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The reduction of aorta histopathological images through inhibition of reactive oxygen species formation in hypercholesterolemia rattus norvegicus treated with polysaccharide peptide of *Ganoderma lucidum*

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ABSTRACT

Objective(s): Atherosclerosis is chronic inflammatory process triggered by oxidative stress. Oxidative stress can increase hydrogen peroxide (H_2O_2) level, which induce atherosclerosis through the processes such as formation of perivascular adipose tissue (PVAT), foam cells, and atherosclerotic plaque. Antioxidant is needed to control negative effects of oxidative stress. One source of antioxidant, which has potential to be developed, is PsP from *Ganoderma lucidum*. This study aims to prove the effect of PsP in decreasing H_2O_2, PVAT, foam cells and atherosclerotic plaque.

Materials and Methods: This study was experimental randomized post-test with control group design using 25 *Rattus norvegicus* Wistar strain rats. Rats were divided into 5 groups (negative control, positive control, and 3 high-fat diet group with PsP dose: 50, 150, 300 mg/kgBW). Measured parameters were H_2O_2, PVAT, foam cell, and atherosclerotic plaques. Analysis of variance (ANOVA) was used for statistical analysis, followed by *post hoc* test.

Results: Mean H_2O_2 levels, PVAT thickness, foam cell numbers, and atherosclerotic plaque were low in negative control group. ANOVA showed that PsP significantly (P<0.05) reduced H_2O_2 levels, PVAT thickness, foam cells numbers and atherosclerotic plaque width.

Conclusion: PsP dose of 300 mg/kgBW has the most significant effect in decreasing H_2O_2 levels, PVAT thickness, number of foam cells, and atherosclerotic plaque width. Based on the results of this research, PsP can be recommended as antioxidant to control pathogenesis of atherosclerosis.

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Introduction

Atherosclerosis is the major cause of cardiovascular disease (CVD) (1). CVD is the leading cause of death in the world. Almost 17.3 million people died worldwide due to cardiovascular disease, and 7.3 million of these deaths are caused by coronary heart disease (CHD). It is estimated that by 2030, deaths from cardiovascular disease will increase up to 23.6 million (2, 3).

Atherosclerosis is a progressive inflammatory disease, mainly found in the intima of many middle sized and large arteries (4, 5). Atherosclerosis causes thickening and hardening of the arteries, which will reduce blood flow and oxygen supply to the tissues (6). Process of atherosclerosis begins when endothelial arteries is damaged by certain factors such as Reactive Oxygen Species (ROS). In low concentration, ROS affects normal vascular function, whereas in high concentration, ROS will induce oxidative stress that causes cellular damage (7). High concentration of ROS contributes to atherosclerotic process through oxidation of low density lipid (LDL) into oxidized LDL (ox-LDL), endothelial dysfunction, migration and proliferation of vascular smooth muscle cell, adhesion and migration of monocytes with the development of foam cells (8).

A certain risk factor of atherosclerosis is dyslipidemia, which is induced by hypercholesterol diet. Hypercholesterol diet can cause imbalance between ROS production and the ability to detoxify those reactive molecules, as well as repairing the damage caused by ROS (9). Due to decreased activity of catalase (CAT), an enzyme that catalyzes the

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decomposition of H_2O_2 into H_2O and O_2, there is an increase in the level of H_2O_2, which is a type of ROS generated by hypercholesterol diet (10).

Hypercholesterolemia is an important factor in the pathogenesis of atherosclerosis, mainly because of high LDL levels (11). Changes in the lipid profile can cause dyslipidemia. Dyslipidemia is lipid metabolism disorder characterized by an increase or decrease in plasma lipid fractions. Major abnormalities of lipid fractions are the increase of total cholesterol, LDL, triglycerides, and the decrease in high density lipoprotein (HDL) (12).

Mechanism of atherosclerosis begins with endothelial dysfunction that can be caused by dysfunction of perivascular adipose tissue (PVAT). In healthy state (no obesity), PVAT has role in regulating blood vessel response (13). PVAT dysfunction is an increase in size and number of adipocyte cells that elevates the mass of PVAT (14). The elevation of PVAT mass is caused by the increase of ROS, which can activate peroxisome proliferator-activated receptor-ɣ (PPAR-ɣ), which is a master regulator of adipogenesis, thus causing expansion and dysfunction of PVAT (15). But this process can be prevented by Wnt signaling pathway (16). Then, PVAT dysfunction can increase the production of adipokines and pro-inflammatory cytokines (17).

When endothelial dysfunction occurs, LDL can be accumulated in the sub-endothelial layer of blood vessels. LDL is susceptible to modification, especially oxidation, resulting in the formation of ox-LDL (18). Macrophage colony-stimulating factor (MCSF) develops monocyte differentiates into macrophage. Then, this macrophage will phagocyte ox-LDL and turn into foam cell (19, 20). Foam cell will continue to expand and stimulate the recruitment of smooth muscle cells from media layer. Thus, the blood vessel walls will become thick, and some parts protrude into the lumen of the arteries called plaque. So, plaque is a pile of fat cells, calcium, smooth muscle, and various inflammatory cell components. Changes in arterial wall histopathology are the end stage of atherosclerosis process, which can be evaluated (21).

Because there is oxidative stress in atherosclerosis, it is necessary to minimize destructive effects of atherosclerosis with antioxidants; one of these antioxidants is polysaccharide peptide (PsP). PsP is a protein-bound polysaccharide found in many classes of fungi, including *Ganoderma. lucidum* (22). One of the substances contained in PsP is β-D-Glucan, which is the largest component in mycelia of *G. lucidum*. PsP has been reported to have ROS scavenging effect (23). Administration of PsP in experimental animals showed decreased lipid peroxide, and increased activity of superoxide dismutase (SOD) and CAT. Increased activity of these enzymes showed that PsP has ROS scavenging activity (24).

Materials and Methods

Study group

The experimental animals were 25 male *Rattus norvegicus* Wistar strain rats; aged 6 weeks with body weight of 100 to 150 g. Rats were obtained from CV. Gamma Scientific Biolab, Malang, Indonesia. Rats were divided into 5 groups: negative control (normal diet), positive control (high-fat diet), and 3 high-fat diet (HFD) group with administration of 50, 150 and 300 mg/kgBW PsP. Administration of PsP was performed with oral gavage once daily. HFD in this study was standard feed plus 1.9% cholesterol, 0.1% cholic acid and 8.9% lard, which were given 40 g per day for 3 months. Measurement of parameters was conducted at the Laboratory of Anatomic Pathology, Faculty of Medicine, University of Brawijaya, after we obtained ethical clearance from the Health Research Ethics Committee with number 461/EC/KEPK/08/2013.

Measurement of parameters

H_2O_2 levels measurement (Abcam, USA)

Colorimetric Hydrogen Peroxide Kit (Assay Design) was used to measure H_2O_2 level in the culture medium. Standard solution was prepared by dissolving 34 µl hydrogen peroxide standard stock with 966 µl diluent, and was referred as standard solution I. Standard solution II was prepared by dissolving 500 µl standard solution I with 500 µl diluent. As well, 6 standard solutions made up to by the same way. Then, 50 µl diluent was added in the first well as blank solution and 50 µl of standard solution I-IV into the next well. Also, 50-µl of each sample solution was put into the well. Then, 100 µl color reagent was added and homogenized using pipette for one second. Incubation was carried out at room temperature for 30 minutes. Next, it was read with ELISA Reader at 450 nm wavelength.

PVAT measurement (Paraffin Block, Hematoxylin-eosin (HE), dotSlide)

Observations of PVAT started with slide preparations. Tissues were blocked and cut, and were deparaffinized, and then HE (Hematoxylin-Eosin) staining was performed. Measurement of PVAT in this study was begun by scanning of histopathological slides using a microscope with 400x magnification, then viewed and measured using *dotslide* software.

Foam cells, atherosclerotic plaques measurement (Frozen Section, Hematoxylin-eosin (HE), dotSlide OlyVia)

Observations of foam cells and atherosclerotic plaque started with placement of tissue on gamit metal specimens and frozen rapidly at approximately -20 °C, so all tissues would harden, then it was frozen and cut with a cryostat that is microtome's part. Pieces were placed on glass slides and stained (usually with hematoxylin eosin) (25, 26). Measurement of foam cells and atherosclerotic plaques in this study

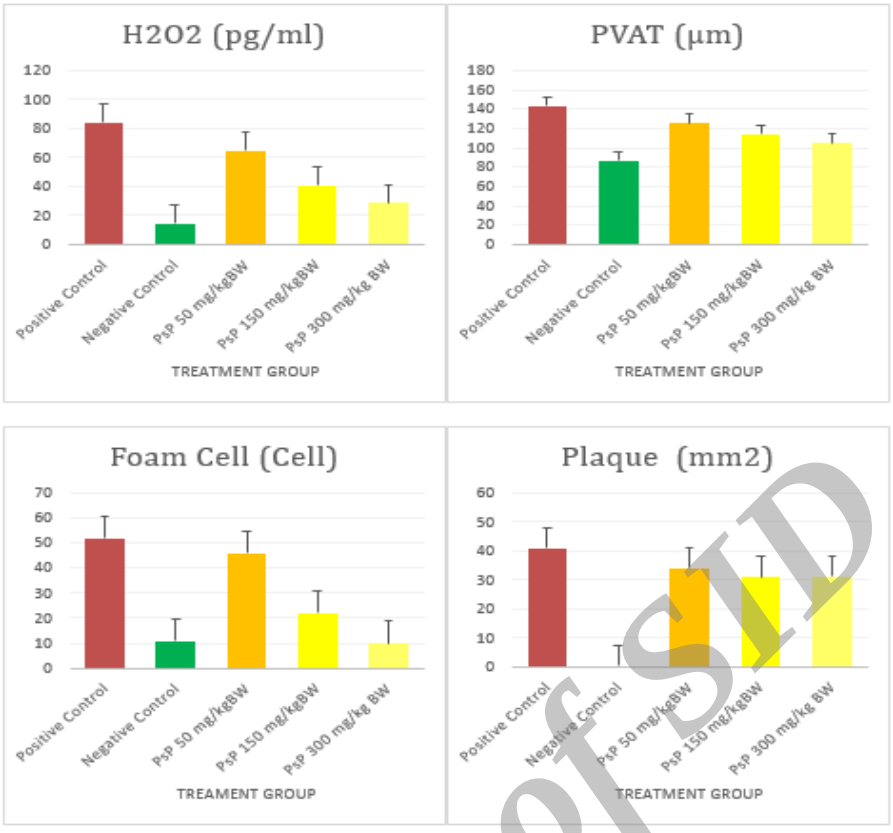

Figure1. Parameters measurement in each treatment group

performed with scanning of histopathological slides using a microscope with 400x magnification, then viewed and measured using *dotslide* software.

Foam cells can be evaluated by measuring tunica media-intima of the vessels. HE staining will appear as a large cell with blue core and empty edge, because fat will be fade or ruptured with HE staining and appears as an empty area between core and cell membrane (27).

Atherosclerosis plaque thickness can be evaluated by measuring tunica media-intima of the vessels. Atherosclerotic plaques contain vascular smooth muscle cells, lipid core, and necrotic debris encroaches on the lumen of the vessel (28).

Statistical analysis

One way analysis of variance (ANOVA) was used in this study to determine the effect of PsP on H_2O_2 level, PVAT, foam cells, and atherosclerotic plaques in *Rattus norvegicus* Wistar strain rats with high-fat diet. Then *Post hoc* test was performed to identify differences between groups. In this study, we used numeric data, so it concluded parametric procedures. Statistical Package for the Social Sciences (SPSS) software version 20 (IBM Corporation, 590 Madison Avenue, New York, USA) was utilized to obtain the data analysis.

Results

H_2O_2 levels in the various treatment groups ranged from 9.25 to 145.50 pg/ ml. Negative control group had the lowest levels of H_2O_2 (9.25 to 18.00 pg/ ml), whereas the highest levels of H_2O_2 were in positive control group (62.13-145.50 pg/ ml). ANOVA test with a 95% confidence level showed that administration of PsP had a significant effect (P=0.003) on reducing the levels of H_2O_2. *Post hoc* test with Duncan method indicated that levels of H_2O_2 in positive control and HFD+PsP 50 mg group differed significantly with normal control and HFD+PsP 300 mg group.

The thickness of PVAT in various treatment groups ranged from 79.67 to 179.34 µm. Negative control group had the lowest PVAT thickness (79.67-94.40 µm), whereas the highest PVAT thickness was found in positive control group (120.62-179.34 µm). ANOVA test with a 95% confidence level showed that administration of PsP had significant effect (P=0.001) on reducing the thickness of PVAT. *Post hoc* test with Duncan method showed that PVAT thickness in positive control group differed significantly with negative control, HFD+PsP 150 mg and 300 mg group, while thickness of PVAT in normal control group did not differ significantly with HFD+PsP 300 mg group.

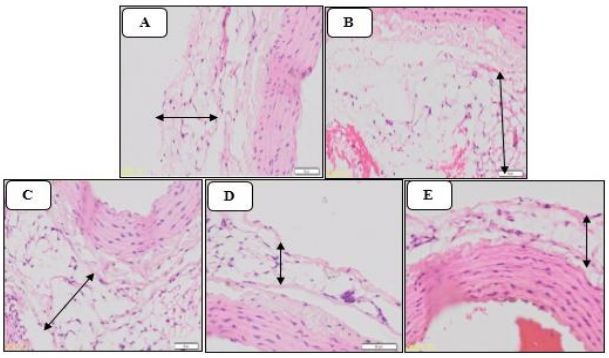

Figure 2. Histopathology of PVAT in different treatment Groups of rats with hypercholesterol. A. Normal Control Group; B. Positive Control Group; C. Hypercholesterol Diet Group and PsP 50 mg/kg BW; D. Hypercholesterol Diet Group and PsP 150 mg/kg BW; E. Hypercholesterol Diet Group and PsP 300 mg/kg BW. (Black arrow show foam cells)

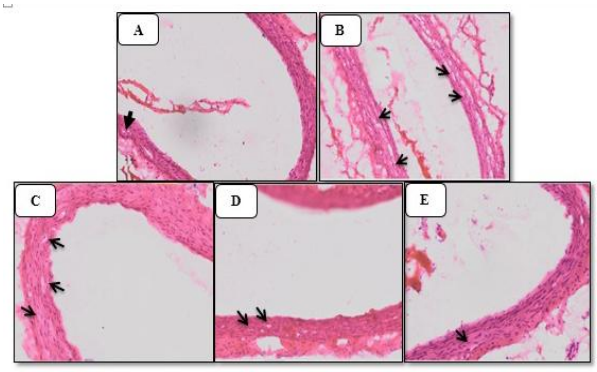

Figure 3. Histopathology of foam cells in different treatment groups of rats with hypercholesterol. A. Normal Control Group; B. Positive Control Group; C. Hypercholesterol Diet Group and PsP 50 mg/kg BW; D. Hypercholesterol Diet Group and PsP 150 mg/kg BW; E. Hypercholesterol Diet Group and PsP 300 mg/kg BW. (Black arrow show foam cells)

Total foam cells in various treatment groups range from 0 to 102 cells. Negative control group had the lowest number of foam cells (0-55 cells), while the highest number of foam cells was found in positive control group (16-102 cells). ANOVA test with a 95% confidence level showed that administration of PsP had significant effect (P=0.024) on reducing the number of foam cells. *Post hoc* test with Duncan method showed that the number of foam cells in negative control and HFD+PsP 300 mg group had no significant difference. But the number of foam cells of negative control differ significantly with positive control and HFD+PsP 50 mg group.

Width of atherosclerotic plaques in various treatment groups range from 0 to 96 mm^2. Negative control group had the narrowest atherosclerotic plaque width (0-2 mm^2), while the widest atherosclerotic plaques were in the positive control group (16-96 mm^2). ANOVA test with a 95% confidence level reveals that administration of PsP had a significant effect (P=0.022) on reducing atherosclerotic plaque width. *Post hoc* test with Duncan method exhibited that atherosclerotic plaque width in negative control group differ significantly with positive control, HFD+PsP 50 mg, 150 mg, and 300 mg group.

Discussion

Atherosclerotic processes that occurred in Wistar strain (*R. norvegicus*) rats with hypercholesterol diet are associated with increase of oxidative stress (ROS). Oxidative stress is a condition where there is an imbalance between the productions and detoxification of ROS. *Post hoc* test with Duncan method showed that levels of H_2O_2 in negative control group differed significantly with positive control group. Increase of ROS can be prevented by administration of antioxidant. Antioxidant is chemical compound that is able to scavenge ROS and other free radicals, so can inhibit oxidative processes (29). It also suppresses blood cholesterol by preventing formation of oxidized LDL, resulting in less adhesion of monocytes, reduced foam cell formation, reduced chemical damage, and reduced toxicity to vascular cells (30).

There are two kinds of synthetic and natural antioxidant. Synthetic antioxidant has harmful side effects that can generate carcinogenesis. Therefore, natural antioxidants are required as alternative resources to prevent the disadvantages of using synthetic antioxidants (31). In this study, PsP, a type of natural antioxidant derived from *Ganoderma lucidum* was administered to animal models and increased the activity of superoxide dismutase (SOD) and catalase (CAT). Increased activity of these enzymes showed that PsP has ROS and free radicals scavenging activity (24). Furthermore, PsP contains a

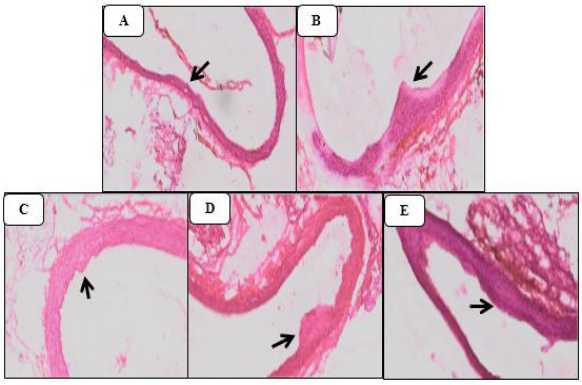

Figure 4. Histopathology of atherosclerotic plaque in different treatment groups of rats with hypercholesterol. A. Normal Control Group; B. Positive Control Group; C. Hypercholesterol Diet Group and PsP 50 mg/kg BW; D. Hypercholesterol Diet Group and PsP 150 mg/kg BW; E. Hypercholesterol Diet Group and PsP 300 mg/kg BW. (Black arrow show foam cells)

bioactive substance called β-D-Glucan that has been suspected of having anti-inflammatory and antioxidant activity in reducing the histopathological images of atherosclerotic damages. In another study, it is mentioned that β-D-Glucan can also be used as an immunomodulator, which is known to reduce inflammation (32).

H_2O_2, a certain type of ROS, is selected as parameter because it is a common model of oxidative stress. It can diffuse from its site of formation, and easily cross cell membranes, and produce cellular oxidative damage. H_2O_2 is involved in two important functions on vascular wall. The first function comes from its ability to produce endothelial barrier dysfunction by either modifying membrane permeability to macromolecules and sodium-potassium pump activity or altering endothelial metabolic function. The second function is derived from its capacity to modify vascular tone, inducing either contraction or relaxation depending on the vascular bed and experimental conditions. Endothelial cells can modulate vascular responses to H_2O_2 by generating vasoactive agents, such as nitric oxide and prostacyclin, and hence protect smooth muscle cells from the oxidative injury mediated by H_2O_2. Therefore, in de-endothelized arteries and in hypertensive conditions such as diabetes and atherosclerosis, higher sensitivity to oxidative stress induced by H_2O_2 can be expected (33).

Based on the data in this study, there were high levels of H_2O_2 in positive control group, and along with the addition of PsP dose, H_2O_2 levels decreased and almost approached the level of negative control group. ANOVA showed that administration of PsP can significantly reduce the levels of H_2O_2 in rats with hypercholesterol diet. Decreased levels of H_2O_2 also followed by inhibition of the atherosclerotic process, which were the decrease of PVAT thickness, amount of foam cells, and atherosclerotic plaque width.

The inhibitory effect of thickening PVAT was supported by the decrease in level of H_2O_2. ANOVA results showed that administration of PsP had a significant role in decreasing the thickness of PVAT in Wistar rats with hypercholesterol diet. PsP dose of 300 mg/kg BW had the most significant effect on decreasing the thickness of PVAT, thus approached the normal control group thickness. It occurred because of decreasing ROS by PsP, so that PPAR-γ receptor was not activated. As a result, differentiation and infiltration of preadipocytes were blocked, so there was no increase in the number of adipocytes that cause PVAT dysfunction.

ANOVA results also showed that administration of PsP had a significant role in decreasing the number of foam cells in Wistar rats with hypercholesterol diet. The most significant dosage in reducing the number of foam cells was showed by PsP dose of 300 mg/kg BW, which caused the amount of foam cell to be similar to the negative control group's. This result was in line with other studies, which reported that administration of antioxidant can reduce oxidative stress, so that it can inhibit atherogenesis by inhibiting the oxidation of LDL and reducing ROS in endothelial cells (34).

In the 95% confidence interval, results of the *post hoc* analysis showed that aortic atherosclerotic plaque of negative control group had a significant difference compared with other groups. While the results of atherosclerotic plaque measurement of HFD+PsP 50 mg, HFD+150 mg, and HFD+300 mg groups showed a decrease in atherosclerotic plaque width, but not very effective. Lack of effectiveness of this PsP therapy occurred because the duration of administration was not long enough and less adequate. Administration of PsP should be given longer because the mechanism of atherosclerotic plaque formation is slower than the rise of other signs of endothelial damage signs.

PsP can be used to prevent atherosclerosis mechanism as an antioxidant in accordance with the theory of inhibition of ROS by antioxidant that can stabilize free radicals by completing electron deficiency and inhibit chain reactions of free radical formation. It causes no formation of activated macrophages, so there will be no release of chemoattractant and cytokines substances that play a role in the migration of smooth muscle cells from media and also fibrous cap formation (35). Therefore, researchers recommend further studies with longer periods in the administration of PsP to get maximum results.

Conclusion

PsP *derived from G. lucidum* has antioxidant effect by decreasing H_2O_2 levels, so it can reduce PVAT thickness, foam cells numbers, and atherosclerotic plaque width in Wistar strain (*R. norvegicus*) rats with hypercholesterol diet. PsP dose of 300 mg/kgBW has the most significant effects on reducing H_2O_2 level, PVAT thickness, foam cells numbers, and atherosclerotic plaque width. Future studies are recommended to explore further using other parameters, so the mechanism of antioxidant effect of PsP can be explained clearly.

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