

Study on iNOS Expression and Levels of Malondialdehyde (MDA) in the White Rat (*Rattus norvegicus*) Exposed with *Aggregatibacter actinomycetemcomitans* (Aa)

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ABSTRACT

Aggregatibacter actinomycetemcomitans (Aa) is gram-negative bacteria composed of lipopolysaccharides (LPS) in the outer membrane. This bacteria is normally found in dental plaque and periodontal pockets causing parotid gland damage when it is present in excess amounts. LPS is an endotoxin and a complex glycolipid composed of polysaccharides (hydrophilic) and lipid A (hydrophobic) which can induces Reactive Oxygen Species (ROS), and increase the levels of malondialdehyde (MDA) and tissue damage. The aims of this research are to find the expression of iNOS parotid gland cell and to determine levels of MDA using thiobarbituric acid (TBA) spectrophotometric test at 533 nm. The results showed a significantly difference ($P < 0.05$) of MDA levels in control rats (3.135 ± 0.0436 ppm) and Aa rats (6.307 ± 0.0473 ppm). iNOS expression is confirmed by the brown spots formed on the parotid gland, affording the enhancements of iNOS expression in Aa rats and control rats of 2.292 ± 0.038 and 0.166 ± 0.003 , respectively ($P < 0.05$).

Key word: *Aggregatibacter actinomycetemcomitans*, Reactive Oxygen Species, malondialdehyde, iNOS

INTRODUCTION

There are some microorganisms in human mouth which have beneficial functions, but there are also some which can be pathogenic. A report explained about 1011 bacteria per milligram of dental plaque can be found in teeth surface, although not all of these bacteria can harm, as they can live in the oral cavity¹. In term of decreasing human immunity, the oral bacteria may turn to be pathogens, and spread in the body causing systemic infections. The progress on identifying of some oral bacteria revealed the relationship between the incidence of infections of the mouth, gums and teeth with various systemic diseases such as heart disease, diabetes and stroke.

Bacterium is one factor which able to cause periodontitis by inducing tissue damage through stimulation of inflammatory cells, and this able to damage tissue by releasing destructive enzymes, reactive oxygen species (ROS) and pro-apoptotic factors². One of various kinds of microorganisms grows in the oral cavity as a source of pathogen is *Aggregatibacter actinomycetemcomitans* (Aa). This bacteria are classified as pathogenic gram-negative bacteria due to their outer membrane is composed of lipopolysaccharide (LPS)¹. LPS is an endotoxin and a glycolipid complex composed of polysaccharides (hydrophilic) and lipid A (hydrophobic)⁴.

Aa bacteria found in dental plaque and periodontal pockets and in excess amounts can cause various diseases. Previous studies confirmed *Aa* was able to impair many types of host cells. Many evidences were reported this organism was able to invade endothelial cells and epithelial³. The *Aa* bacteria induced reactive oxygen species (ROS) in the oral cavity by producing free radicals to affect increasing levels of MDA, tissue damage and changing of protein profiles.

Production of ROS in cell its causes the oxidation of protein, DNA oxidation and changes in the constituent bases of DNA, which lead to triggering cell and tissue damages¹⁰. ROS also causes peroxidation of membrane lipid which one of its final products is MDA. One of the tissues found in the oral cavity possibly affected directly because of excessive ROS production by bacteria *Aa* in the parotid gland tissue. Tissue of parotid gland is the largest salivary gland located under the ear around the maxilla. Excess amount of ROS especially in can directly stimulate the tissue damage. The *Aa* bacteria in the body react with the blood, and affecting the whole system of the body. In this paper was reported the effect of *Aa* bacteria on the expression of iNOS and MDA level of parotid gland using white rats (*Rattus norvegicus*)⁵.

EXPERIMENTAL

Treatment of Animals

A 10 males of white rats aged 3 months with weight of 150-180 grams and adapted for 12 days were used. Samples were divided into 2 groups, consisting of 5 rats for each group. Group 1 is the control and treated by injecting 200 μ L of physiological NaCl (0.9%) whereas group 2 is the *Aa* rats and treated by injecting 200 μ L of bacteria *Aa* with the dose of 10^8 CFU/mL. Injection is done twice on day 1 and day 7 in the upper gum located at the left gingiva. On the day 13, the white rat samples were surgered and subsequently their parotid gland and blood serum were harvested. All conditions and handling of animals in this study were conducted with protocols approved by the Brawijaya University Committee on Animal Use and Care (UB 106-UB-PEM).

Harvesting the parotid glands

After surgery on the day 13, parotid gland tissues of white rats are divided into two sections; $\frac{1}{4}$ part is treated with 10% paraformaldehyde (PFA) as a preparative sample while the remaining part is treated with a phosphate buffer saline-azide (PBS-azide) pH 7.4 for isolation of a protein tissue.

Protein isolation

Parotid gland is cut into small pieces and subsequently added with 1 mL PMSF solution, small amount of quartz sand, and crushed with a mortar placed on the ice block. Then, the homogenates are added with 2 mL PSMF solution and transferred into a sterilized polypropylene tube. The mixture is then homogenized with a vortex vibrator for 10 minutes, sonicated for 10 minutes, and centrifuged at 6000 rpm for 15 min. Furthermore, the supernatant is taken and added with cold absolute ethanol with a ratio of 1:1. The mixture is left overnight for the precipitate formation. After centrifugation for 15 minutes at 10,000 rpm, the precipitate is taken and dried to remove ethanol. Then the precipitate is homogenized with cold Tris-HCl (0.02 M, pH 6.5) with the ratio of 1:1 (w/v).

Preparation of Standard Curve of MDA

Each 100 μL of Kit MDA standard (density 0.997 mg/mL) with a series concentration of 0, 1, 2, 3, 4, 5, 6, 7 and 8 mg/mL is transferred into micro-tubes, and followed by addition 550 μL of distilled water. Then, into each tube was added 100 μL of 10% TCA, 250 μL of 1N HCl, and 100 μL of Na-thio 1%. These solutions are homogenized by vortex and incubated in a water bath at 100 ° C for 30 min, followed by cooling at room temperature. Measurements of each solution were conducted at the wavelength of 533 nm.

Measurement of MDA using Thio Barbituric Acid test (TBA)

Parotid gland is cut into small pieces, crushed using a mortar placed on an ice block and added with 1 mL of NaCl 0.9%. After homogenization, the mixture is transferred into micro tubes and centrifuged at 8000 rpm for 20 min. Then, 100 μL of the parotid gland supernatant is taken, and subsequently added with 550 μL of distilled water, 100 μL of 10% TCA, 250 μL of 1N HCl, and 100 μL of Na-thio1%. Every addition of reagent solution, the mixture is homogenized by vortex, followed by centrifugation at 500 rpm for 10 min. The supernatant was taken and then incubated in a water bath at 100 ° C for 30 min. After cooling the sample solution to room temperature, the absorbance is measured at 533 nm. The absorbance is then plotted on a standard curve to calculate sample concentrations.

Expression of iNOS Immunohistochemistry method

Staining maxillary parotid tissue preparations involves the binding of antigen-antibody (avidin-biotin). The addition of primary antibody (antibody NOS) conducted for 1 hour at room temperature, and washed in PBS pH 7.4 for 3 x 5 min. Further, secondary antibodies Anti-Rabbit IgG Biotin Labeled were added for 1 hour at room temperature, and washed in PBS pH 7.4 for 3 x 5 min. SA-HRP (Strep Avidin-Horseradish Peroxidase) were added for 30-60 minutes at room temperature and washed in PBS pH 7.4 for 3 x 5 min. Furthermore, chromogen DAB (3,3-diaminobenzidine tetra hydrochloride) were added for 10-20 minutes at room temperature and washed in distilled water for 3 x 5 min. Then counterstain was conducted with Hematoxylen for 5 minutes at room temperature, washed in distilled water for 3 x 5 min, followed by the mounting with entellan. The observation was preformed using a microscope with 400x magnification. Expression of iNOS *Aa* is noticeably from a brown color on the tissue preparation.

RESULTS AND DISCUSSION

Levels of MDA white rats parotid gland (*Rattus norvegicus*) were exposed to *Aa*

MDA is a major reactive aldehyde resulting from the lipid peroxidation reaction and usually used as a marker of oxidative stress. In a condition of high oxidative stress, MDA levels also increase significantly. Analysis of MDA level in rat parotid gland is be measured from each group of rats. MDA level in the parotid gland of rats exposed by *Aa* bacteria increases due to the LPS existing in the outer membrane of bacteria.

The incorporation of LPS with LBP is generated by dihydrogen phosphate ($\text{R-H}_2\text{PO}_4$) group of LPS and ammonium group (R-NH^{3+}) derived from LBP. At physiological pH (6-7) of the parotid, hydrolysis of $\text{R-H}_2\text{PO}_4$ occurs by releasing hydrogen ion (H^+) to form hydrogen phosphate (R-HPO_4^-). The loss of one hydrogen ion causes a negatively charged oxygen ion (O^-). This anion tends to bind with ammonium group (R-NH^{3+}) of LBP to form a new complex called LPS-LBP as displayed in **Figure 1**. This complex will interact with

specific receptors of macrophages to stimulate the production as well as release inflammatory cells⁷.

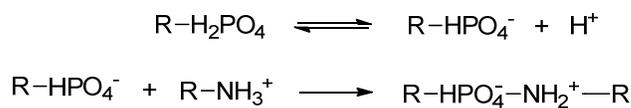


Figure 1. Reaction mechanism of formation of LPS-LBP complex

Table 1. MDA levels in parotid gland of white rats (n = 5)

Treatment group	Level of MDA (ppm)
Control	3.135 ± 0.0436
<i>Aa</i>	6.307 ± 0.0473

The damage of the parotid gland tissue, which is caused by bacteria *Aa* exposure, would trigger ROS. ROS can deteriorate lipids and proteins as the major components of the tissue. Consequently, a lipid peroxidation reaction occurred and thus the levels of MDA in the parotid gland tissue increased. As shown in **Table 1**, the MDA level in *Aa* rats is significantly ($P < 0.05$) rise in comparison to that of control rats.

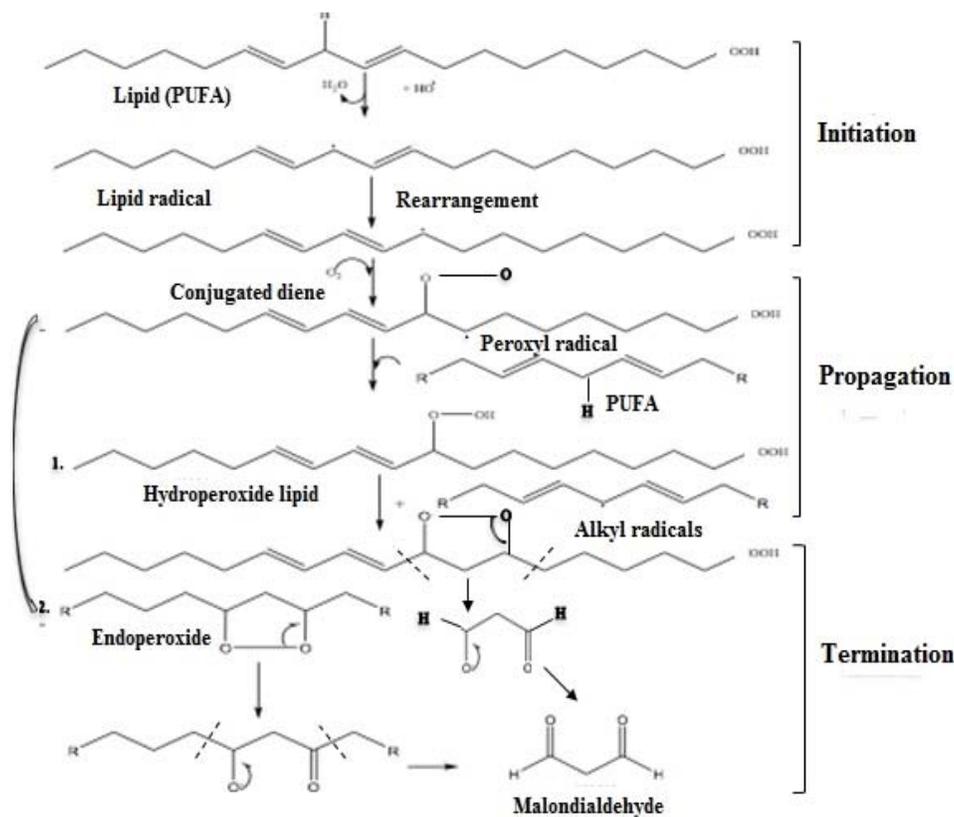


Figure 2. MDA formation through lipid peroxidation

Phagocytosis may stimulate the excessive production of ROS, which causes the oxidative stress, leading to inflammation. Oxidative stress is an imbalance situation between the amount of oxidants (free radicals) and antioxidants in the body. Meanwhile, ROS are molecules or atoms that are formed due to the oxygen reduction reaction (O₂) and it can be

classified based on its radical activity, such as superoxide (O_2^\bullet), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$). ROS serves to damage cells infected by a virus or bacteria. Highly reactive radicals can cause biochemical changes and damage to various components of living cells, such as proteins, lipids, carbohydrates and nucleic. The attachment of free radicals to cells membrane would lead to lipid peroxidation, which is indicated by the increase in MDA level. The latter compound is highly toxic to the cells since it is able to changes cell membrane fluidity and function. The mechanism of MDA formation is shown in **Figure 2**.

The C=C bonds of polyunsaturated fatty acid (PUFA) as the component of lipid in cells membrane is attacked by ROS. The double bonds weaken C-H bonds in lipid, causing the reduction of H due to the attachment of OH^\bullet . As the result, the lipid radicals are formed. Moreover, the lipid radical is oxidized by O_2 to form a peroxy radical. Newly formed peroxy radical can react with another PUFA by removing H, producing lipid hydro peroxides and other lipid radicals. This process is continuously propagated in a chain reaction. Lipid hydro peroxide is an unstable compound and it will produce MDA when fragmentation occurred. Additionally, peroxy radical can also undergo cyclization reaction to produce cyclic peroxide. This radical can be reduced to form hydro peroxide or subsequent cyclization reaction to form the bicyclic peroxide which then produces a molecule analogous to endoperoxide. The latter compound generates MDA formation⁹. So, the excessive numbers of free radicals increase the lipid peroxidation production followed by the increasing number of MDA.

Expression of iNOS white rats parotid gland (*Rattus norvegicus*) were exposed to Aa

The expression of inducible nitric oxide synthase (iNOS) causes inflammation in many types of cell as a response to the foreign substances such as virus and bacteria. The catalysis of NO by iNOS normally involves the reaction of L-arginine to form L-sitruilin in the presence of NADP (**Figure 3**).

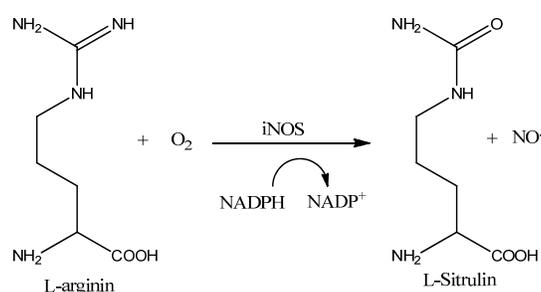


Figure 3. Catalysis reaction of NO^\bullet formation by iNOS

Excess production of NO could damage the cells. iNOS showed NO production in large quantities for immune defense. The presence of iNOS in a tissue can be described by immunohistochemistry, which involves a combination of immunological and histological methods. In immunohistochemistry, method of staining substances or active ingredients in the tissue is based on the basic principles of immunology (binding of the antigen with antibodies). The results of antigen and antibody can be identified on the specimen since the

antibody is bound by a marker for visualization, which can indicate the presence of the active ingredient in the tissue.

LPS could stimulate macrophage to produce and release inflammatory cells that causes pathophysiological effects such as infection up to tissue damage. Inflammatory processes in the cell will cause the migration of macrophages and produce cytokines⁸. Initially, LPS as an exogenous NO will activate macrophages. Then, the activated macrophages produce pro inflammatory cytokines and excessive inflammatory cells. Consequently, the increase in the effectiveness of the inflammatory enzyme iNOS is obtained. Moreover, iNOS could trigger the formation of free radicals endogenous NO* through the reaction catalyzed by NOS **Figure 4**. Additionally, the expression formed between antigen and antibody indicates the condition of the infected tissue pathology in the expression of iNOS. As shown in **Table 2**, it was found that there is a significantly (P<0.05) increase of iNOS expression in *Aa* rats in comparison to the rats control.

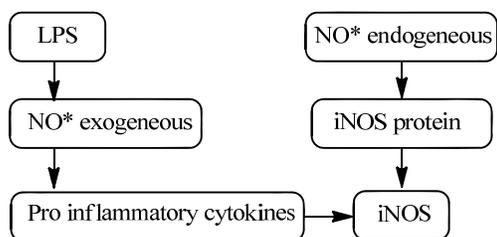


Figure 4. The mechanism of endogenous NO* formation catalyzed by iNOS as a result of inflammation

Table 2. Profile of the number of cells expression of iNOS in *Aa* rats and control rats

Treatment group	The average number of cells expressed iNOS (%)
Control	0.166 ± 0.003
<i>Aa</i>	2.292 ± 0.038

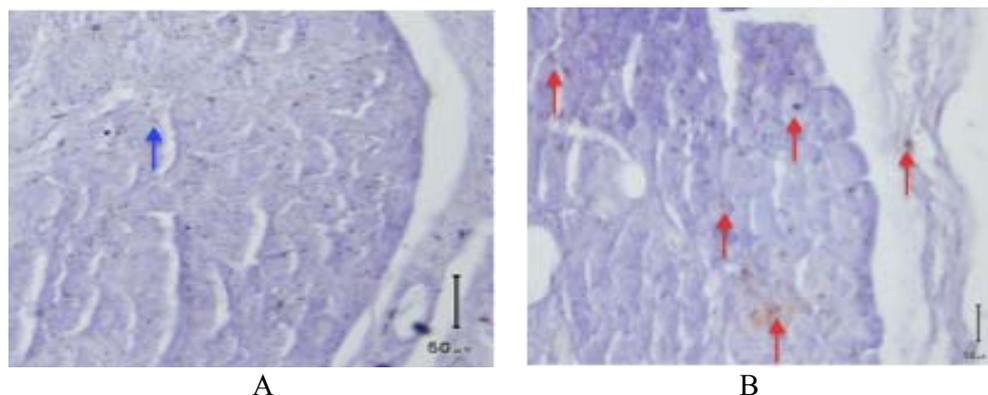


Figure 5. Parotid gland (*Rattus norvegicus*) by immunohistochemistry method 200x magnification (Blue arrow, A: Control rats; Red arrow, B : *Aa* rats).

Figure 5 showed expression of iNOS in cells of control rats (A) and *Aa* rats (B). It was found that there is a small number of at least the brown color shown on the tissue of control rats. This result reveals that there is always a process of regeneration of cells iNOS endogenous to produce NO in maintaining homeostasis in the body. In *Aa* rats (B), lots of

brown spots were detected as the result of high expression of iNOS. The high expression of iNOS in *Aa* rats indicates pathological conditions as a consequence of *Aa* bacteria exposure.

CONCLUSION

Based on the exposure to bacteria *Aa* known by relation between MDA and iNOS expression in the parotid gland. Levels of MDA for control and *Aa* rats by T-test respectively, 3.135 ± 0.0436 ppm and 6.307 ± 0.0473 ppm. Meanwhile the numbers of cells that express iNOS is $0.166 \pm 0.003\%$ for control rats and increase $2.292 \pm 0.038 \%$ for *Aa* rats.

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