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Porphyromonas gingivalis vesicles reduce MDA-LDL levels and aortic wall thickness in high fat diet induced atherosclerosis rats

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KEYWORDS
Atherosclerosis; Vaccines;

Abstract  Background: Recently, atherosclerosis-associated disease has been reported simultaneously increased. Whereas, to date, no atherosclerosis vaccine is available. Since the epitope mimicry between malondialdehyde low-density lipoprotein (MDA-LDL) and arginine
Introduction

Atherosclerosis, narrowing of the arteries caused by plaque deposits, contributes to a high number of morbidity and mortality of cardiovascular and cerebrovascular diseases globally. In 2015, there were 17.7 million deaths associated with cardiovascular diseases and representing 31% of all global deaths. Although atherosclerosis management has been established, the mortality rates due to atherosclerosis-associated diseases have increased in the last two decades. This because the management of atherosclerosis is a complex, involving various aspects. Therefore, management with a prevention approach such as vaccination is likely to bring better outcome. Several atherosclerosis vaccines have been studied and each of them has a different target site antigen, such as malondialdehyde low-density lipoprotein (MDA-LDL), native LDL or copper oxidized-LDL, and p210. Of these antigens, MDA-LDL is the most widely studied. MDA-LDL is the most important oxidized LDL (ox-LDL) and considered more atherogenic than LDL. A study found that the induction of antibodies against MDA-LDL was associated with reduced atherosclerotic lesion formation and lower serum cholesterol level.

Recently, the correlation between infectious pathogens and the diseases has been proposed to eliciting the potency of vaccination, for example; *Streptococcus pneumoniae* and *Salmonella typhimurium* were shown to have an effect on the decreased risk of atherosclerosis; influenza vaccination were found to be associated with reduced risk of stroke, and pneumococcal polysaccharide vaccine had been disclosed beneficial for reduction of acute coronary syndrome risk. Periodontitis is an inflammation of the periodontal and supporting structures of the teeth, associated with polymicrobial infections including *Porphyromonas gingivalis*, one of the major contributors of this disease. Scientific evidence revealed that periodontopathogens such as *P. gingivalis* during periodontitis have a pivotal role in inducing in the development of atherosclerosis. This correlation is probably due to the structure mimicry between MDA-LDL and arginine specific epitope gingipain (Rgp) on the vesicles of *P. gingivalis*, structures with high immunogenicity. Theoretically, two high immunogenic properties having similar fragment size have the potency to generate cross-immunity. Based on this, this study sought to evaluate the potency of *P. gingivalis* vesicles to be an atherosclerosis vaccine in rats model assessed by MDA-LDL, visceral fat, body weight, and aortic wall thickness. The results of this study provide a primary data the potency of *P. gingivalis* vesicles as atherosclerosis vaccine candidate.

Methods

Animal and *P. gingivalis* outer membrane vesicles

Male albino wistar rats at eight weeks of age were purchased from Physiology Laboratorium, Brawijaya University. Ten of them were randomly assigned into each study group. All animal protocols in this study were approved by the Ethical Committee of Brawijaya University, Malang, Indonesia and were carried out in strict accordance with the Indonesian law and guidelines on the use of experimental animals. The study was conducted in animal facilities at Biomedical Laboratory, Brawijaya University.

*P. gingivalis* strain (ATCC® 33277™), kindly provided by Supriyono Hasan from Microbiology Laboratory, Faculty of Dentistry, Airlangga University, was used. The outer membrane vesicles of *P. gingivalis* vesicles were isolated according to previous study. Briefly, after separating from the culture media, the bacterial cells were mixed with 40% ammonium sulfate for two hours and centrifuged at 20,000 g for 40 min. The pellet was suspended with Tris buffer (50 mM, pH 9.5) containing 0.5 mM dithiothreitol (DTT) (ThermoFisher, MA, USA) and was dialyzed overnight.
The vesicles were then collected by centrifugation (27,000 g for 40 min) and resuspended in 10 ml of Tris buffer. Finally, the suspension was re-centrifuged at 27,000 g for 40 min and the vesicles were resuspended in 1.5 ml of Tris buffer (pH 7.2) and stored at 4 °C for immunization.

High fat diet

High fat diet (HFD) was adapted from previous study with some modifications. Briefly, the animals were fed with HFD consisted of 4% cholesterol, 1% cholic acid, 0.5% thio-uracil, 10% pork oil, and 5% duck egg yolk. The diet was provided ad libitum. HFD was given at 10 weeks of age in the negative control group and three treatment groups.

P. gingivalis vesicle challenge

Male wistar rats were divided into five groups: three intervention groups and two control groups. The first intervention group (T1) was given a HFD and injected intraperitoneally with 100 µl P. gingivalis vesicles and adjuvants (antigen to adjuvants ratio was 1:1). The second group (T2), was at a HFD and injected with 100 µl vesicles only while the third group (T3) was at a HFD and injected with adjuvants only. A group of rats (PC) were injected with aquabidest while another group (NC) were given HFD only. A booster dose of P. gingivalis vesicles (100 µl) was administered two weeks following the initial dose and repeated until four times in fortnight interval. During initial and booster dose, complete (CFA) and incomplete Freund adjuvants (IFA) were used, respectively. All rats were euthanized after eight weeks on HFD.

MDA-LDL measurement

MDA-LDL serum levels were measured using ELISA as described previously with some modifications. ELISA plates were coated with the capture antibody for MDA-LDL (Abcam, San Francisco, USA) at 10 µg/ml concentration in bicarbonate buffer (pH 9.6) then the plates were incubated at 4 °C overnight. Coating solution was removed and plates were washed with PBS solution. Protein binding sites in coating wells were blocked overnight at 4 °C with 1% skim milk in PBS with 0.5% Tween 20 (PBS-T). Each serum sample was serially diluted with 1% skim milk in PBS-T, added to the well (100 µl each) and incubated for 90 min at 37 °C. The wells were then incubated for one hour at 37 °C with alkaline phosphatase-conjugate goat anti-mouse antibody (Abcam, San Francisco, USA) at a dilution 1:1000. Subsequently, MDA-LDL was detected by chromogenic development using para-nitrophenyl phosphate as the alkaline phosphatase substrate. MDA-LDL was measured in ELISA Reader with absorbance at OD 405 nm.

Measurement of visceral fat and body weight

A digital scales (Ohaus CS200, NJ, USA) was used to measure body weight of rats. While for visceral fat measurements, after euthanized using isoflurane anesthesia, epididymal white adipose depots of rats were separated from the epididymis.
with molecular mass approximately 40 kDa and 28 kDa (Fig. 1A). Western blot showed that the sera from P. gingivalis vesicle-immunize rats were significant immunoreactive against both 40 kDa and 28 kDa protein band (Fig. 1B).

**MDA-LDL level**

To characterize the specific immune response induced by P. gingivalis vesicles, circulating MDA-LDL were determined by ELISA. Our results showed that MDA-LDL level of PC, T1, T2, T3 and NC group were 24.53 ± 7.65 µg/ml, 22.11 ± 3.59 µg/ml, 36.09 ± 10.28 µg/ml, 42.74 ± 15.71 µg/ml, and 44.73 ± 7.67 µg/ml, respectively. There was significant difference among groups (p = 0.015). The level of MDA-LDL among rats in T1 group was significantly lower compared to NC group (22.11 ± 3.59 µg/ml vs. 44.73 ± 7.67 µg/ml, p = 0.037).

**Visceral fat, body weight, and aortic thickness**

The mean weight of visceral fats for PC, T1, T2, T3, NC group were 0.98 ± 0.76 g, 2.66 ± 0.37 g, 2.80 ± 0.25 g, 1.93 ± 0.49 g, and 3.13 ± 0.84 g, respectively. There was significant difference among groups (p = 0.001). However, post hoc analysis suggested no significant difference between T1 and NC group (p = 0.789), T2 and NC group (p = 0.929) and T3 and NC group (p = 0.072) (Table 1). Body weights of animal from PC, T1, T2, T33, and NC group were 67.25 ± 19.77 g, 69.75 ± 14.86 g, 100.50 ± 17.02 g, 89.75 ± 9.11 g, and 91.00 ± 13.74 g, respectively. Our analysis using one way ANOVA showed that there was significant difference among groups (p = 0.031). However, post hoc analysis suggested no significant difference between NC and T1 (p = 0.329) or T2 (p = 0.901) or T3 (p = 1.000).

As expected, our results showed that the lowest aortic wall was in PC (26.83 ± 1.39 µm) and the highest was in NC (32.91 ± 2.28 µm) and there was significant difference among groups (p = 0.001). However, our data showed that the significant difference was observed only between T1 and NC group, with T1 was lower than NC (29.01 ± 1.37 µm vs. 32.91 ± 2.28 µm, p = 0.016).

**Discussions**

Although atherosclerosis-associated diseases are reported to increase, up to date, vaccine to prevent atherosclerosis is not available. Since P. gingivalis is associated with inflammation and ox-LDL modification, this species is considered a potential candidate for atherosclerosis vaccine. Our study was conducted to assess the potency of P. gingivalis to prevent atherosclerosis in rats model by measuring four atherosclerosis indicators: the level of MDA-LDL, visceral fat, body weight, and aortic thickness.

MDA-LDL plays an important role in the development of atherosclerosis. Several studies had reported that elevated circulating MDA-LDL levels were found in atherosclerosis-associated disease including heart disease and peripheral artery disease. Our study found that P. gingivalis vesicles challenge decreased the level of MDA-LDL. This achieved among rats that were treated with both vesicles and adjuvants, but not with vesicle or adjuvant only (Table 1). This suggested that immunization with P. gingivalis vesicles with adjuvant had a protective effect against atherosclerosis, by assessing MD-LDL level, the most important ox-LDL in the pathogenesis of atherosclerosis. The mechanism underlying this association is complex and remains largely undefined. However, this could be explained as follow. MDA-LDL uptake by macrophages would lead to the formation of foam cells and activated foam cells may lead to the upregulation of macrophage receptors, adhesion molecules, the expression of tissue factor, growth factor production, and increase the production of free radicals and inflammatory mediators. These lead to the thickening of blood vessel walls, defined as atherosclerosis. There are four possible mechanisms that bridge between P. gingivalis immunization and atherosclerosis. First, Rgp44 on adhesin-hemagglutinin domain of P. gingivalis vesicles are shown to have a high immunogenicity as well as both P1-linear peptide and P2-cyclic peptide on MDA-LDL epitope. The mimicry of fragment size between Rgp44, P1-linear peptide, and P2-cyclic peptide allegedly raises a cross-immunity. As the result, specific antibodies against Rgp44 on the bacterial vesicles may cross-react with peptides on MDA-LDL. Second, immunization with P. gingivalis may be able to induce specific IgG serum against atherosclerosis. Third, C-reactive protein (CRP) and cytokines may be induced by P. gingivalis immunization. Fourth, P. gingivalis immunization may stimulate general activation of B1 cell and interleukin-5 (IL-5) production against atherosclerosis. Because of these possible mechanisms, MDA-LDL uptake by macrophages and foam cell formation may be

![Image](image_url)

**Figure 1** SDS-PAGE and western blot analysis in P. gingivalis vesicles. (A) SDS-PAGE illustrated protein profiles in P. gingivalis vesicles. (B) Western Blot indicated specific binding of serum IgG from rats immunized with P. gingivalis vesicles to the membrane-bound protein component of P. gingivalis vesicles at molecular weight 40 kDa and 28 kDa.
Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group (mean ± SD)</th>
<th>P Among groups</th>
<th>P (T1 vs. NC)</th>
<th>P (T2 vs. NC)</th>
<th>P (T3 vs. NC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-LDL (mg/ml)</td>
<td>24.53 ± 7.65</td>
<td>0.015</td>
<td>0.041</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Visceral fat (gram)</td>
<td>0.98 ± 0.76</td>
<td>0.001</td>
<td>0.329</td>
<td>0.016</td>
<td>0.099</td>
</tr>
<tr>
<td>Body weight (gram)</td>
<td>67.25 ± 19.77</td>
<td>0.031</td>
<td>0.329</td>
<td>0.016</td>
<td>0.099</td>
</tr>
<tr>
<td>Aortic thickness (μm)</td>
<td>26.83 ± 1.39</td>
<td>0.001</td>
<td>0.235</td>
<td>1.000</td>
<td>0.867</td>
</tr>
</tbody>
</table>

Note: SD, standard deviation; MDA-LDL, Malondialdehyde low-density lipoprotein; HFD, high fat diet; PC, Positive control (aquabidest); T1, Treatment 1 (HFD + vesicles + adjuvants); T2, Treatment 2 (HFD + vesicles); T3, Treatment 3 (HFD + adjuvants); NC, Negative control (HFD).

Currently, a number of studies have focused on atherosclerosis vaccine and most studies assumed that MDA-LDL had a great potency to be a target for atherosclerosis vaccine. Concerning atherosclerosis vaccines with target site at MDA-LDL, our findings confirm and extend the previous studies that immunizations targeting at MDA-LDL have protective effect against atherosclerosis. Moreover, several studies had reported the potency of *P. gingivalis* as an atherosclerosis vaccine using various antigens such as 40-kDa outer membrane protein, with different route of administration for example subcutaneous, nasal, and oral. Although their studies had a very complete and specific design, however, most of them used *P. gingivalis* to induce atherosclerosis. This leads to the assumption that the vaccines they designed were only effective for atherosclerosis triggered by *P. gingivalis*. Therefore, the effectiveness of their vaccines against non-infectious atherosclerosis is still questionable. In our present study, we used HFD to induce atherosclerosis. Therefore, it might be assumed that our vaccines had a good effectiveness not only against *P. gingivalis*-induced atherosclerosis but also non-infectious atherosclerosis. Furthermore, they used only negative controls. Our present study did not only use negative control but also positive control. Therefore, the best and worst data limits could be known. Of those studies, our study is most similar to study by Turunen et al. They evaluated MDA-LDL immunization in mice challenged with *P. gingivalis* and the levels of IgG, IgM, IL-5, IL-10, and interferon-γ (IFN-γ) were measured. They showed that MDA-LDL-immunized mice had elevated IgM and IgG levels to MDA-LDL and increased IL-5 production compared to controls. Overall, they found that MDA-LDL immunization in mice induced with *P. gingivalis* challenge had significantly reduced aortic plaque formation compared to mice without immunization. However, reduction of plaque formation in MDA-LDL-immunized mice was not significant compared to mice that given HFD only, although there were some declines in aortic lipid deposition. Furthermore, the use of LDL modification as atheroprotective immunogen seems to be impractical for generalized use. On the other hand, whole bacteria utilization, despite its effectiveness to elicit antibody response against disease, is still controversial following some studies that linked its infection to accelerate several side effects.

Our results found that freund's adjuvants, both CFA and IFA alone, had no protective effects against atherosclerosis. Previous studies had shown the protective effect of adjuvants in atherosclerosis. The mechanism of adjuvants as atheroprotective includes their capacity to increase IgG and IgM against MDA-LDL. Our results were contrast with those previous studies. Further studies are required to elucidate this difference. However, atheroprotective effect of adjuvants is a complex involving several factors. A study revealed that atheroprotective
effect of adjuvants is CD4-dependent.\(^\text{12}\) Therefore, this may explain our results, adjuvants alone may not confer protection against atherosclerosis, and the combination of adjuvants and \(P.\) gingivalis vesicles may provide protection against atherosclerosis as reported in our study. However, this reason is not a final. In the near future, we expect there will be studies investigating how the precise mechanism of adjuvants in atherosclerosis.

We also evaluated the effect of \(P.\) gingivalis vesicles challenge on aortic wall thickness. Our data indicates that administration of vesicles with adjuvants decrease aortic wall thickness significantly compared to negative control group. This could be because of cross reaction response caused by \(P.\) gingivalis immunization. Foam cells accumulation caused by MDA-LDL uptake by macrophages is the basic mechanism for aortic wall thickness and atherosclerosis.\(^\text{39}\) Therefore, prevention of foam cell accumulation stimulated by \(P.\) gingivalis immunization\(^\text{26}\) has the important role to prevent the thickening of aortic wall. Our study found that \(P.\) gingivalis vesicles did not associate with reduction of visceral fat and body weight. One of the plausible reasons is visceral fat and obesity indeed have been linked to atherosclerosis risk factors, however they are chronic and prolonged process.\(^\text{57,58}\) Therefore, with a short time period of our study, it is understandable that \(P.\) gingivalis immunization has no effect on visceral fat and body weight of rats.

Our study demonstrated a good efficacy of \(P.\) gingivalis as an atherosclerosis vaccine. Our study was consistent to previous studies concerning \(P.\) gingivalis efficacy as an atherosclerosis vaccine in animal models. Further investigation with more specific design or involving genetic approach or in higher level of trials needs to be performed. Moreover, to evaluate the response of society regarding atherosclerosis vaccines, the acceptance and or willingness to pay studies may be needed.

There were several limitations in our study. We did not assess other variable that likely to have effect on atherosclerosis such as the level of triglyceride, cholesterol, fasting blood sugar, CRP, and LDL. In addition, the possibility of a false negative remains due to the small number of tested animals.

**Conclusion**

Our data suggest that \(P.\) gingivalis vesicles have protective effect in preventing atherosclerosis by reducing the level of MDA-LDL and aortic wall thickness in animal model. However, further studies are required to elucidate the mechanisms of these effects.

**Author contributions**

Designed the experiments = AIM. Performed the experiments = AIM. Analyzed the data = AIM, JFK, TH. Contributed reagents/material/analysis tools = AIM. Wrote the manuscript = AIM, JFK, HH, TH. Reference collection and data management = AIM, JFK, HH, TH. Statistical analyses and paper writing = AIM, JFK, HH, TH. Revised manuscript = SRP, EW, MSR, KM, BSP, YP.

**Conflict of interest**

The authors declared that there is no conflict of interest regarding the publication of this paper.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.artres.2018.05.008.

**References**

Porphyromonas gingivalis as an atherosclerosis vaccine


