IL-6 expression was significantly increased in NASH patients and was positively correlated with serum IL-6 concentration (1).

Research by Oliva et al. showed that bortezomib could reduce fat accumulation and steatosis in liver cells of rats induced using ethanol due to a decrease in fatty acid synthesis, triglycerides, and cholesterol synthesis. These results indicate the potential of bortezomib to be used as therapy in alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH) (10). This current research aimed to investigate the effect of bortezomib on the fatty liver of the atherosclerosis rat model and to analyze its correlation with serum IL-6 concentration.

MATERIALS AND METHODS

Research design

This research has received ethical clearance from the Medical/Health Research Ethics Committee, the Faculty of Medicine, Riau University. This experimental study used 18 male Wistar rats aged ten weeks. The experimental animals were randomly divided into three groups, namely Group I (atherosclerosis), group II (atherosclerosis with proteasome inhibitors), and group III (control).

Treatment of experimental animals

The rats were kept indoor with a 12-hour dark and light cycle, 70% humidity, and at 26 °C with adequate ventilation. Food and drink were checked daily and given ad libitum. Atherosclerosis induction was performed by administering vitamin D3 (700,000 IU/kg) on the first day, and followed by an atherogenic diet (2% cholesterol, 0.2% cholic acid, goat fat) (5). The proteasome inhibitor used was bortezomib (50 μg/kg BW), intraperitoneally given twice, on day 1 and day 3 (11). After 4 days of treatment, blood and liver were taken after the rats were anesthetized using ether. Blood was taken from the heart, placed in tubes, and centrifuged at 3000 rpm to obtain the serum, which was then stored in a refrigerator at -80 °C until the measurement time. The thoracic aorta and liver were removed and placed in plastic pots filled with formalin buffer. The thoracic aorta and liver were subsequently examined histopathologically with hematoxylin-eosin (HE) staining. Atherosclerotic lesions in the thoracic aorta of the white rats were assessed qualitatively by an anatomical pathologist by assessing the atherosclerotic lesions in the form of macrophages, foam cells, smooth muscle proliferation, medial lipid infiltration, fibrosis/calcification, and surface defects (fissure/ulceration/ hematoma/thrombus). The evaluation was performed in 9 fields of view with a light microscope at 400x magnification (5). The level of changes in the fatty liver was measured in percentage. The fatty liver percentage was obtained from the sum of microvascular steatosis and macrovascular steatosis divided by the total number of cells. The total number of cells was the result of the sum of normal cells, microvascular steatosis, and macrovascular steatosis. Normal means that the cell has a nucleus in the middle and a polygonal shape, microvesicular means that the nucleus is in the middle and a small vacuole is formed, and macrovesicular means that large vacuoles are formed and the nucleus is pushed aside. The evaluation was performed by light microscope in 5 fields of view at 400x magnification (12).

Measurement of serum IL-6 concentration

Blood draws were performed with cardiac punctures. Serum was obtained by centrifuging blood at 1000 g for 15 minutes. Further, serum IL-6 was measured using enzyme-linked immunosorbent assay kits (CSB-E04640r, Cusabio, Wuhan, China).

Statistical analysis

The one-way ANOVA test was used to determine differences in fatty liver between treatment groups. Spearman’s rank correlation was performed to assess the correlation between serum IL-6 concentration and fatty liver. The statistical test was said to be significant if the p-value was lower than 0.05.
RESULTS

Effects of bortezomib on atherosclerosis

Atherosclerosis induction using 700,000 IU/kg BW vitamin D3 and a high-fat diet in this study succeeded in obtaining atherosclerosis model rats. In group I, foam cells, calcification, smooth muscle cell proliferation were seen, while in group II there were atherosclerotic lesions with a lesser degree. Group III showed normal blood vessels with clear boundaries between intima, media, and adventitia. Smooth muscle cells were neatly arranged and parallel to the internal elastic membrane (Fig. 1).

Effect of bortezomib on fatty liver

The results of liver histopathology observations in this study showed that the highest percentage of fatty liver (38.33%) was found in group I, while the lowest percentage was in group III (5.83%). Intraperitoneal injection of bortezomib 50 μg / kgBW on day 1 and 3 significantly inhibited fatty liver (Table 1).

In group I, liver cells were with small vacuoles attaching together to form larger vacuoles so that the nucleus was pushed aside (macrovesicular steatosis) and liver cells with small vacuoles, but the nucleus remained in the middle (microvesicular steatosis). In group II, macrovesicular steatosis and microvesicular steatosis were also seen but in a smaller percentage. The histopathological image of the liver in the control group was dominated by normal liver cells with the nucleus in the middle (Fig. 2).

Table 1. Mean score of liver histopathological image

<table>
<thead>
<tr>
<th>Replication</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44%</td>
<td>31%</td>
<td>4%</td>
</tr>
<tr>
<td>2</td>
<td>35%</td>
<td>32%</td>
<td>5%</td>
</tr>
<tr>
<td>3</td>
<td>43%</td>
<td>34%</td>
<td>7%</td>
</tr>
<tr>
<td>4</td>
<td>33%</td>
<td>23%</td>
<td>7%</td>
</tr>
<tr>
<td>5</td>
<td>39%</td>
<td>28%</td>
<td>6%</td>
</tr>
<tr>
<td>6</td>
<td>36%</td>
<td>27%</td>
<td>6%</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>38.33% ± 4.5%</td>
<td>29.17% ± 1.6%</td>
<td>5.83% ± 0.5%</td>
</tr>
</tbody>
</table>

Eighteen Wistar rats were used and divided into three groups, namely Group I (atherosclerosis), group II (atherosclerosis with proteasome inhibitors), and group III (control). a = p <0.05 I vs II, b = p <0.05 I vs III.
Steatosis is classified as normal if it is below 10%, mild if between 11-33%, moderate if between 34-66%, and severe if higher than 66% (13). Steatosis in group I was classified as moderate, while steatosis in group II was classified as mild. These conditions show that the induction of atherosclerosis in experimental animals causes steatosis, and the administration of proteasome inhibitors, i.e. bortezomib, can reduce steatosis that occurs due to atherosclerosis induction.

**Correlation between fatty liver and serum IL-6 concentrations**

Fig. 3 shows that the bivariate analysis found a significant correlation between the increasing concentration of serum IL-6 and the steatosis degree in the liver of atherosclerosis model rats ($r = 0.544$). So that conclusions can be drawn that atherosclerosis induction can cause steatosis parallel to the increasing serum IL-6 concentration.
DISCUSSION

The administration of a high dose of vitamin D3 and an atherogenic feed in this study succeeded in obtaining a model of atherosclerosis. It can be seen from the presence of foam cells, calcification, smooth muscle cell proliferation in the groups of atherosclerosis-induced rats. A high dose of vitamin D3 was given to stimulate the proliferation of vascular smooth muscle cells and increase calcification. Cholesterol and triglycerides content in the atherogenic feed results in hypercholesterolemia and hypertriglyceridemia conditions, while cholic acid reduces HDL levels and increases LDL (5).

Atherosclerosis induction in this study also induced steatosis. The results of this study are in line with the research by Kampschulte et al., that also obtained steatosis in apolipoprotein E/low-density lipoprotein receptor double-knockout mice using atherosclerosis induction method, which is different from this study. Dyslipidemia that occurs in the experimental animals is a risk factor for the occurrence of NAFLD (14). NAFLD is histopathologically characterized by steatosis, lobular and portal inflammation, hepatocyte injury (ballooning and apoptosis), and fibrosis. Simple steatosis is Non-Alcoholic Fatty Liver (NAFL). If the process continues, inflammation and injury in the liver will occur and lead to Non-Alcoholic Steatohepatitis (NASH), that ultimately lead to the hepatic cirrhosis phase. Not only fibrosis, but the process can also progress to hepatocellular carcinoma (15).

Interleukins have an important role in the inflammatory response at each atherosclerosis stage (16). This study shows the role of interleukins in pro-atherogenic processes, such as the appearance of adhesion molecules on endothelial cells, macrophage activation, and smooth muscle cell proliferation. Interleukin 6 is a pro-atherogenic inflammatory marker interleukin that becomes the target of atherosclerosis therapy (17). IL-6 also has an important role in the pathogenesis of NASH (18). IL-6 is secreted by adipocytes, immune cells, fibroblasts, endothelial cells, and monocytes. Research shows that IL-6 concentrations are increased in people who are obese and insulin resistant. IL-6 will stimulate an increase in liver gluconeogenesis, followed by hyperglycemia and hyperinsulinemia. Liver IL-6 expression is significantly increased in NASH patients and was positively correlated with inflammation and fibrosis (1).

The administration of bortezomib in this study can reduce the occurrence of steatosis in atherosclerosis model rats. The results of this study are in line with research conducted by Oliva et al. that proteasome inhibitors can reduce fatty liver in rats induced with steatosis using ethanol. Research by Oliva et al. stated that the bortezomib mechanism in reducing fatty liver is by reducing the synthesis of fatty acids, cholesterol, and triglycerides. Significantly, bortezomib has the effect of lowering the levels of acyl-glycerol-3-phosphatase acyltransferase (AGPAT) and diacylglycerol acyltransferase (DGAT) enzymes which play a role in triacylglycerol synthesis (10). In this study, the mechanism of action of bortezomib in reducing fatty liver was correlated with a decrease in serum IL-6 with moderate correlation strength. This suggests that the anti-inflammatory activity of bortezomib has an effect on fatty liver and atherosclerosis.

CONCLUSION

The administration of bortezomib (50 μg/kg BW) in atherosclerosis model rats can reduce the occurrence of fatty liver, and there is a correlation between fatty liver and serum IL-6 concentration.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

REFERENCES