ORIGINAL ARTICLE

Bali Medical Journal (*Bali MedJ*) 2022, Volume 11, Number 1: 391-396
P-ISSN.2089-1180, E-ISSN: 2302-2914

Effect of proteasome inhibitor on serum 8-OHdG and aortic SOD2 in a rat model of atherosclerosis

Ismawati[1*], Ilhami Romus[2], Mukhyarjon[1], Jihan Salsabilqis[3], Nadia Wulandari[3]

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

*Corresponding author:
Ismawati;
Department of Biochemistry, Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia;
ismawati75@yahoo.com

Received: 2022-01-18
Accepted: 2022-04-20
Published: 2022-04-30

ABSTRACT

Background: Numerous studies have been performed to analyze the effect of proteasome inhibitors on atherosclerosis. However, there is still controversy and the mechanism of action of proteasome inhibitors is still not clearly understood. This study aimed to analyze the effect of proteasome inhibitor on 8-hydroxy-2'-deoxyguanosine (8-OHdG) level in the serum of atherosclerotic rats and the antioxidant expression of Superoxide Dismutase 2 (SOD2) in the aorta.
Methods: The sample was 18 rats with the strain Wistar rats aged 2-3 months. The sample was grouped into normal (N) group got a standard feed, A1 induced by atherosclerosis, and A2 induced by atherosclerosis and given proteasome inhibitor. The proteasome inhibitor was a bortezomib dose of 50 µg/kg BW/day given on days one and three. After four days of treatment, rats were sacrificed, and the aorta removal was done for analyzing the tunica intima-media thickness (IMT) and SOD2 expression assessment using immunohistochemistry, and serum 8-OHdG measurement was done using the ELISA method. SOD2 expression assessment was carried out quantitatively using Adobe Photoshop.
Results: We established a decrease in IMT in the A2 group compared to the A1 group and an enhancement of SOD2 expression and a decrease in 8-OHdG levels in the A2 group compared to the A1 group, although not statistically significant.
Conclusion: In our findings showed bortezomib can prevent thickening tunica intima-media in the aorta, although does not reduce serum 8-OHdG levels and did not significantly increase SOD2 expression in the aorta.

Keywords: atherosclerosis, bortezomib, 8-OHdG, proteasome, SOD-2.
Cite This Article: Ismawati., Romus, I., Mukhyarjon., Salsabilqis, J., Wulandari, N. 2022. Effect of proteasome inhibitor on serum 8-OHdG and aortic SOD2 in a rat model of atherosclerosis. *Bali Medical Journal* 11(1): 391-396. DOI: 10.15562/bmj.v11i1.3126

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in the first half of the century 20th in the world[1] and has become the main cause of death worldwide. Coronary heart disease occurs due to narrowing or blockage of coronary blood vessels in the heart resulting from atherosclerosis.[2] Atherosclerosis sometimes leaves serious sequelae that impact the quality of life.[3] Clinical manifestations of CHD may vary, ranging from stable angina, unstable angina, and myocardial infarction, to sudden death.[2]

Atherosclerosis is an inflammatory process in the arteries that occurs slowly and gradually. This process is marked by fat deposits, leukocyte accumulation, the formation of form cell, migration, smooth muscle cells proliferation, and extracellular matrix deposits that makes blood vessels thicker and stiffer.[4] The atherosclerosis process occurs for a long duration, gradually, and based on the stages of occurrence that are divided into the initiation, progression, and complication stages.[5] It can be assessed by the intima-media thickness (IMT).[6]

The three main mechanisms of atherosclerosis involve are inflammation, proliferation, and apoptosis. Proteasome, a subcellular enzyme complex, plays a role in all three processes.[7] Emerging study suggests that impairment of proteasome function is sufficient to cause several cardiac diseases, such as heart failure, cardiomyopathies, hypertrophy, atrophy, ischemia-reperfusion, and atherosclerosis.[8] Research by Ismawati *et al.* (2016) showed increasing proteasome expression at each stage of atherosclerosis, and the highest escalation occurs at the progression stage.[9] The data about increased proteasome expression in atherosclerosis open the opportunities for developing proteasome inhibitors as a therapy for atherosclerosis.[7]

Proteasome inhibitors are compounds that can inhibit the proteasome pathway. The effect of proteasome inhibitors on atherosclerosis varies that somehow are beneficial and detrimental. It is suspected by reducing the inflammation process due to proteasomal activity. The inflammation process is also known to be the main mechanism in atherosclerosis progression.[10]

This variance may be due to dissimilar organ responses, stages of atherosclerosis, cell type, route of administration, and dose of proteasome inhibitors used against atherosclerosis.[7] Bortezomib has become a proteasome inhibitor first developed and used for cancer therapy in 2003.[11] Administration of the proteasome inhibitor bortezomib 50 µg/kg for six weeks in LDLR -/- mice suppressed the formation of early atherosclerotic lesions.[12] However, administration of the

same dose of bortezomib in LDLR -/- mice with advanced atherosclerotic lesions did not provide a therapeutic effect.[12] Therefore, it is important to analyze the effect of bortezomib at a certain stage of atherosclerosis, in this case, the progression stage.

Oxidative stress contributes to atherosclerosis.[13–16] Cell component damage becomes the source of it. Nowadays, the interaction of the C8 double bond on the guanine base will form 8-hydroxy-2′-deoxyguanosine (8-OHdG). It is a relatively stable product of DNA repair and is associated with various pathological conditions, such as cancer and cardiovascular disease.[17] In addition, 8-OHdG is one of the most widely recognized biomarkers of oxidative damage of DNA in atherosclerosis.[18]

Little research has been done on analyzing the antioxidant effects of proteasome inhibitors, and the results are still varied. Results are beneficial, and some are disadvantageous. Superoxide Dismutase 2 (SOD2) is the body's first defense against superoxide, a product of the electron transport chain. SOD2 deficiency causes mitochondrial dysfunction, increases mitochondrial DNA damage, and accelerates atherosclerosis in ApoE-KO mice.[19] The research conducted by Ludwig *et al.* (2009) obtained the antioxidant effect of low doses of a proteasome inhibitor in reducing superoxide and malondialdehyde (MDA) levels in a rat model of hypertension.[7] In vitro research by Dreger *et al.* also obtained that proteasome inhibitors protect vascular cells against oxidative stress by increasing the antioxidant superoxide dismutase (SOD) expression.[20]

The potential development of proteasome inhibitors as a therapy for atherosclerosis makes the effects of therapy and its mechanism of action very interesting to analyze. However, It is necessary to concern with the dose and stage of atherosclerosis. The current study has been designed to figure out more about the antioxidant effect of bortezomib on serum 8-OHdG concentrations and the expression of aortic superoxide dismutase (SOD) antioxidant in atherosclerotic progression-stage rats. In addition, evaluating the formation of atherosclerosis in certain stages, and the effect of atherosclerosis modality is accurately using the histological feature. This research becomes a basis for the development of proteasome inhibitors for atherosclerosis therapy. Thus, this study aimed to analyze the effect of proteasome inhibitor on 8-hydroxy-2′-deoxyguanosine (8-OHdG) level in the serum of atherosclerotic rats and the antioxidant expression of Superoxide Dismutase 2 (SOD2) in the aorta.

MATERIAL AND METHODS

Research design

This experimental study with a posttest-only control design used 18 male Wistar rats aged 2-to 3 months that were classified into three groups equally. The total sample was using Kemas sampling calculation. The formula has been used was (t-1) (r-1) > 15. The normal group (N) was rats given standard food only, A1 was the group of rats induced by atherosclerosis, and group A2 was the group of rats induced by atherosclerosis and given proteasome inhibitor. For minimizing the bias, all of the samples have been standardized and the treatment of samples in certain groups is similar.

Experimental animal treatment

The treatment of experimental animals in this study followed the Helsinki convention. The rats were kept in proper cages, well ventilated, and kept clean. Food and drink were provided ad libitum, checked, and supplemented daily. Atherosclerosis was induced using an atherogenic diet (2% cholesterol, 5% goat fat, 0.2% cholic acid) and oral administration of vitamin D3 (700,000 IU/kg). Vitamin D3 was given orally on day 1 through gastric intubation. The treatment was given for four days to obtain atherosclerosis in the progressive stage.[9] Bortezomib 50 µg/kg BW/day was administered intraperitoneally on the first and third days.

Intima-Media Thickness (IMT) assessment

Abdominal aortic sampling was performed after the anesthesia process using ether. Abdominal aortic fixation was performed using a formalin buffer. Hematoxylin-eosin (HE) staining was performed for the IMT assessment. The analysis was carried out using a light microscope at 5 points, and the mean value was calculated. The examination was carried out at 100X magnification using an application on the microscope (Leica).[21]

Serum 8-OHdG assessment

Blood was taken from the heart and stored in tubes. The blood was then centrifuged at 3000 rpm to obtain the serum and then stored in a refrigerator at -80 °C until measurement. Serum 8-OHdG concentration was measured using an ELISA kit (E-EL-0028, Elabscience Biotechnology Co., Ltd, Wuhan, China).

Immunohistochemical assessment

SOD2 expression in the aorta was assessed using an immunohistochemical technique following the procedure (ABclonal, MA, USA). The primary antibody used was a polyclonal antibody SOD2 (A1340, ABclonal, MA, USA). For the negative control was using phosphate-buffered saline (PBS).

Images in 2D were taken at 400x magnification, and seven images of each preparation were taken by using a microscope camera (Leica). Further, the percentage of area and intensity were assessed using Adobe Photoshop CS3 software. The percentage area shows the expression breadth, and the intensity describes the concentration.[22]

Statistical analysis

Statistical analyses for SOD2 intensity, percentage of SOD2 area, IMT, and 8-OHdG levels were using the ANOVA test. For the percentages of SOD2 area and IMT, post hoc analysis using LSD was conducted. The p-value below 0.05 was considered statistically significant.

RESULTS

Effects of proteasome inhibitor on IMT

The assessment results showed that the highest IMT was in the atherosclerosis group (A1), while the lowest was in the normal group (N). Administration of bortezomib 50 µg/kg BW/day on the first and third day could reduce the thickening of tunica intima-media, and the reduction was statistically significant (Table 1).

Microscopic examination showed thickening tunica intima-media in the atherosclerosis group (A1). Meanwhile, in the atherosclerosis group given bortezomib, the thickness of the tunica intima-media was nearly the same as the normal group (N). Histopathological examination results in the atherosclerosis-induced groups showed smooth muscle proliferation and calcification. The atherosclerotic group given bortezomib showed milder atherosclerotic lesions (figure 1).

Effect of proteasome inhibitor on 8-OHdG concentration

The mean concentration of serum 8-OHdG in each group was shown in figure 2. The normal group had the lowest mean concentration of serum 8-OHdG, equal to 7.31 ng/mL, and the highest concentration of serum 8-OHdG was in the atherosclerosis group, equal to 10.56 ng/mL. The increasing 8OHdG concentration in the atherosclerosis group was the opposite in the atherosclerotic group given bortezomib, although this difference was not statistically significant.

Table 1. Intima-Media Thickness (IMT) in all groups.

Group	IMT (mm)	p-value
N	0.072±0.002	
A1	0.093±0.003	<0.05 [a]
A2	0.075±0.003	<0.05 [b]

The value is the mean±SD. (a)Compared to group N. (b)Compared to group A1

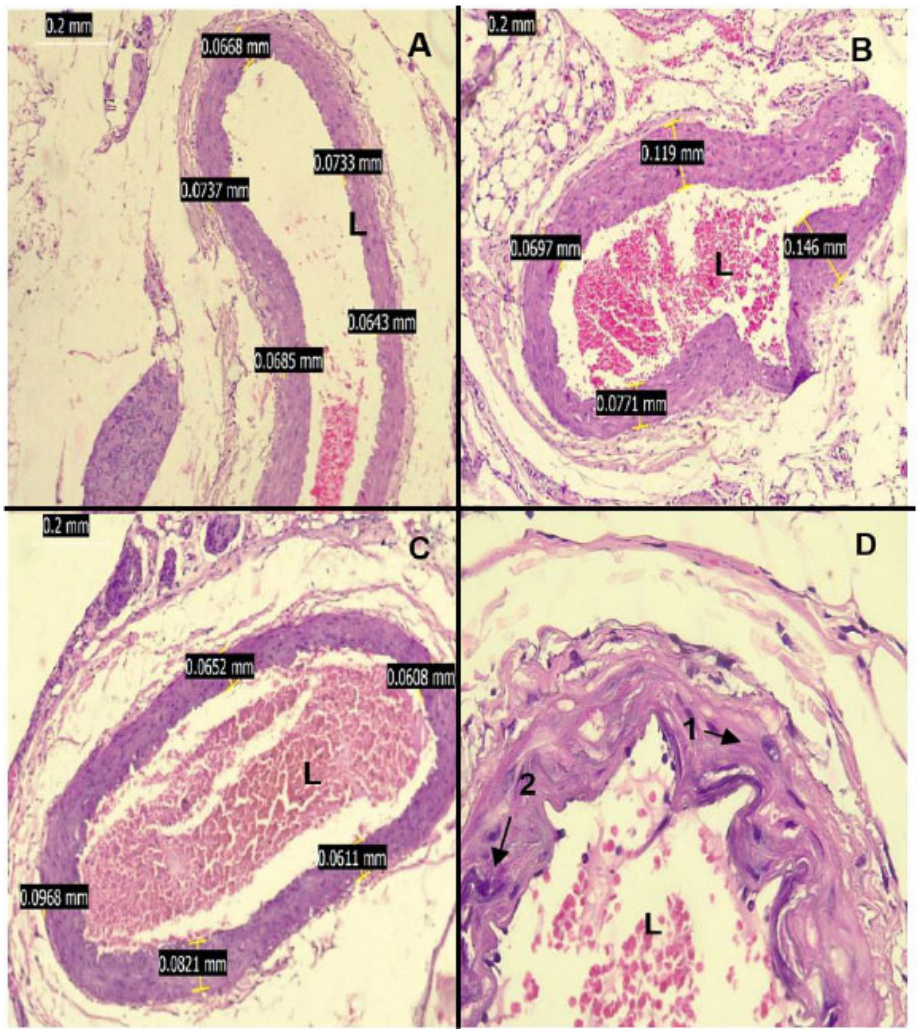

Figure 1. Aortic histopathology, hematoxylin-eosin stain (A,B,C: x100 original magnification, D:x400 original magnification). A: N (normal group); B: A1 (atherosclerosis-induced group); C: A2 (atherosclerosis-induced group and given bortezomib.). 1: smooth muscle proliferation; 2: calcification.

Effect of proteasome inhibitor on SOD2 expression

The decrease in SOD-2 expression was significant in the atherosclerosis group compared to the normal group in this study. This decrease can be seen from the difference in the area percentage in the atherosclerosis group compared to the normal group, although the intensity is similar. The expression of SOD2 in the atherosclerosis+bortezomib group was higher than that in the atherosclerosis group but was not statistically significant (Figure 3).

The expressions of SOD2 were seen in the endothelium, intima, and tunica media in all three groups, but in the atherosclerosis group, it was more concentrated in the tunica media. (Figure 4).

DISCUSSION

The atherosclerosis model in this study had developed and established in the previous study. Induction of atherosclerosis using an atherogenic diet (2% cholesterol, 5% goat fat, 0.2% cholic acid) and oral administration of vitamin D3 (700,000 IU/kg) for four days succeeded in obtaining atherosclerotic lesion at the progression stage.[9] In the current study, histopathological examination results in the atherosclerosis-induced groups showed smooth muscle proliferation and calcification which are the characteristics of the atherosclerosis progression stage.

In this study, a decrease in IMT was found in the atherosclerosis group given proteasome inhibitors, which showed the potential anti-atherosclerosis effect of this proteasome inhibitor. The thickness of the tunica intima-media reflects the atherosclerosis process and is used as a predictor of cardiovascular disease.[6] The histopathological assessment also showed a decreasing lesion formation in the atherosclerotic group treated with bortezomib. Similar to Wilck *et al.*'s study that the formation of early atherogenic

lesions was suppressed by administering the bortezomib 50 μg/kg for 6 weeks in LDLR -/- mice.[12]

The serum 8OHdG concentration in the atherosclerosis group was increasing compared to the normal group, although not statistically significant. This result is different from the results of a study by Xiang *et al.* that serum 8-OHdG concentrations increased in coronary artery disease (CAD) patients, related to the severity of coronary artery stenosis.[23] This difference may be due to the different stages of atherosclerosis studied. This study was still in the early stages of atherosclerosis progression so the increased oxidative stress might still be compensated by various antioxidants available. Oxidative stress depends on increased ROS production and antioxidant defense mechanisms.[16,24]

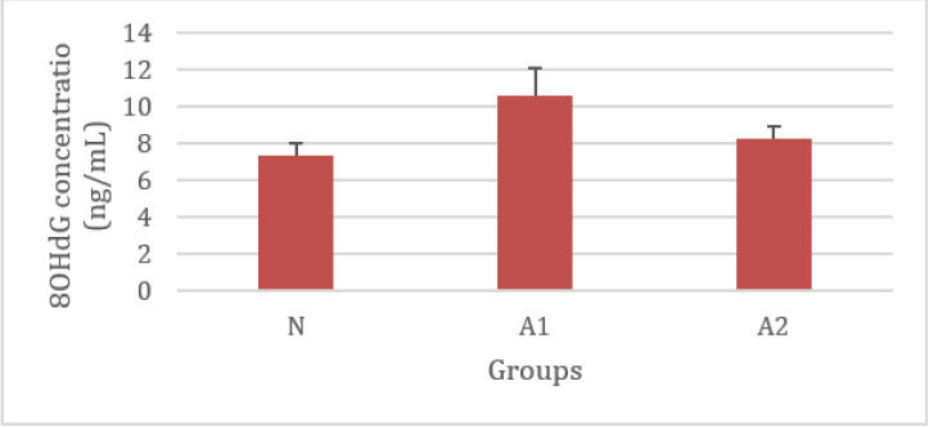

Figure 2. Graph of 8OHdG concentrations in various groups.
Note: N: normal group; A1: atherosclerosis-induced group; A2: atherosclerosis-induced group and given bortezomib.

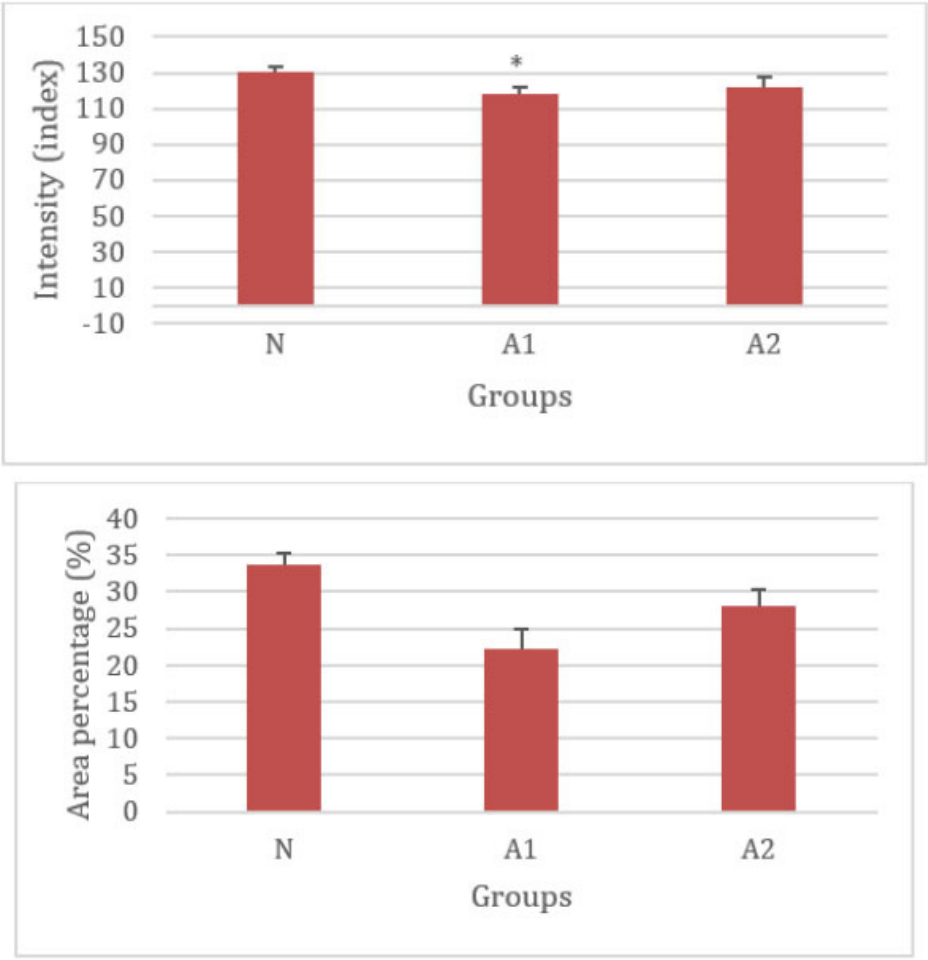

Figure 3. Graph of SOD2 expression in various groups.
Note: N: normal group; A1: atherosclerosis-induced group; A2: atherosclerosis-induced group and given bortezomib.
* = $p < 0.05$ to the N group

Studies analyzing the effect of proteasome inhibitors (bortezomib) on oxidative stress markers, particularly 8-OHdG in atherosclerosis, are still very limited. Research by Wilck *et al.* found that bortezomib administration at a dose of 50 μg/kg Bortezomib in LDLR-/- mice could reduce the formation of superoxide, MDA, and protein carbonyl.[12] Different results in this study showed that the administration of proteasome inhibitor did not significantly reduce the serum 8-OHdG concentration. The differences in results might due to the different animal models used and stages of atherosclerosis.[7]

In this study, there was a decrease in the expression of the antioxidant SOD2 in the atherosclerosis group than in the normal group. These results are in line with studies on ApoE -/- mice that showed an increase in SOD2 followed by a decrease. SOD2 expression increases only in living cells but decreases in apoptotic cells or atherosclerotic lesions.[25] It might be because the increased oxidative stress in atherosclerosis reduces the expression of antioxidants, including superoxide dismutase (SOD).[26]

The administration of bortezomib in atherosclerotic rats in this study has not increased SOD2 expression significantly. Contrary to what was obtained by Dreger *et al.*, proteasome inhibitors could protect vascular cells against oxidative stress by increasing the antioxidant superoxide dismutase (SOD1) expression in vitro. This difference might be due to differences in the type and administration method of proteasome inhibitors. In addition, the measurement of SOD2 expression in the aorta in this study also has limitations

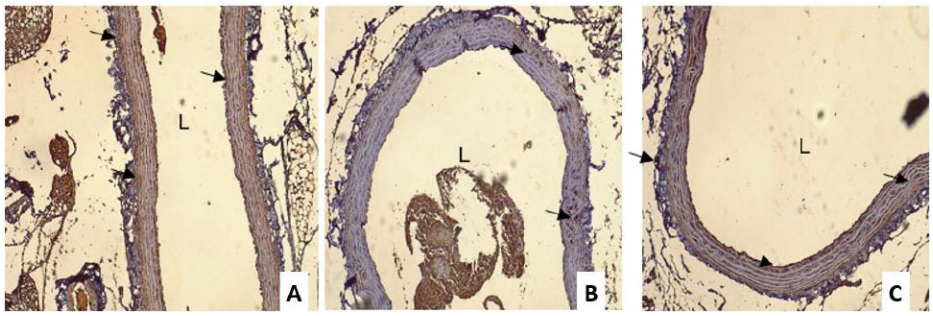

Figure 4. Immunoreactivity of SOD2 in the aorta of rats.
Diaminobenzidine stains the SOD2 brown (black arrows). Slides were counterstained with hematoxylin, x100 original magnification.
Note: A: group N (normal group); B: Group A1 (atherosclerosis group); C: Group A2 (atero+bortezomib group); L: lumen side.

in assessing the function of an enzyme since assessing enzyme activity is more important. In measuring the enzyme expression or concentration, the inactive enzymes are also measured. In cancer, for example, the loss of SIRT will decrease SOD2 activity due to increased acetylation but does not affect the concentration of the SOD2 protein.[16]

Although not statistically significant in intensity and area expression of SOD2, there are differences in the distribution of SOD2 expression between the atherosclerosis group and the atherosclerosis group given bortezomib. This study found that in the atherosclerosis group, SOD2 expression was dominant in tunica media, but in atherosclerosis given bortezomib, SOD2 expression was seen in the endothelium, intima, and tunica media. This indicates a possible role of SOD2 in overcoming atherosclerosis.

This study indicates that the main mechanism of action of bortezomib as an anti-atherosclerosis at the atherosclerosis progression stage is might not through the antioxidative pathway but the anti-inflammatory pathway. A study by Wilck *et al.* showed that bortezomib has an anti-inflammatory effect through a decrease in VCAM-1 expression in atherosclerotic mice, as seen by a decrease in plasma MCP-1 and IL-6.[12] Another possibility is the bortezomib effect on the increase of other antioxidants in blood vessels or other tissues so that the overall role in compensating for the increased oxidative stress at this stage of atherosclerosis.[16] Research in normal mice shows that SOD2 is highly expressed in the heart and muscles.[27]

The limitation of our study is this study was conducted with post-group only, thus we do not know the baseline level of 8-OHdG serum. The stages of atherosclerosis are not involved in variable evaluation in this study, thus we could not know whether there are variations in levels at each stage.

CONCLUSION

Our finding showed that there was no effect on serum 8OHdG concentration and SOD2 expression in the aorta but could suppress the thickening tunica intima-media in the aorta of atherosclerotic rats after administered bortezomib 50 µg/kg for 4 days.

DISCLOSURE

Conflicts Of Interest
None.

Author Contribution
All of the authors are contributed to processing this article.

Ethical Clearance
This study has been approved by the ethical review board for medicine and health research medical faculty of Universitas Riau by the number ethic was B/046/UN19.5.1.1.8/UEPKK/2021.

Fundings
This research was sponsored by Universitas Riau through a scientific research scheme under the contract number 680/UN.19.5.1.3/PT.01.03/2021

REFERENCES

1. Barquera S, Pedroza-Tobías A, Medina C, Hernández-Barrera L, Bibbins-Domingo K, Lozano R, et al. Global Overview of the Epidemiology of Atherosclerotic Cardiovascular Disease. Arch Med Res. 2015;46(5):328–38.
2. Malakar AK, Choudhury D, Halder B, Paul P, Uddin A, Chakraborty S. A review on coronary artery disease, its risk factors, and therapeutics. J Cell Physiol. 2019;234(10):16812–23.
3. Mensah GA, Wei GS, Sorlie PD, Fine LJ, Rosenberg Y, Kaufmann PG, et al. Decline in Cardiovascular Mortality: Possible Causes and Implications. Circ Res [Internet]. 2017 Jan 20;120(2):366–80. Available from: https://pubmed.ncbi.nlm.nih.gov/28104770
4. Robbin basic pathology. 9th ed. 2013.
5. Andreou I, Sun X, Stone PH, Edelman ER, Feinberg MW. miRNAs in atherosclerotic plaque initiation, progression, and rupture. Trends in Molecular Medicine. 2015.
6. Nezu T, Hosomi N, Aoki S, Matsumoto M. Carotid Intima-Media Thickness for Atherosclerosis. :18–31.
7. Wilck N, Ludwig A. Targeting the ubiquitin-proteasome system in atherosclerosis: Status Quo, challenges, and perspectives. Antioxidants and Redox Signaling. 2014.
8. Shukla SK, Rafiq K. Proteasome biology and therapeutics in cardiac diseases. Transl Res. 2019;205:64–76.
9. Ismawati, Oenzil F, Yanwirasti, Yerizel E. Changes in expression of the proteasome in rats at different stages of atherosclerosis. Anat Cell Biol. 2016;49(2):99.
10. Wilck N, Fechner M, Dan C, Stangl V, Stangl K, Ludwig A. The Effect of Low-Dose Proteasome Inhibition on Pre-Existing Atherosclerosis in LDL Receptor-Deficient Mice. Int J Mol Sci [Internet]. 2017 Apr 7;18(4):781. Available from: https://pubmed.ncbi.nlm.nih.gov/28387708
11. Nunes AT, Annunziata CM. Proteasome inhibitors: structure and function. Semin Oncol. 2017;44(6):377–80.
12. Wilck N, Fechner M, Dreger H, Hewing B, Arias A, Meiners S, et al. Attenuation of early atherogenesis in low-density lipoprotein receptor-deficient mice by proteasome inhibition. Arterioscler Thromb Vasc Biol. 2012;32(6):1418–26.
13. Gimbrone MA, García-Cardeña G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. Circ Res. 2016;118(4):620–36.
14. Forman HJ, Zhang H. Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. Nat Rev Drug Discov. 2021;20(9):689–709.
15. Sharifi-Rad M, Anil Kumar N V., Zucca P, Varoni EM, Dini L, Panzarini E, et al. Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases. Front Physiol. 2020;11(July):1–21.
16. Halliwell, Barry; Gutteridge JMC. Free Radicals in Biology and Medicine. 5th ed. Oxford University Press; 2015.

17. Kroese LJ, Scheffer PG. 8-Hydroxy-2′-Deoxyguanosine and Cardiovascular Disease: a Systematic Review. Curr Atheroscler Rep. 2014;16(11):1–8.
18. Di Minno A, Turnu L, Porro B, Squellerio I, Cavalca V, Tremoli E, et al. 8-Hydroxy-2-deoxyguanosine levels and cardiovascular disease: a systematic review and meta-analysis of the literature. Antioxid Redox Signal. 2016;24(10):548–55.
19. Förstermann U, Xia N, Li H. Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. Circ Res. 2017;120(4):713–35.
20. Dreger H, Westphal K, Wilck N, Baumann G, Stangl V, Stangl K, et al. Protection of vascular cells from oxidative stress by proteasome inhibition depends on Nrf2. Cardiovasc Res. 2010 Jan;85(2):395–403.
21. Al-Joufi F, Al-Ani IM, Saxena AK, Talib NA, Mokhtar RH, Ku-Zaifah N. Assessment of the anti-atherosclerotic effect of Eurycoma longifolia extract on high-fat diet model in rats. I: Histological study. Eur J Anat. 2016;20:131–6.
22. Lehr HA, Mankoff DA, Corwin D, Santeusanio G, Gown AM. Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. J Histochem Cytochem. 1997;
23. Xiang F, Shuanglun X, Jingfeng W, Ruqiong N, Yuan Z, Yongqing L, et al. Association of serum 8-hydroxy-2′-deoxyguanosine levels with the presence and severity of coronary artery disease. Coron Artery Dis. 2011;22(4):223–7.
24. Mahat RK, Singh N, Rathore V, Arora M, Yadav T. Cross-sectional correlates of oxidative stress and inflammation with glucose intolerance in prediabetes. Diabetes Metab Syndr Clin Res Rev. 2019;13(1):616–21.
25. Tabas I, Wang Y. Emerging roles of mitochondria ROS in atherosclerotic lesions: causation or association? J Atheroscler Thromb. 2014;23929.
26. Dennis JM, Stocker R. Actions of antioxidants in the protection against atherosclerosis. Free Radical Biology and Medicine. 2012;53:863–84.
27. Kim JM, Kim HG, Son CG. Tissue-specific profiling of oxidative stress-associated transcriptome in a healthy mouse model. Int J Mol Sci. 2018;19(10).