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RESEARCH ARTICLE

Effects of Proteasome Inhibitor on Catalase Expression and Intima-media Thickness in the Aorta of Atherosclerotic Rats

Ismawati Ismawati,[1] Enikarmila Asni,[1] Ilhami Romus,[2] Mukhyarjon Mukhyarjon,[1] Winarto Winarto,[3] Muhammad Fadhillah Arif,[4] M Derillovyandra Dwi Anugrah[4]

[1]Department of Biochemistry, Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia,
[2]Department of Pathology Anatomy, Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia,
[3]Department of Histology, Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia,
[4]Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia

Abstract

Various studies have been carried out to obtain proper management for atherosclerosis. Proteasome, a subcellular enzyme complex, is a potential therapeutic target for atherosclerosis. However, the effect of proteasome inhibitors on atherosclerosis still needs to be explored. It was an experimental study with a post-test-only control group design conducted at the Faculty of Medicine, Universitas Riau in Juni–November 2021. This study aimed to analyze the effects of proteasome inhibitors on catalase expression and intima-media thickness (IMT) in the thoracic aorta of atherosclerotic rats. Fifteen male Wistar rats were randomly divided into three groups (five rats per group), namely rats given standard feed (control, group I), rats induced atherosclerosis (group II), and rats induced atherosclerosis and given proteasome inhibitor (group III). The proteasome inhibitor, bortezomib, 50 μg/kgBW/day was given intraperitoneally on days one and three. After 4 days, rats were terminated, and the thoracic aorta was taken for the IMT analysis and catalase expression assessment using immunohistochemistry. Catalase expression was carried out quantitatively using Adobe Photoshop software. Analysis of variance test was used to compare the expression of catalase and IMT. A p value<0.05 was considered statistically significant. The results showed a significant decrease in IMT in group III compared to group II and an increase in catalase expression in group III compared to group II but not statistically significant. This study concludes that administration of bortezomib 50 μg/kgBW in atherosclerotic rats could inhibit thickening tunica intima-media in the thoracic aorta, although not significantly increasing the catalase expression.

Keywords: Atherosclerosis, bortezomib, catalase, intima-media thickness, proteasome inhibitor

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in developed and developing countries.[1] In 2019, according to the World Health Organization, CVD became the first cause of death in the world, and 17.9 million people have died from coronary heart disease (CHD). About 32% of all global deaths are caused by CHD.[2,3] Coronary heart disease occurs due to the atherosclerosis process.[4] Atherosclerosis is a chronic inflammatory process that causes plaque buildup on the artery walls.[5] This atherosclerosis process occurs gradually over a long time, based on the stages (initiation, progression, and complication stages).[6] Atherosclerosis process can be evaluated from the thickness of blood vessel walls, namely the thickness of tunica intima and tunica media.[7]

Studies have been carried out to obtain appropriate management of atherosclerosis.[4] Proteasome, a subcellular enzyme complex, is a potential therapeutic target for atherosclerosis.[8] The previous research by Ismawati et al.[9] revealed an increase in proteasome expression in blood vessels at every stage of atherosclerosis, and the highest growth occurs at the progression stage. The effects and mechanisms of proteasome inhibitors' action that inhibits proteasome are essential and interesting to be analyzed in atherosclerosis.[8] Bortezomib, the first developed proteasome inhibitor, has been used for cancer therapy since 2003.[10] The bortezomib dose of 50 μg/kgBW for six weeks in low-density lipoprotein (LDL) receptor (LDLR−/−) deficient mice suppresses the formation of the early atherosclerotic lesion.[11] Different results are obtained in advanced atherosclerosis studies using the same dose and experimental animals but do not show therapeutic effects.[12]

Several studies have shown the antioxidative effect of low-dose proteasome inhibitors in

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Correspondence: Dr. dr. Ismawati, M.Biomed. Department of Biochemistry, Faculty of Medicine, Universitas Riau. Jln. Diponegoro No. 1, Pekanbaru 28133, Riau, Indonesia. E-mail: ismawati75@yahoo.com

inhibiting atherosclerosis, but none are based explicitly on the stages of atherosclerosis. For example, Ludwig et al.[13] suggested that the administration of low doses of a proteasome inhibitor in rats induced by hypertension could reduce the levels of superoxide and malondialdehyde (MDA). Similarly, the study by Li et al.[14] also showed that proteasome inhibitors could protect vascular cells against oxidative stress by increasing the expressions of antioxidants such as superoxide dismutase (SOD) and catalase. Catalase, a cellular antioxidant that plays a vital role in overcoming oxidative stress, catalyzes the conversion of hydrogen peroxide to oxygen and water ($2H_2O_2 \rightarrow 2H_2O+O_2$).[15] Mammals, including humans and mice, express catalase in every tissue, and the highest concentrations of catalase are found in the liver, kidneys, and erythrocytes.[16] However, the protective role of catalase in atherosclerosis still requires further research.

This study aimed to analyze the effects of proteasome inhibitors on catalase expression and intima-media thickness (IMT) in the thoracic aorta of atherosclerotic rats. It is essential to conduct this study since it will serve as a basis for developing proteasome inhibitors for atherosclerosis therapy.

Methods

It was an experimental study with a post-test-only control group design conducted at the Faculty of Medicine, Universitas Riau, in Juni–November 2021. This study used 15 male Wistar rats from the Sekolah Tinggi Ilmu Farmasi Riau in Pekanbaru, Indonesia. Some requirements were given to rats to make them homogeneous. They are 10–12 weeks old and weigh ±200 grams. They also have no macroscopic abnormalities.

Rats were placed in cages in rooms with proper ventilation, humidity, and temperature between 20–26°C. All rats were fed with standard feed for one week to be adapted to the environment. Rats were divided randomly into three groups (five rats per group). Group, I was a group of rats given only standard feed, group II was a group of rats induced with vitamin D3 and given an atherogenic diet, and group III was a group of rats also given bortezomib in addition to an atherogenic diet and induced with vitamin D3. The treatment of experimental animals in this research was consistent with the Helsinki Convention. All procedures were approved by the Medical and Health Research Ethics Unit, Faculty of Medicine, Riau University, with letter number B/046/UN19.5.1.1.8/UEPKK/2021.

Atherosclerosis induction was carried out using an atherogenic diet (5% goat fat, 2% cholesterol, 0.2% cholic acid) for four days and a high vitamin D3 (700,000 IU/kg) through gastric intubation on day one.[9] Bortezomib was administered intraperitoneally at 50 μg/kgBW/day.[13]

Thoracic aortic samplings were performed after the rats were anesthetized using ether. The thoracic aorta was then fixed using a formalin buffer. The IMT assessment was performed on hematoxylin-eosin (HE) slides. All slides were observed under a light microscope at 100× magnification. The examination was carried out using an application on the microscope (Leica) at five points, and then the mean value was taken.[17]

Expression of catalase in the aorta was assessed by an immunohistochemical technique based on the procedure (ABclonal, MA, USA). The primary antibody was the catalase monoclonal antibody (A11220, ABclonal, MA, USA). Phosphate-buffered saline (PBS) was used as a negative control. Seven 2D images at 400× magnification were taken on each preparation using a microscope camera (Leica). Adobe Photoshop CS3 software was used to evaluate the percentage of area and intensity. The percentage area showed the breadth of expression, and the intensity showed the concentration.[18]

Statistical analyses for the intensity and percentage of catalase area and IMT were done using the ANOVA test. For IMT, it was followed by post hoc analysis using LSD. The p value below 0.05 is statistically significant.

Results

IMT measurement results show that the highest IMT was in the atherosclerosis group (II), while the lowest was in the control group (I). Administration of bortezomib 50 μg/kgBW/day in rats induced by atherosclerosis can inhibit the thickening tunica intima-media, which was statistically significant (Table 1).

Microscopic examination in the atherosclerosis group (II) showed thickening tunica intima-media, but the atherosclerosis group given bortezomib (III) almost had the same thickness of tunica intima-media as the control group (I). In addition, the atherosclerosis-induced group

Table 1 Intima-Media Thickness (IMT) in Groups at the End of the Study

Replication	IMT of Groups (mm)			p*
	I	II	III	
1	0.0700	0.1070	0.0820	0.001
2	0.0762	0.0950	0.0790	
3	0.0670	0.0920	0.0640	
4	0.0818	0.1070	0.0799	
5	0.0768	0.0910	0.0640	
Mean±SD	0.0744±0.003	0.0984±0.004[a]	0.0737±0.004[b]	

Note: I: control group; II: atherosclerosis-induced group; III: atherosclerosis-induced group and given bortezomib; *ANOVA test, values are presented as mean±SD; [a]p<0.05 compared to group I; [b]p<0.05 compared to group II

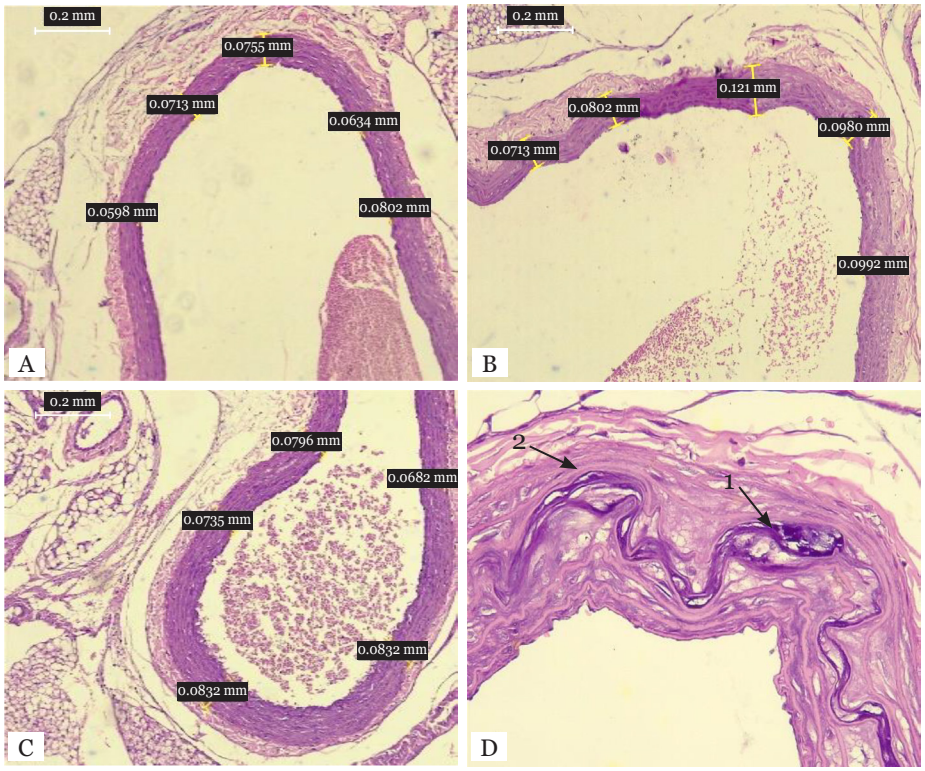

Figure 1 Histopathology of Thoracic Aorta

(**A**) Control group (I); HE, 100× magnification. (**B**) Atherosclerosis group (II); HE, 100×. (**C**) Atherosclerosis+ bortezomib group (III); HE, 100×. (**D**) Atherosclerotic lesions; HE, 400×. 1: calcification. 2: smooth muscle proliferation

revealed vascular smooth muscle cells (VCMC) proliferation and calcification as characteristics of the progression stage.[9] The atherosclerosis group given bortezomib showed milder atherosclerotic lesions (Figure 1).

Catalase expression was seen in all groups in the tunica intima, media, and adventitia. However, there was a difference in the distribution of catalase expression between the control and atherosclerosis groups. The catalase expression in the control group was more concentrated in the tunica adventitia. In contrast, it was more evident in the tunica intima and media in the atherosclerosis group. The distribution of catalase expression in the atherosclerosis group treated with bortezomib was similar to that in the atherosclerosis group (Figure 2).

In this study, catalase expression in the atherosclerosis group was lower than in the control group. This decrease can be seen from

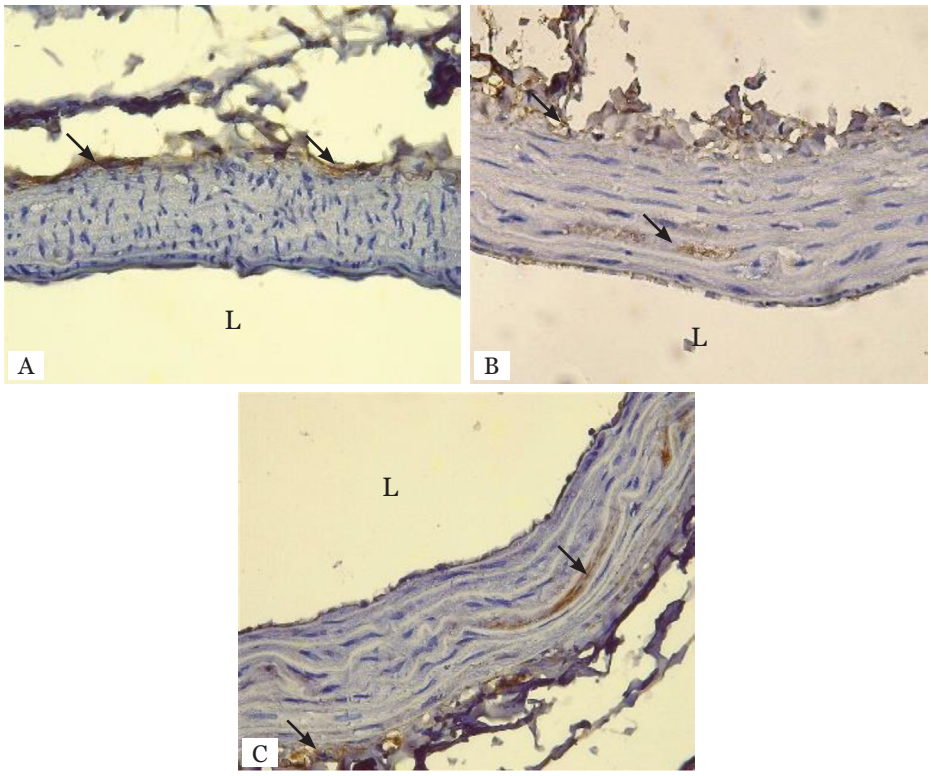

Figure 2 Catalase Expression in the Aorta by Immunohistochemical Examination (Arrow)
HE, 100× magnification. (**A**) Control group (I). (**B**) Atherosclerosis group (II). (**C**) Atherosclerosis+bortezomib group (III). L: lumen side

Table 2 Frequency of Foods Containing Vitamin D that Most Consumed by Respondents

Characteristics	Groups			p*
	I	II	III	
Percentage area (%)	3.09±0.75	1.63±0.32	2.43±0.0.58	0.577
Intensity	86.23±6.31	86.21±11.48	92.26±3.53	0.425
Mean±SD	0.0744±0.003	0.0984±0.004a	0.0737±0.004b	

Note: I: control group; II: atherosclerosis-induced group; III: atherosclerosis-induced group and given bortezomib; *ANOVA test; [a]p<0.05 compared to group I; [b]p<0.05 compared to group II

the difference in the percentage of area in the atherosclerosis group compared to the control group, although the intensity is not different. On the other hand, catalase expression in the atherosclerosis+bortezomib group was higher than in the atherosclerosis group, but this increase was not statistically significant (Table 2).

Discussion

Atherosclerosis induction in this study succeeded in obtaining atherosclerotic lesions at the progression stage with macrophages, foam cells, smooth muscle proliferation, and calcifications. vitamin D3 administration contributes to increasing vascular calcification and stimulating the proliferation of vascular smooth muscle. In addition, the administration of an atherogenic diet, which contains cholesterol and goat fat, increases LDL, and hypercholesterolemia occurs. Further, cholic acid gives a more atherogenic lipoprotein picture by increasing LDL levels and reducing HDL, causing endothelial damage.

The decrease in IMT in the atherosclerosis group given the bortezomib indicated the potential anti-atherosclerosis effect of the proteasome inhibitor. The IMT reflects the atherosclerotic process and predicts cardiovascular events.[7]

This decreasing IMT was also confirmed by histopathological examination that showed a decrease in lesions formed in the atherosclerosis group given bortezomib. A study by Wilck et al.[11] in administering bortezomib 50 µg/kgBW for six weeks in LDLR−/− mice also obtained similar results, suppressing the formation of early atherosclerosis lesions.

Atherosclerosis is initiated by the entry of low-density lipoprotein (LDL) into the artery wall, then this LDL is oxidized and turns into oxidized LDL (oxLDL). The oxidized lipoprotein components induce a local inflammatory response. Further, oxidative stress occurs as the effect of oxLDL on endothelial cells, characterized by increasing pro-oxidant enzymes (NADPH oxidase, xanthine oxidase) and decreasing antioxidant system (superoxide dismutase, catalase, glutathione peroxidase).[19]

As a result, significant differences did not occur in catalase expression in atherosclerotic rats compared to the control group. This result is different from an in vitro study, which obtained an increase in catalase expression in cells exposed to oxLDL.[20] This difference is probably because, in an in vitro study, it occurred at the initiation of atherosclerosis, whereas in this study, atherosclerosis was in the stage of atherosclerosis progression. Research on transgenic mice showed increased expression of catalase associated with reduced cardiac pathology, reduced oxidative stress, and increased life expectancy.[21] Oxidative stress and damage in advanced stages of atherosclerosis are some of the causes of antioxidant failure. Research on CHD patients showed a decrease in the concentration of the antioxidant glutathione (GSH).[22]

Only a few studies are analyzing the antioxidant effect of proteasome inhibitors, especially bortezomib.[23] In this study, the administration of proteasome inhibitors to atherosclerosis-induced rats increased catalase expression, although it was not statistically significant. It is different from a survey by Dreger et al.[23] that in vitro administration of proteasome inhibitor MG132 to myocytes significantly increased catalase expression.This difference may be due to differences in type, method of administration of proteasome inhibitors (in vitro vs in vivo), and the stage of atherosclerosis. In addition, the measurement of catalase expression in this study also has limitations in assessing the function of catalase as an enzyme to assess its activity. Measurement of enzyme expression or concentration will also measure the inactive enzymes.[15]

The difference in catalase expression in this study is not statistically significant. However, the difference in the distribution of catalase expression in the atherosclerosis group, the atherosclerotic group was given bortezomib, and the control group indicates a possible role of catalase in overcoming oxidative stress. In addition, this study found that in the control group, catalase expression was dominant in the tunica adventitia. In contrast, catalase expressions were prevalent in the tunica intima and tunica media in the other two groups.

The anti-atherosclerosis effect of a proteasome inhibitor in this study might not go through the antioxidative pathway but through the anti-inflammatory course. A survey by Wilck et al.[11] showed that bortezomib had a decreasing effect on plasma MCP-1 and IL-6 through an anti-inflammatory mechanism, namely a decreasing VCAM-1 expression in mice in the early stages of atherosclerosis.[12] Another possibility is the effect of bortezomib on increasing catalase or other antioxidants in other tissues, which also plays a role in overcoming oxidative stress at this stage of atherosclerosis. The highest concentrations of catalase are found in the liver, kidneys, and erythrocytes.[16]

Conclusions

In short, administering bortezomib 50 µg/kgBW for four days in rats induced by atherosclerosis could inhibit the thickness of the tunica intima-media. However, no change was in catalase expression. Therefore, further research is needed to analyze the expression of antioxidant catalase in other organs, such as the liver and kidneys.

Conflict of Interest

We have no conflict of interest.

Acknowledgment

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