

Effect of Proteasome Inhibitor on Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Adhesion Molecule-1 (ICAM-1) Expressions in Rat Model of Atherosclerosis

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Abstract

Background: The effect of proteasome inhibitors on atherosclerosis is known to vary depending on the atherosclerosis stage. Previous studies have shown that the highest proteasome expression in atherosclerotic lesions is at the progression stage. Adhesion molecules play a role in the progression stage of atherosclerosis, but no studies have analyzed the effect of proteasome inhibitors on the expression of adhesion molecules at this stage.

Methods: This experimental study aimed to analyze the effect of a proteasome inhibitor, namely bortezomib, on the vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expressions in blood vessels of rat model of atherosclerosis at the progression stage. This study used 18 male Wistar rats divided into three groups, i.e. group I that is the control group given standard feed, group II induced by atherosclerosis, and group III induced by atherosclerosis and given bortezomib. Atherosclerosis induction was performed using vitamin D3 (700,000 IU/kg) orally by gastric intubation on the 1st day and atherogenic feed given for four days. Bortezomib 50 µg/kgBW/day was administered intra-peritoneally. The expression of VCAM-1 and ICAM-1 molecules was measured using immunohistochemistry and analyzed quantitatively using Adobe Photoshop software.

Results: The statistical test showed differences in VCAM-1 expression between atherosclerosis + Bortezomib group and atherosclerosis group, but there were no differences in the expression of ICAM-1 and atherosclerotic lesions between the groups.

Conclusions: Administration of bortezomib 50µg/kg for four days in progressive atherosclerosis model rats can inhibit VCAM-1 expression, although it does not affect ICAM-1 expression and cannot inhibit atherosclerotic lesion formation.

Keywords: Atherosclerosis, Bortezomib, Proteasome, VCAM-1, ICAM-1.

Introduction

Cardiovascular diseases (CDVs), consisting of coronary heart disease and cerebrovascular disease (particularly ischemic stroke), are currently a global health problem. Coronary heart disease and stroke, correspondingly, are the main (84.5%) and third (28.2%) causes of death in the world (1). Cardiovascular diseases are caused mainly by atherosclerosis, which is an

inflammatory process mostly occurring in medium-sized arteries, such as the coronary arteries, carotid arteries, thoracic aorta, and abdominal aorta. Atherosclerosis is a process that happens over a prolonged time and gradually so that the patients were unaware until later clinical manifestations, such as unstable angina, acute myocardial infarction, or sudden death, occur.

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The atherosclerosis stage begins with the initiation stage, then the progression stage, and ends with the complication stage. The clinical symptoms will arise in the complication stage (2).

Various studies have shown that atherosclerosis is preceded by an injury in the endothelium or a low-density lipoproteins (LDL) accumulation on the artery walls that subsequently undergo oxidation or modification (3,4). Endothelial injury is characterized by inflammation that activates the upregulation of adhesion cell molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and selectin. These molecules facilitate the monocytes attachment to endothelial cells, and afterwards, the monocytes will migrate (5,6).

Proteasome, a subcellular particle that plays a role in every stage of atherosclerosis, is involved in various vascular biological processes and pathologies, such as oxidative stress, inflammation, foam cell formation, and apoptosis (7). Proteasomes become a factor in NF-kb activation, a transcription factor that plays a role in regulating the expression of approximately 400 genes, including various enzymes (COX-2 and iNOS), cytokines (TNF, IL-1, IL-6, IL-8, and chemokines), adhesion molecules, such as VCAM-1, ICAM-1, cell cycle regulatory molecules, and angiogenic factors (8). A study by Ismawati *et al.* (2016) found increases in proteasome expression in blood vessels at each stage of atherosclerosis, and the highest occurred at the progression stage (9).

Various studies have been conducted to obtain an ideal atherosclerosis therapy. Atherosclerosis management should focus on anti-inflammatory properties; however, statins and aspirin have been used as atherosclerosis therapy but do not work precisely as an anti-inflammatory (10). Besides, severe side effects, such as gastric swelling and pain as well as high medical costs, urge the need to develop better therapies (11).

Proteasomes are potential targets for atherosclerosis therapy. Several studies have been conducted to determine the effect of proteasome inhibitors on atherosclerosis. Bortezomib is a proteasome inhibitor that was

first developed and has been used for cancer therapy since 2003 (12,13). Administration of a proteasome inhibitor at the initiation stage of atherosclerosis is known to suppress atherosclerotic lesions; such as a study by Wilck *et al.* (2012) that found administration of bortezomib 50 µg/kg for six weeks reduced VCAM-1 expression and suppressed the formation of early atherosclerotic lesions in LDLR *-/-* mice (14). A study by Ludwig (2009) found that proteasome inhibitors, MG132 and MG262, can inhibit TNF-α in inducing the expression of VCAM-1 and ICAM-1 proteins invitro (15). However, no study analyzes the effect of proteasome inhibitors on the expression of adhesion molecules at the progression stage of atherosclerosis. Therefore, this study aims to analyze the effect of proteasome inhibitor administration on the expression of VCAM-1 and ICAM-1 adhesion molecules in the blood vessels of progressive atherosclerosis model rats. This research is important as a basis of a proteasome inhibitor development as atherosclerosis therapy.

Materials and Methods

Research Design

This research is an experimental research design with posttest only with control. A total of 18 male Wistar rats aged 2-3 months were divided into three groups. Group I was the control group given only standard feed, group II was induced by atherosclerosis, and group III was induced by atherosclerosis and given bortezomib.

Treatment of experimental animals

The treatment of experimental animals followed the Helsinki convention and has obtained ethical approval from the Medical and Health Research Ethics Committee, the Faculty of Medicine, Riau University through the Ethic Approval number B/029/UN.19.5.1.1.8/UEPKK/2020. The rats were kept in cages placed in a well-ventilated room and given wood shavings to absorb rat droppings. The base of the cage was changed twice a week. Food and drinks were provided *ad libitum*, checked, and added daily. Atherosclerosis induction was performed by administering vitamin D3 (700,000 IU / kg)

orally through gastric intubation on day one and atherogenic feed (2% cholesterol, 5% goat fat, 0.2% cholic acid) for four days (9). Bortezomib 50 µg/kgBW/day was given intra-peritoneally on the 1st day and the 3rd day.

Atherosclerosis assessment

After four days of treatment, blood and aorta were taken. Rats were formerly anesthetized using ether. The thoracic aorta was taken and stored in a plastic pot containing a formalin buffer for further histopathological examination using hematoxylin-eosin (HE) staining. The assessment was carried out by an anatomical pathologist with a light microscope at 9 fields of view at 400 x magnification. Each field of view was assessed by scoring as follows: 0: intact; 1: macrophages, foam cell formation; 2: smooth muscle proliferation, medial lipid infiltration and fibrosis or calcification; 3: surface defect (fissure, ulceration, hematoma, or thrombus). The scores of each microscopic slide were summed and averaged (9,16).

Immunohistochemical examination

VCAM-1 and ICAM-1 expressions in the aorta of white rats were assessed through an immunohistochemical examination using polyclonal antibodies VCAM-1 (Fine Biotech Co., Ltd, Wuhan, China) and ICAM-1 (Fine Biotech Co., Ltd, Wuhan, China). All preparations were examined with a light

microscope at 400x magnification, and 2D images were taken using a microscope camera (Leica) as many as 9 images each preparation. The expressions of VCAM-1 and ICAM-1 aortic tissues were assessed in terms of area percentage and intensity using Adobe Photoshop CS3 software. The percentage of the area shows the extent of expression, and the intensity describes the concentration (17).

Statistical analysis

Statistical analysis for atherosclerotic lesions and VCAM-1 intensity were using the Kruskal Wallis test and the Mann Whitney test for post hoc analysis. The differences in the mean of percentage of VCAM-1 areas between groups were analyzed using the ANOVA test and the LSD for post hoc analysis. The differences in percentage area and intensity of ICAM-1 expression were analyzed using the ANOVA test. The p-value lower than 0.05 was statistically significant.

Results

Effects of bortezomib on atherosclerosis

The results showed that the highest atherosclerosis score was in the atherosclerosis group, while the lowest was in the control group. Administration of bortezomib 50 µg/kgBW/day on day 1 and day 3 can reduce the formation of atherosclerosis lesions, although not statistically significant (Table 1).

Table 1. Mean score of aortic histopathological features.

Repetition	I (control) (n= 6)	II (atero) (n= 6)	III (atero+bort) (n= 6)	p value
1	0.00	2.00	0.33	0.004
2	0.11	0.67	0.33	
3	0.22	2.00	0.00	
4	0.00	2.00	2.00	
5	0.00	0.89	0.22	
6	0.00	0.67	0.78	
Mean±SE	0.06±0.04	1.37±0.3 ^a	0.61±0.3	

*a = p <0.05 compared to group I, using Kruskal Wallis test and the Mann Whitney test for post hoc analysis.

The distribution of atherosclerotic lesions in the thoracic aortic study showed that most (66.67%) of the control group (group 1) had a score of 0 (normal) (Table 1). There were clear vascular structures in normal conditions, and the boundaries between the intima, media, and adventitia were clear. The internal elastic

membrane and smooth muscle cells were neatly arranged and parallel to the internal elastic membrane (Fig. 1A). The atherosclerosis-induced group (group 2) were mostly in the progression stage (Table 1), marked by the manifestation of foam cells, calcification, proliferation, and disruption of smooth muscle fibers and

connective tissue proliferation (fibrosis) (Fig. 1D). However, these lesions were not found in all field of view. Most of the groups induced by atherosclerosis and given bortezomib were in the initiation stage marked by the presence of foam cells.

VCAM-1 Expression

In this study, VCAM-1 expressions were increasing in the atherosclerosis group compared

to the control group. VCAM-1 expressions were seen on the endothelium, intima, and tunica media. This increase was seen from the differences in the area percentage in the atherosclerosis group compared to the control, although the intensity was similar. VCAM-1 expression in the atherosclerosis + bortezomib group was lower than that in the atherosclerosis group (Table 2, Fig. 2), and this decrease was statistically significant.

Table 2. VCAM-1 expression in the treatment groups.

Characteristic	I (control) (n= 6)	II (atero) (n= 6)	III (atero+bort) (n= 6)	p value
Area percentage (%)	5.0±1.9	16.9±1.8 ^a	11.8±1.4 ^{a,b}	0.001*
Intensity	34.8±10.5	56.5±6.3	56.9±1.6	0.169**

Values are means±SE. *using ANOVA test, **using Kruskal Wallis test. ^ap< 0.05 vs normal, ^bp< 0.05 vs atherosclerosis.

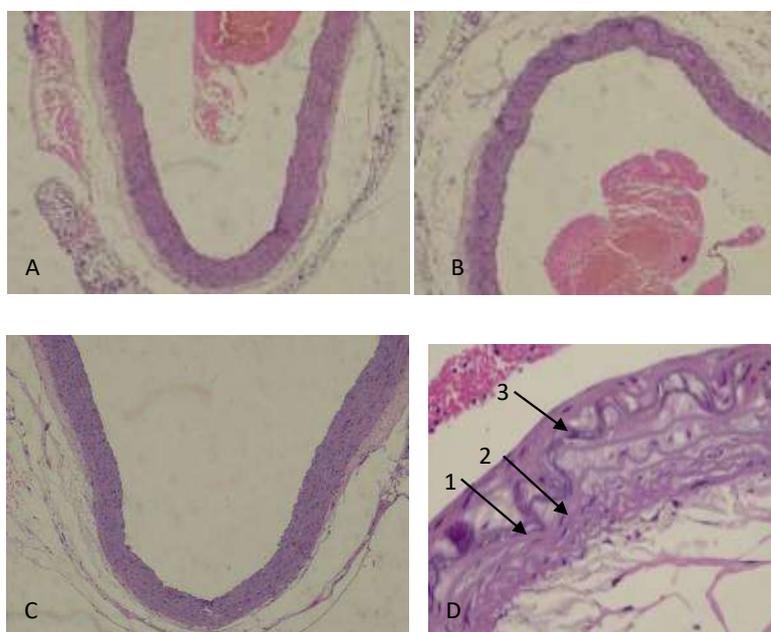


Fig. 1. Histopathological image of aorta of rats (HE staining, A, B, C 100x, D 400x).

A: control group; B and D: atherosclerosis groups; C: atherosclerosis + bortezomib group
1: foam cells; 2: fibrosis; 3: calcification. L; lumen side.

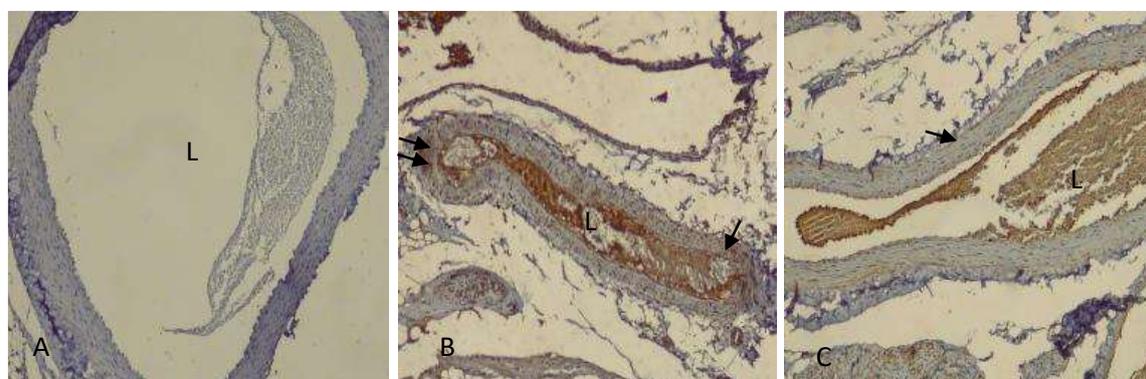


Fig. 2. VCAM-1 expression in the aorta by immunohistochemical examination.

A: Group I (control group); B: Group II (atherosclerosis group); C: Group III (atherosclerosis + Bortezomib group); L: lumen side. Black arrow: VCAM-1 expression (brown color).

ICAM-1 expression

ICAM-1 expressions were increasing in the atherosclerosis group compared to the control group, that there was a difference in the area percentage. There were no differences in

ICAM-1 expressions between the atherosclerosis group given Bortezomib and the atherosclerosis group (Table 3). Figure 3 shows that ICAM-1 is expressed on the endothelium, intima, and media.

Table 3. ICAM-1 expression in the treatment groups.

Characteristic	I (control) (n=6)	II (atero) (n=6)	III (atero+bort) (n=6)	p value
Area percentage (%)	3.94±1.3	8.33±0.9 ^a	4.99±1,1	0.036
Intensity	56.23±4.6	63.21±0.8	66.12±6.9	0.349

Values are means ± SE. using the ANOVA test and the LSD for post hoc analysis. ^ap< 0.05 vs normal.

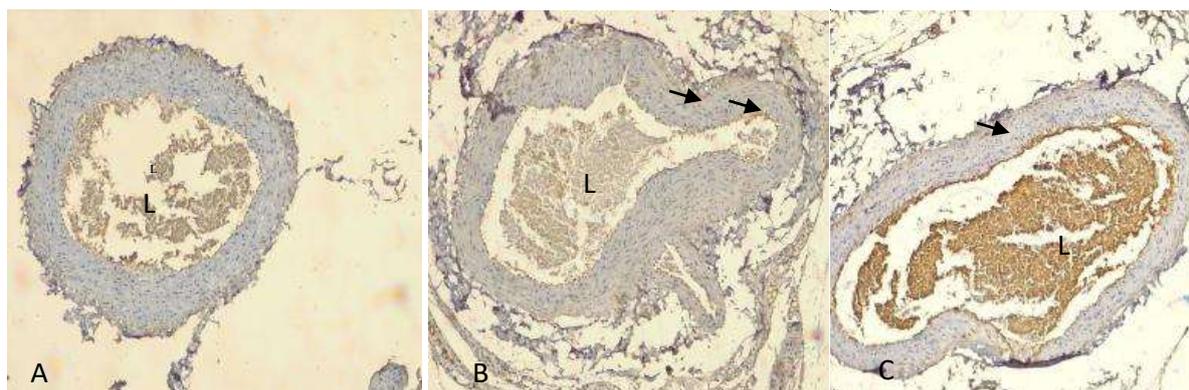


Fig. 3. ICAM-1 expression in the aorta by immunohistochemical examination. A: Group I (control group); B: Group II (atherosclerosis group); C: Group III (atherosclerosis + bortezomib group); L: lumen side. Black arrow: ICAM-1 expression (brown color).

Discussion

In this study, the score of atherosclerotic lesions in the atherosclerosis group given bortezomib was decreasing, although not significant. It shows that the administration of bortezomib 50 µg/kg/day for four days does not suppress the atherosclerotic lesion formation in the atherosclerotic group. This result opposes the research of Wilck et al. (2012) that revealed bortezomib 50 µg/kg administration for six weeks in LDLR -/- mice suppressed the formation of early atherosclerotic lesions (14). However, the research of Wilck et al. (2017) support the results of this research that the administration of bortezomib dose of 50 µg/kg for six weeks in LDLR -/- mice with advanced atherosclerotic lesions (complication stage) did not provide a therapeutic effect (18). The use of proteasome inhibitors can have varying results depending on the dose, administration duration, and the atherosclerosis stage since this study used the same dose of bortezomib (50 µg/kg), but the administration was different (for four

days), and most of the experimental animals were in the progression stage of atherosclerosis. The progression stage of atherosclerosis is characterized by the formation of a foam cell layer and the proliferation of smooth muscle cells (9,19). The same inhibitor dose, when used at different stages of atherosclerosis, will give different results. The administration of a low dose of proteasome inhibitor will have a beneficial effect, but this effect will be lost if the dose is increased (20). Increased proteasome activity resulted in reduced eNOS activity and increased NFκβ activity, but it may also have a protective effect to destroy damaged proteins. Therefore, total inhibition of proteasome function can have a detrimental impact. A dose of 50 µg/kgBW is known to inhibit proteasome function partially.

The VCAM-1 and ICAM-1 adhesion molecules have the same structure and function. Their expression is regulated by the activation of NF-κB. The active form of NF-

kB is a heterodimer complex consisting of a P50 subunit (NF- κ B1) and a P65 subunit (RelA) (21). Under basal conditions, p65 is bound by I κ B α . When there is stimulation by pro-inflammatory cytokines, I κ B α will be drained by the proteasome and release p65. Further, p65 and p50 will bind to certain gene promoters, including ICAM-1 and VCAM-1 (22).

The administration of bortezomib 50 μ g/kgBW on day 1 and 3 for four days in this study can suppress the extent of VCAM-1 expression. This result is in line with the research of Wilck (2012), which found a decrease in VCAM-1 expression in LDLR $-/-$ mice by administering bortezomib 50 μ g/kg for six weeks in the early stages of atherosclerosis. The study of Ludwig *et al.* (2009) also showed a decrease in VCAM-1 expression by administering the same dose of bortezomib for eight weeks in hypertension-induced rats (15). There are several possible mechanisms for bortezomib to decrease VCAM-1 expression. The antioxidative ability of bortezomib is the underlying mechanism for inhibition of VCAM-1 expression in hypertension-induced mice. It is related to the ability of bortezomib to reduce serum thiobarbituric acid reactive substances (TBARS) and aortic superoxide levels (15). Research by Wilck *et al.* proposed an anti-inflammatory effect of bortezomib that reduces VCAM-1 expression in atherosclerosis model mice, seen from decreasing plasma MCP-1 and IL-6 (14).

In contrast to VCAM-1, ICAM-1 expression in this study was not affected by bortezomib administration. Research by Ludwig (2009) found that the proteasome inhibitors MG132 and MG262 can inhibit TNF- α in inducing VCAM-1 and ICAM-1 protein expression *in vitro* in vascular cells, but this inhibition is more noticeable in VCAM-1 (15). These differences in results may be due to differences in the type of proteasome inhibitor used and the type of study, *in vitro* versus *in vivo*. However, there is one similarity that the proteasome inhibitor

effect is more prominent in VCAM-1 than ICAM-1.

The administration of bortezomib 50 μ g/kgBW on day 1 and 3 for four days in this study could not prevent the formation of atherosclerotic lesions, possibly because bortezomib can only inhibit the expression of VCAM-1, but not ICAM-1. It is probably because most of the experimental animals in the atherosclerosis group are in the progression stage, and the proteasome expression at the progression stage is higher than the atherosclerosis initiation stage so that a proteasome inhibitor with a higher or longer dose is needed. If the dose of proteasome inhibitor given is higher, it is likely to cause adverse effects; so, another option is to provide a lower dose of proteasome inhibitor for a more extended time. The atherosclerosis model in this study uses atherosclerosis induction for four days for the progression stage, and the progression stage atherosclerosis model is still obtained if the treatment is continued for less than two weeks (9). Another possibility is that there are other mechanisms besides the proteasome that regulate the activation of ICAM-1, such as autophagy which plays a role in the activation of VCAM-1 via slow-phase I κ B α degradation (22).

In short, administration of Bortezomib 50 μ g/kg for four days in progressive atherosclerosis model rats can inhibit VCAM-1 expression even though it does not affect ICAM-1 expression and has not been able to inhibit the formation of atherosclerotic lesions. The results of this study are essential for the development of bortezomib as a therapeutic agent in atherosclerosis, although further research is needed.

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Authors have no conflict of interest.

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