POLYSACCHARIDE PEPTIDE: A NOVEL ANTI-INFLAMMATION IN REDUCING INTIMA MEDIA PROLIFERATION IN DIABETIC RATS

Joko Hermawan*, Ria Ashriyah, Widyo Mahargo, Yunita Leksono, Djanggan Sargowo, Titin Andri Wihastuti, Muhammad Aris Widodo

*Department of Cardiology and Vascular Medicine, University of Brawijaya-Saiful Anwar Hospital, Malang, Indonesia
Department of Cardiology and Vascular Medicine, University of Brawijaya-Saiful Anwar Hospital, Malang, Indonesia
Department of Cardiology and Vascular Medicine, University of Brawijaya-Saiful Anwar Hospital, Malang, Indonesia
Department of Cardiology and Vascular Medicine, University of Brawijaya-Saiful Anwar Hospital, Malang, Indonesia
Department of Biomedicine, Faculty of Medicine, Brawijaya University, Malang, Indonesia
Department of Pharmacology, Faculty of Medicine, Brawijaya University, Malang, Indonesia

Abstract

Diabetes Mellitus (DM) is a risk factor equivalent to Coronary Artery Disease (CAD). DM and CAD involves a chronic inflammatory process. Inflammatory cytokines are involved in both diseases, either DM or CAD. This study was aimed to evaluate polysaccharide peptide (PSP) of the extract of Ganoderma lucidum as an anti-inflammatory that inhibits the process atherogenesis in diabetic rats model. The study was experimental post-test with control group design. Thirty Wistar rats were divided into 5 groups: 1 group of normal rats as control group and 4 groups with High Fat Diet (HFD) and low dose streptozotocin (30 mg/kgBW) injection sc, treated with placebo and PsP dose 50,150,300 mg/KgBW respectively. Parameters measured are level of interleukin-6 (IL-6), high sensitive C-reactive protein (hsCRP), and intima media thickness (IMT). After 5 weeks of treatment there were significant lower levels of IL-6 (p<0.000), atherosclerosis plaque area (p=0.000), IMT (p=0.000) and lower of hsCRP level (p=0.079) descriptively in the treatment group of PSP 150 and 300 mg/kgBW. Based on this study, PsP is a novel promising anti-inflammation to inhibit the process of atherogenesis in diabetes mellitus rats model.

Introduction

The high prevalence of diabetes mellitus (DM) is a health problem in the world. Both in the developed and developing countries. The total number is estimated at 171 million people affected by diabetes in 2000 and will increase to 366 million in 2030. Indonesia was ranked 4th in the world in 2000 with a prevalence of 8.4 million people with diabetes. In Indonesia, in 2030, is estimated to reach 21.3 million people(1). While the results of Riset Kesehatan Dasar (Risksedas) in 2007, the prevalence of DM in Indonesia based on the diagnosis by health personnel was 1.1% and found that the proportion of causes of death from diabetes in the age group 45-54 years in urban areas (14.7%). And in rural areas, DM ranks 6th (5.8%)(2).

Many studies state that diabetes is a risk factor equivalent to coronary artery disease (CAD)(3-5). One of them Goraya, 2002 showed that the high prevalence of diabetes is related to atherosclerosis. In diabetic patients without clinical symptoms of CAD, nearly three quarters had a high grade coronary atherosclerosis, and more than half had multi vessel disease. Whereas in patients without clinical symptoms of CAD, DM is associated with a surge in global CAD and the prevalence of high grade atherosclerosis is the same as in the non DM patients with clinical symptoms of CAD(3).
Basically DM and CAD involves chronic inflammatory processes. CAD process is coordinated by local secretion of adhesion molecules, chemotactic factors, and cytokines which are an expression in vascular injury. While many metabolic abnormalities accompany DM, including hyperglycemia, free fatty acid excess, insulin resistance, which cause endothelial abnormality. Endothelial abnormality cause dominance of vasoconstriction, pro-thrombotic factors, and release of inflammatory cytokine and chemokine. DM has a low grade chronic inflammatory factors that associated with the state of obesity, which is a response of the innate immune system. In this state, pro-inflammatory cytokines are involved in both, either DM or CAD. Nowadays, herbal medicine as a complementary or optional therapy in patients with CAD and DM has been developed. *Ganoderma lucidum*, a mushroom which is used as traditional herbal medicine in Asia especially in oriental country, has benefits in heart disease. A previous studies have shown that *Ganoderma lucidum* is useful on CAD, improving clinical complaints, lowering total serum cholesterol, improves ECG and blood pressure. In Scandinavian study also showed that, extracts of the fungus *Saccharomyces cerevisiae* wall containing β-glucan given to patients undergoing CABG, decreased the inflammatory response and protect the heart from reperfusion injury. And several studies in rats showed use of *Ganoderma lucidum* as antihyperglycemia. But only limited study focus in the effect of *Ganoderma lucidum* in chronic inflammation in atherosclerosis especially in diabetes mellitus. Aim of this study was to evaluate polysaccharide peptide (PsP) of the extract of *Ganoderma lucidum* as an anti-inflammation that inhibits the process atherogenesis in diabetes rats model.

**Materials and methods**

**Animals**

Experimental animals in this study were rats (rattus norvegicus) obtained from CV Gamma Scientific Biolab, Malang. Inclusion criteria were male rats, approximately 3 months of age, weight about 150-200 grams, healthy condition and no anatomical abnormalities. While the exclusion criteria were rats had diarrhea during the study period were marked by feces is not formed and or lose weight, dead and sick during the treatment period. Rats drop out if match with exclusion criteria and replaced with other rats in accordance with the inclusion criteria, in order to get the number of rats in accordance with the sample needed. This research was conducted at the Central Laboratory of Life Sciences Brawijaya (LSIH) University of Brawijaya, Malang.

**Ganoderma lucidum-Polysaccharide Peptide Preparation**

PsP preparation was made by Sahabat Lingkungan Hidup Surabaya, a biopharmaceutical company. PsP extracted from mycelia of *Ganoderma lucidum*. PsP is prepared in dry powder form of capsules containing 250 mg of an extract of *Ganoderma lucidum*. Each gram of PsP contained 200 mg of β-D-glucan. PsP was administrated to the animal models daily via oral gavage in the last 4 weeks of the study.

**Experimental Design**

The study was experimental study with post test design. A total of thirty rats acclimated 2 weeks with normal food. Then the rats were divided randomly into 5 groups: 1 group of normal rats as control group and 4 groups with High Fat Diet (HFD) and low dose streptozotocin (STZ) injection sc, treated with placebo and PsP dose 50, 150, 300 mg/KgBW respectively. Rats for the control group were given a normal diet. High fat diet was given along study period (for 12 weeks). Streptozotocin injection was given at 5th week. Diet was made every day, given as a daily diet of 50g/rat/day. Diet was given at the same time during the day at 12.00 am-02.00 pm. A normal diet consisted of chicken feed/Pars with wheat flour containing 3.43 cal/gram. Whereas HFD consisted of chicken feed/Pars, wheat flour, cholate acid, cholesterol, and lard containing 4.03 cal/gram.

**Induction of Diabetes Mellitus**

The rats were adapted to HFD for 4 weeks before induction of DM. After a 24-hour fasting, diabetes was subsequently induced in the rats through intraperitoneal (ip) administration of STZ (BioWorld Products Inc. Visalia CA, USA), at a low dose of 30 mg/kg body weight. STZ was freshly prepared in an ice-cold citrate buffer and immediately injected into the animals (within 5 min). Three days later, high and steady blood glucose levels were observed in STZ-induced rats. At this point, the STZ-induced rats with high blood glucose levels (>11.1 mmol/L) were selected as diabetic models. Measurement of blood glucose levels was carried out by use of Glucometer (Terumo Medisafe Mini, Tokyo, Japan).

**Parameter Analysis**

Tissue sample was taken for parameter analysis. Blood was drawn from cardiac puncture, while aorta was surgically taken. Measurement of inflammation marker IL-6 (Komabiotech, Korea), hsCRP (Elabscience, Beijing, China) used ELISA method. Aorta were pathologically examined with hematoxylin eosin or HE staining. Slide pictures were taken using Scan Dot Slide Olyvia software (Olympus America Inc., Center Valley, PA).
PA, USA) in 40x magnification under microscope. Intima media thickness was measured by calculating length (µm) of thickness.

**Ethics**

We obtained ethical approval for the animal treatment and experimental processes in this study from the Ethics Committee for Health Research Number 462/EC/KEPK/08/2013.

**Statistical Analysis**

The data were analyzed with the SPSS version 17.0 (IBM Corporation, New York, NY, USA). Descriptive analysis that will be presented include the mean value, standard deviation, the lower and upper 95% confidence interval, and the minimum value of the maximum temptation. Bivariat analysis using One way Anova was used to identify significant difference of IL-6, hsCRP, and IMT between at least the two treatment groups in all parameters. Post Hoc Duncan test will be done if one way anova showed significant difference (p < 0.005) between at least two treatment groups to identify doses of PsP that affects the parameters.

**Results**

**Inflammation Marker**

After 5 weeks treatment of PsP, IL-6 level was lower significantly (p=0.000) (table). The lowest experimental results was in DM with PsP 300 mg/kgBW group. Post Hoc analysis showed that mean value of IL-6 level in group DM+PsP 50, 150 and 300 mg/kgBW was significantly different with DM+placebo group. It seemed that IL-6 level reduction was related with PsP dose. The higher PsP dose, the lower IL-6 level. Interestingly, PsP has no significant reduction effect to hsCRP level (p=0.079). But descriptively, hsCRP was lower with PsP treatment as shown in figure 1. The highest hsCRP level was found in the DM group while the lowest was found in normal group. hsCRP level lowered progressively with increasing PsP dose, which at dose 300 g/kgBW hsCRP level is the lowest (206.6 ng/ml).

**Table. Result of parameters measurement, descriptive statistic, and one way anova**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetes Mellitus</th>
<th>Diabetes Mellitus + PsP 50 mg/kgBW</th>
<th>Diabetes Mellitus + PsP 150 mg/kgBW</th>
<th>Diabetes Mellitus + PsP 300 mg/kgBW</th>
<th>sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>832.6±57.7</td>
<td>1707.1±492.3</td>
<td>1294.8±307.0</td>
<td>1007.2±94.6</td>
<td>972.4±104.3</td>
<td>0.000</td>
</tr>
<tr>
<td>hsCRP (pg/mL)</td>
<td>841.6±400.6</td>
<td>2166.6±859.9</td>
<td>1976.6±535.6</td>
<td>1847.2±809.8</td>
<td>1461.6±978.9</td>
<td>0.079</td>
</tr>
<tr>
<td>IMT (µm)</td>
<td>70.46±4.7</td>
<td>92.6±6.8</td>
<td>86.3±5.5</td>
<td>72.6±8.0</td>
<td>69.0±5.0</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* Data are presented as mean ± standard deviation (range) values. All the values of the parameters have been corrected into International Standard of Mathematics (decimals).

*P < 0.05 indicates the significant difference.
PsP: polysaccharide peptide; IL-6: interleukin-6; hsCRP: high sensitive C-reactive protein; IMT: intima media thickness

**Intima Media Thickness**

Atherogenesis in DM rats is inhibited by PsP in this research. Intima media thickness was significantly lower in PsP treatment (p=0.000). Post hoc analysis showed that PsP dose of 150, and 300 mg attenuated the atherosclerotic process and could reduce the intima media thickness as compared with normal group. Lower inflammation markers in this study was linear with a better lipid profile in the treatment groups given PsP. Cholesterol total and triglyceride level significantly lower (p=0.010 and p=0.001) but LDL and HDL were descriptively lower with increasing PsP dose (Data not shown).

**Discussion**

*Ganoderma lucidum* has been used since hundreds of years ago in various countries in the world, but the effectiveness and side effects is still in ongoing research. There was limited study focus in the effect of *Ganoderma lucidum* in chronic inflammation especially in atherogenesis process with background DM, to our knowledge, we conduct the first in this field.

Metabolic conditions in DM such as hyperglycemia state, insulin resistance, and free fatty acid induced alteration of function and structure of blood vessel. These condition lead to oxidative stress, and also activation of nuclear factor kappa B (NF-kB). Activation of NF-kB cause release of cytokines and chemokines that leads to endothelial activation and recruitment of inflammatory cells(6, 17). Thus, primary cytokine (tumor nuclear factor-a and interleukin-1) stimulates the production of IL-6, that acts as messenger cytokine. This IL-6 finally, induces expression of hepatic genes encoding acute phase reactants, including CRP that found in blood(18). In this study, giving 50, 150, 300 mg PsP level of IL-6 was lower significantly in diabetes mellitus rats. While there were lower of inflammatory cytokines hsCRP descriptively between the negative control group, atherosclerosis, and the treatment group which treated with PsP. Level of hsCRP decrease incrementally as with increase of PsP dose, whereas with PsP 300 mg/kgBW the lowest level of hsCRP was reached.

Previous studies are still debatable. In healthy individuals, *Ganoderma lucidum group* showed no significant difference in hsCRP levels with placebo group(19). Li's in 2007, in a population of patients suffering from rheumatoid arthritis (RA) with disease-modifying antirheumatic therapy drugs (DMARDs) eg hydroxychloroquine, sulfasalazine, methotrexate, and leflunomide, also showed no difference of CRP level with placebo groups given additional therapy extract ganoderma 4 g in combination with other herbs for treatment duration of 24 weeks(20). Otherwise, several studies showed different result. *Ganoderma lucidum* increases IL-10 level(21-23), increases TNF-α(24) and no correlation with IL-6 level in cancer patients(25). But none of the previous studies focused on inflammatory aspect of atherogenesis process in diabetes mellitus.

Atherogenesis is clearly inhibited by PsP treatment. Intima media thickness as results of chronic inflammation and oxidative stress was lower significantly (p=0.000). Giving PsP with maximum dose 300 mg/kgBW tended to be followed by a decline average of intimal media thickness. This result strengthened study by Yin and Lin in 2002, that *Ganoderma lucidum* polysaccharides peptide could inhibit atherogenesis, proven by reduce of foam cell formation and necrosis of macrophages(26).

There are several reasons of inhibition of atherogenesis in DM rats in this study. First, by inhibition of chronic inflammation. There was a study that stated ganoderma lucidum ethanol extract in lipopolysaccharide (LPS)-stimulated murine BV2 microglia could inhibit and suppress NF-kB translocation and transcriptional. It also proved that inhibition of NF-kB followed by inhibition formation of pro inflammatory cytokine such as IL-1β and TNF-α, and also excessive production of NO (27). This study showed that inflammation is inhibited with PsP treatment. We suggest that PsP inhibit and suppress NF-kB formation, thus downstream pathway including activation, and release of inflammatory cytokine is inhibited. Second, by inhibition of oxidative stress. There have been various mechanisms that can explain how hyperglycemia causes vascular complications. There are several pathways which get activated through hyperglycemia and can potentiate each other. The basis for the activation of these pathways is most likely the overproduction of ROS in mitochondria induced by hyperglycemia. Which activates polyol, DAG/PKC, AGE, hexosamine pathway, and finally increases oxidative stress. This process leads to endothelial dysfunction as induction of vascular complication in DM(28). Some studies showed that *Ganoderma lucidum* in DM has a potent antioxidant effect(16, 29). This study also showed
that *Ganoderma lucidum* decrease oxidative stress by increasing anti oxidant (superoxide dismutase) and reducing level of oxidant (malondialdehyde)(30). Third, by lowering hyperglycemia and insulin resistance. Studies showed that ganoderma lucidum (crude or extract) had hypoglycemic effect (15, 31). Hyperglycemia affects the normal endothelial function that stimulates the decrease in NO production, the activation of NF-kB, and the increase in proinflammatory and prothrombotic mediators(32), and this process is inhibited by PsP as hypoglycemia agent. In this study as reported previously by Heriansyah et al, PsP treatment reduced insulin resistance in diabetic rats model(33).

Limitation of this study is short duration (5 weeks) of treatment of PsP, whereas other study usually used at least 8 weeks of treatment(16). Also, PsP administration given at 9th week, after 8 weeks rats fed with HFD, means chronic inflammation and oxidative stress is longer than treatment given.

**Conclusion**

PsP administration at dose 50,150,300 mg/kgBW is a novel promising prevention or treatment as anti-inflammation thus inhibiting atherogenesis in diabetes mellitus. Further research is needed to evaluate safety and efficacy of PsP in human by conducting toxicity and cohort study.

**Acknowledgements**

We acknowledge the Sahabat Lingkungan Hidup Surabaya for preparing the PsP extract of *Ganoderma lucidum*. And this study was funded by Directorate of High Education through Brawijaya University Indonesia.

**References**

22. Sun LX, Lin ZB, Duan XS, Li J, Ge ZH, Li XJ, Li M, Xing EH, Jia J, Lan TF, Li WD. Ganoderma lucidum polysaccharides antagonize the suppression on lymphocytes induced by culture supernatants of B16F10 melanoma cells. J Pharm Pharmacol 2011 May;63(5):725-35 doi: 101111/j2042-7158201101266x.
Author Bibliography

Joko Hermawan, MD
Senior resident of cardiologist in Saiful Anwar Hospital-University of Brawijaya, Malang, Indonesia from 2011-now. Graduated from Faculty of Medicine University of Brawijaya in 2007. Second winner of Young Investigator Award in 23rd Annual Scientific Meeting of The Indonesian Heart Association in Conjunction with 7th Asia Pacific Congress of Heart Failure in Bali, Indonesia 2014. Presented poster presentation T2 Weighted Image & Contrast Enhanced Magnetic Resonance Imaging Differentiate Acute and Chronic Myocarditis in 27th Weekend Course on Cardiology in Jakarta 2015.