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Effect of Bortezomib on hs-CRP Concentration in the Progression Stage of Atherosclerotic Rats

Ismawati[1]*, Ilhami Romus[2], Mukhyarjon[1], Ariza Julia Paulina[3]

[1]Department of Biochemistry, Faculty of Medicine, University of Riau, Indonesia
[2]Department of Pathology Anatomy Faculty of Medicine, University of Riau, Indonesia
[3]Biomedical Study Program, Faculty of Medicine, University of Riau, Indonesia

Corresponding author:
E-mail:
ismawati75@yahoo.com

ABSTRACT

The mechanism of anti-atherosclerosis of bortezomib is thought to be through its anti-inflammatory. High-sensitivity C-Reactive Protein (hs-CRP) is an inflammation marker and an independent risk factor for atherosclerosis. This study aimed to analyze the effect of bortezomib on serum hs-CRP concentration in the progression stage of atherosclerotic rats. This study was an experimental study with a post-test only with a control design. This study used 15 male Wistar rats divided into three groups; group I was only given standard feed, group II was induced by vitamin D3 and an atherogenic diet, and group III was induced by vitamin D3 and given an atherogenic diet and bortezomib. Bortezomib 50 g/kg BW/day was administered on the first and third days. Serum hs-CRP concentration was measured using the ELISA technique. Statistical analyses to compare serum hs-CRP concentrations between groups were done using the ANOVA test followed by a post-hoc LSD test; $p < 0.05$ was considered statistically significant. The results showed that the induction of atherosclerosis resulted in 100% of atherosclerotic lesions in the progression stage. The highest hs-CRP concentration was found in group II (an average of 66.84 ng/mL) , followed by groups I (an average of 59.32 ng/mL) and III (an average 49.43 ng/mL). The hs-CRP concentration in group III was significantly lower than in group II. It can be concluded that the administration of bortezomib could reduce hs-CRP concentration in the progression stage of atherosclerotic rats.

Keywords: Atherosclerosis, bortezomib, hs-CRP, Progression Stage

Introduction

Atherosclerosis is a process underlying atherosclerotic plaque formation that obstructs blood flow. This process disrupts the transport of oxygen and muscle metabolism products to the heart, resulting in myocardial ischemia (Filla, 2015). Globally, atherosclerosis complication is the leading cause of morbidity and mortality. In the last two decades, some evidence showed that inflammation played a vital role in developing atheroma (Ridker, 2016). Various inflammatory markers have been developed. Compared to other inflammatory markers, CRP is relatively easy and inexpensive for laboratory examinations and offers more stable analytes, availability of commercial tests, standardization, and test precision (Filla, 2015). High-sensitivity C-Reactive Protein (hs-CRP) is a highly sensitive CRP assay developed to detect CRP at deficient levels, between 0.5 - 10.0 mg/L (Filla, 2015; Ridker, 2016; Niknezhad et al., 2020). Hs-CRP is the most significant predictor of the risk of cardiovascular events. CRP value measurement helps determine disease progression or assess the effectiveness of therapy (Filla, 2015).

Proteasomes are enzyme complexes that play a role in the selective degradation of 80-90% of intracellular proteins in eukaryotic cells. Proteasomes are found mainly in two forms,

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constitutive proteasomes, and immunoproteasomes. Constitutive proteasomes are expressed throughout the body and are responsible for degrading proteins in tissues, including the heart, kidneys, and liver. Immunoproteasomes are primarily expressed in immune cells. Proteasome dysfunction has been proven to be associated with tumors, immune diseases, infectious diseases, and others (Xi et al., 2019). Ubiquitin-proteasome system (UPS) dysfunction is also known to be involved in atherosclerosis (Wilck et al., 2017). Therefore, inhibiting proteasome function in atherosclerosis is one of the potential therapeutic targets (Xi et al., 2019).

The inhibition function of proteasome has been used as a therapeutic target in cancer. One type of proteasome inhibitor, bortezomib (Velcade, PS-341), was approved in 2003 by the Food and Drug Administration (FDA) to treat relapsed and multiple refractory myelomas. Other agents, such as carfilzomib (Kyprolis) in 2012 and ixazomib (Ninlaro) in 2015, have also received FDA approval for treating multiple myeloma. Marizomib is used for glioblastoma patients (Nunes & Annunziata, 2017; Kisselev, 2021; Gandolfi et al., 2017). These proteasome inhibitors act mainly on subunit 5, whereas at higher concentrations, proteasome inhibitors also inhibit other subunits, such as β2 and β1 ((Nunes & Annunziata, 2017).

Proteasomes involve various biological processes, such as inflammation, proliferation, and apoptosis, that are essential in atherosclerosis initiation, progression, and complication stages (Shukla & Rafiq, 2019). Contrary to the use of proteasome inhibitors in cancer, the use of proteasome inhibitors in atherosclerosis has unknown effects, some are beneficial, but some are detrimental. Besides the differences in organ response, cell type, method of administration, and dose of proteasome inhibitors, atherosclerosis stages are also very influential (Wilck et al., 2012). Studies on the mechanism of action of proteasome inhibitors in atherosclerosis remain very limited, one of which is the anti-inflammatory effect of proteasome inhibitors. Research conducted by Ismawati et al. showed that administering bortezomib 50 g/kg BW in atherosclerosis rats could reduce fatty liver formation by reducing the inflammatory process by decreasing IL-6 formation (Romus et al., 2022).

Differences in proteasome expressions of tissues at various stages of atherosclerosis open the possibility for elaborating proteasome inhibitors as atherosclerosis therapy specific to atherosclerosis stages (Ismawati et al., 2016). Therefore, the researchers were interested in further analyzing the effect of bortezomib (a proteasome inhibitor) on serum hs-CRP concentrations in rat models of progression-stage atherosclerosis. This research is an essential basis for developing proteasome inhibitors for atherosclerosis therapy.

Material and Methods

Experimental animal treatment

This study has received ethical approval from the ethics committee of the Faculty of Medicine, Riau University with number B/046/UN19.5.1.1.8/UEPKK/2021_Adendum. This experimental study used a post-test-only control group design on 15 male Wistar rats aged ten weeks. Experimental animals were divided into three groups; 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. Group III was a group of rats induced with vitamin D3 and given an atherogenic diet and bortezomib. The rats were kept in cages in a well-ventilated room. Rats were induced with vitamin D3 orally (700,000 IU/kg) on day one by gastric intubation and continued with an atherogenic diet (0.2% cholic acid, 2% cholesterol, 5% goat fat) for four days until obtaining the atherosclerosis progression stage. Bortezomib (50 μg/kg BW/day) was given intraperitoneally (Ludwig et al., 2009).

Atherosclerosis assessment

Coronary arteries and the heart were taken simultaneously, placed in plastic pots containing formaldehyde buffer in 0.1 M phosphate buffer, and fixed for ±24 hours. Atherosclerosis assessment of the coronary arteries was performed using hematoxylin-eosin staining based on a

scoring system. The assessment was carried out on nine fields of view with 400x magnification based on the scores of 0: intact; 1: macrophages, foam cells; 2: medial lipid infiltration, smooth muscle proliferation, calcification/fibrosis; 3: surface defect (ulceration/ fissure/ thrombus/ hematoma). The score taken was the highest value from the nine fields of view ((Ismawati et al., 2016).

Measurement of hs-CRP concentration

Blood taken from the heart was stored in tubes, then centrifuged at around 3000 rpm to gain the serum and stored at -80 °C until the measurement step. Serum hs-CRP concentrations were measured using ELISA kits (WR1048, Wuhan Fine Biotech., Ltd, Wuhan, China).

Statistical analyses

Statistical analyses to compare serum hs-CRP concentrations between groups were done using the ANOVA test followed by a post-hoc LSD test; $p < 0.05$ was considered statistically significant.

Results and Discussion

Histopathological observations on coronary arteries showed that all rats in the group induced by atherosclerosis (group II) were in the progression stage (Table 1). This group showed structural changes in the endothelial layer compared to the standard layer; through a magnification of 100x, atherosclerotic lesions were found, namely calcification of blood vessels (score 2) and proliferation of smooth muscle cells (score 2) (Figure 1). Unlike group I, most samples showed standard layers of tunica intima and tunica media, consisting of layers of smooth muscle cells arranged orderly. Bortezomib 50 µg/kg BW given on day one and day three could reduce the formation of atherosclerotic lesions in group III, only 60% of experimental animals were in the progression stage, and the rest were at the initiation stage (Table 1).

Table 1. Distribution of atherosclerotic lesion scores in all groups

Score	Group I (n=5)	Group II (n=5)	Group III (n=5)
0	4	0	0
1	1	0	2
2	0	5	3
3	0	0	0

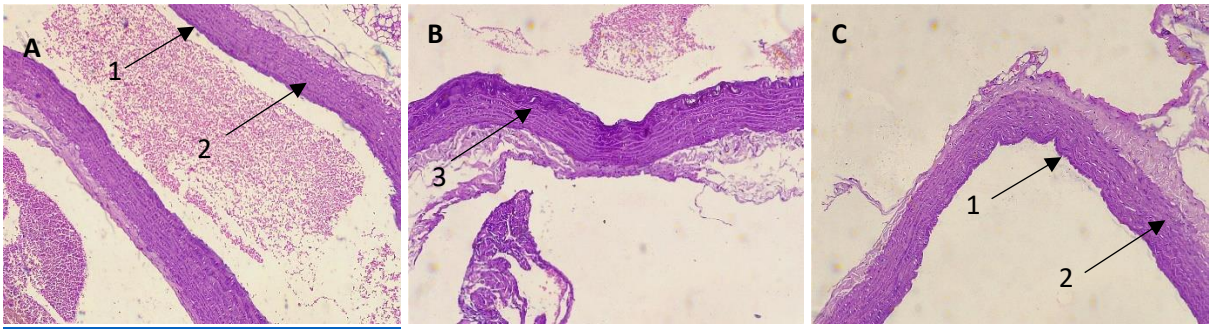
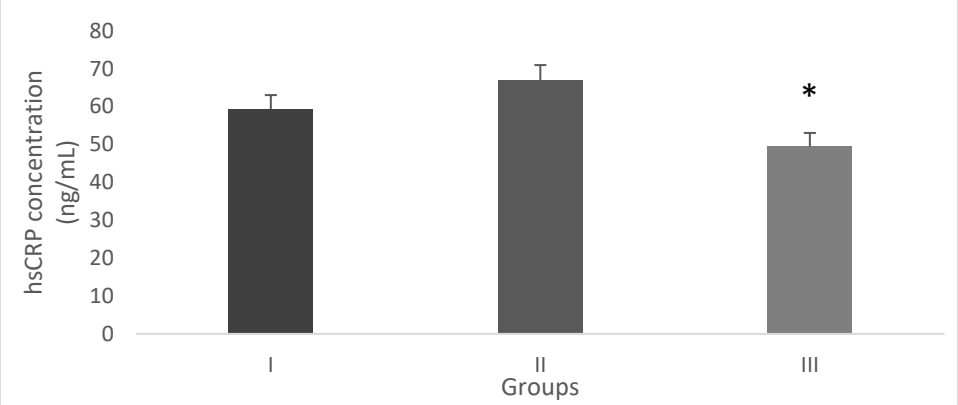


Figure 1. Histopathology of coronary artery (hematoxylin-eosin stain, magnification 100x)
A: group I (standard), B: group II (atherosclerosis), C: group III (atherosclerosis+bortezomib). 1: tunica intima, 2: tunica media, 3: calcification

C-reactive protein (CRP) is an acute-phase protein in humans used to monitor infection, trauma, response to antibiotics, and autoimmune conditions (Nunes & Annunziata, 2017). CRP is synthesized in the liver due to stimulation by IL-6 and other proinflammatory cytokines. CRP

consists of a 23 kDa subunit that plays an essential role in the natural immune response. The level of CRP will increase significantly in the inflammatory process and tissue damage (Filla, 2015). The two main functions of CRP are activating the classical complement pathway and binding to human immunoglobulin receptors with subsequent ligand opsonization for macrophages. CRP contributes to foam cell formation in atherogenesis. Recent studies have found that CRP is produced by vascular smooth muscle cells in coronary artery disease. This CRP production directly causes the release of several mediators in the atherothrombotic process (Blinc et al., 2020).

The results of this study showed that the hs-CRP concentration in group II (an average of 66.84 ng/mL) was higher than in group I (an average of 59.32 ng/mL) (Figure 2). In this study, the hs-CRP concentration in the group of rats induced by atherosclerosis increased compared to the standard group but was not statistically significant (Figure 2). These results were in line with a study by Razban et al. that showed no significant relationship between serum hs-CRP concentrations with severity and coronary artery angiography in patients with stable angina (Razban et al., 2016). In contrast, the results from a study by Habib and Masri show a significant difference in hs-CRP concentration in patients with coronary heart disease compared to normal, and the increase in hs-CRP is related to the degree of severity (Habib & Al-Masri, 2013). Guruprasad et al. argue that increasing hs-CRP concentration increases the risk of coronary heart disease independently (Guruprasad et al., 2012). The absence of a significant difference in hs-CRP concentration in the atherosclerotic group with the standard group in this study is possibly due to the differences in the atherosclerosis stage, which in this study atherosclerosis was still in the progression stage (has not shown any symptoms).



Note: Experimental animals were divided into three groups; group I was a group of rats given 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 induced with vitamin D3 and given an atherogenic diet and bortezomib. * $p<0.05$ vs. group II.

Figure 2. Serum hs-CRP concentration in various groups

In this study, the average hs-CRP concentration in group III was 49.43 ng/mL, which was significantly lower than group II with an average of 66.84 ng/mL (Figure 2). It indicates that administering bortezomib 50 g/kg BW/day could reduce the increasing serum hs-CRP concentrations in atherosclerotic rats. This study is in line with the research by Ismawati et al. that bortezomib could reduce inflammation in atherosclerotic rats by suppressing IL-6 formation. Other studies on the anti-inflammatory effect of proteasome inhibitors have been carried out in malignancy conditions or other inflammatory diseases. Research conducted by Silswal et al. showed that resveratrol as a proteasome inhibitor could be used for therapeutic interventions in chronic inflammatory diseases, such as arthritis, atherosclerosis, sepsis, and neurodegenerative (Silswal & Qureshi, 2017). Selective immunoproteasome inhibitors inhibit cytokine release in vitro and anti-inflammatory activity in vivo (Kisselev, 2021). Selective inhibitors of

immunoproteasome (KZR-616) are being developed for treating autoimmune diseases (Wang et al., 2021).

Conclusion

It can be concluded that administering bortezomib could reduce hs-CRP concentration in the progression stage of atherosclerotic rats. Further research is needed to analyze inflammation in other organs such as the heart, blood vessels, liver, and others.

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