ORIGINAL ARTICLE

Effect of LTA *Lactobacillus Plantarum* (LP) Exposure Duration in Inflammatory Response (as Study Model of Dental Pulp Inflammation Wistar Rat)

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ABSTRACT

Introduction: *Lactobacillus plantarum* (Lp) were found lots in deep dental caries. Lipoteichoic acid (LTA) Lp induces inflammatory response by activation p38 MAPK expression in mitogen-activated protein kinase pathway. The nature and extent of altered inflammatory response are depended on intensity and duration exposure. To verify that duration exposure LTA Lp associated with inflammatory response in dental pulp. **Methods:** 45 samples of right upper molar of wistar rats were randomly grouping based on the type of injury. Control negative group, control positive group were given mechanically perforated, experimental group were given mechanically perforated and given LTA Lp 10 µg/ml. Observed in 24 h, 48 h, and 72 h and counting the amount of p38 MAPK expression. **Results:** there is increasing significantly of p38 MAPK expression between 24 h and 72 h. **Conclusion:** duration exposure LTA Lp associated with inflammatory response in dental pulp characterized by significantly increased of p38 MAPK expression.

Keywords: Inflammation, Infectious disease, Lipoteichoic acid, Macrophages, P38 MAPK

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INTRODUCTION

Dental caries is known the most infectious disease in oral cavity (1). Dental caries could develops and destrucs the tooth tissue. This decay occurs because of the interaction between teeth, biofilm, carbohydrate, and time on the tooth which influenced by bacteria. The main etiologic of dental caries is cariogenic bacteria which have the main role in initiation dan development of dental caries. The most dominant bacteria in dental caries and involved directly in progression of deep caries and tooth decay is *Lactobacillus*. *Lactobacillus* has 2 cariogenic properties, there are adhesion and acid-producing ability (2).

Lactobacillus is in saliva, dorsum tongue, mucus membrane, hard palate, dental plaque, and small amount on the surface of teeth (2). Lactobacillus is gram positive bacteria, has a rod-shape, includes to bacillus species, anaerob facultative, and nonspore (1,2).

In the oral cavity there are some species of colonizes bacteria in dental plaque. Those bacteria including *Streptococcus mutans* and Lactobacillus. Lactobacillus are adequate to produce acid, that lead demineralization process of dental hard tissue (1,2). Lactobacillus metabolism glucose that leads the environment of oral become acid. Those bacteria could cause decrease of pH value of less than 4.5 in the oral environment and are able to survive at a pH of up to 2.2. This acid environment could dissolve tooth enamel and may lead bacteria invasion into dentin and pulp. Critical concentration of Lactobacillus in saliva which can cause dental caries is ¬10¬5 CFU/ml. One of the predominant bacteria in dental caries in children is *L. plantarum* (1).

L. plantarum has a virulence factor called lipotechoic acid (LTA), which is an amphiphilic molecule that contains glycolipids and stick to the cytoplasmic membrane of bacterial cells and glyserol units or ribitol phosphate (1,3). LTA has a pleiotropic role in gram positive bacteria and it is the main surface component of immunocompetent cells which usually causes an inflammatory response from antigen- presenting cells such as macrophages (3).

Macrophages works as antigen precenting cell in the first line as a defense to against the pathogens. Macrophages as antigen preenting cells capable to recognize microbial ligands through the pattern recognition receptor (PRR) (4). There are several PRR classes that divided based on structure and specificity for various types of stimuli, one of them is Toll-like receptors (TLR) (5). TLRs are the main pattern recognition receptors of the innate immune system that provide various signals include Pathogen Associated Molecular Pattern (PAMP) (6).

LTA initiates the series of reaction by binding to specific receptors or nonspecific receptors. CD14 and TLR2 include to the specific receptors (7). LTA is a PAMP signal that will be recognized by TLR2 on macrophage cells (5). After recognition and binding of PAMP, TLR induces a cascade of intracellular signalling that leads in activator protein activation (6).

Bonding between LTA and TLR 2 will induce a signalling pathway. There is various signalling pathway, one of them is MyD88 dependent (7,8). Furthermore, MyD88 dependent will lead to the activation of mitogen - activated protein kinase (MAPK) (8). MAPK signalling pathway is mediator extracellular stimulation that leads to cellular response. The p38 MAPK signalling pathway is participated in important and crucial cellular processes, for example proliferation, differentiation, motility, apoptosis, and survival (9).

The series of MAPK pathways consists of MAPK kinase kinase (MKKK), MAPK kinase (MKK) in the form of MKK 3/6, and p38 MAPK. Activation of p38 MAPK depends on sequential phosphorylation of MKKK and MKK (8). Activation of p38 MAPK will result in stimulation of transcription factors including activator protein-1 (AP-1) and cAMP response element binding protein (CREB) (6).

The p38 protein is a class of MAPK that has an important role during the inflammatory response, especially in macrophage cells (6,7). The members of the p38 family are classified into four subtypes, there are α (MAPK14), β (MAPK11), γ (MAPK12 / ERK6), and δ (MAPK13 / SAPK4). p38α and p38δ are widely expressed in macrophages. p38α is involved in the expression of proinflammatory mediators in macrophage cells such as IL-1β, TNF-α, PGE2, IL-12, COX-2, IL-8, IL-6, IL-3, IL-2, and IL-1. Number of studies have shown that p38 has an important role in macrophage-mediated inflammatory disease (6).

Acute inflammation is an important part of cell defence mechanism, because it causes widening and increased permeability of blood vessels, migration of phagocytes, and tissue repair (1). The cells that most involved in the inflammatory response are phagocytic cells consisting of polymorphonuclear leukocyte (PMN) which accumulate within 30-60 minutes. PMN will phagocyte pathogens or damaged tissue and release lysosomal enzymes to destroy pathogens. If within 4 - 6 hours the cause of inflammation exists, the areas that contain microorganisms or external substances will be infiltrated by macrophages and lymphocytes (3).

The acute inflammatory process is relatively fast, which it takes 3 - 5 days (8). Neutrophil will increased in the first 24 hours, and then substitute with macrophage in 48 hours. After 48 hours, in 72 hours, neutrophil and macrophage decreased and the acute inflammatory process ended. Alteration in the nature and level of immunity based on many factors, one of which is the intensity and duration of exposure to the injury (4).

Based on the data research related to the properties of Lactobacillus palantarum in dental caries, pattern of acute inflammatory cells in 24, 48 and 72 hours and inflammation reaction through MAPK signaling pathway, the author needs to to verify effect of LTA Lactobacillus plantrum exposure duration in dental pulp inflammatory responses by analyzing the expression of p38 MAPK.

MATERIALS AND METHODS

Ethics Statement

This type of research is a true experimental laboratory that uses experimental animals as research objects. All procedures that have been carried out in this study are ethical, approved by the Ethics Commission of the Faculty of Dentistry, Airlangga University and have obtained a certificate number 300 / HRECC.FODM / VI / 2020.

Animals

This study used 45 male wistar rats (Rattus novergicus), aged 8-12 weeks, weigh 270-350 grams, and healthy rats. Feeding with Hi-pro-vite type 593 as much as 20 grams / day for each rat and given water ad libitum (always available for rats). Before treatment, the rats were kept for two months for adjustment of body weight and environmental conditions and for stress recovery. Rats were kept in plastic cages measuring 30 cm x 25 cm x 15 cm which were covered with husks. Each cage is filled maximum of 2 rats.

The number of research subjects was 45 rats and random allocation was carried out for the division of groups of rats based on the type of injury. There are divided into 3 groups. First, control group (-): no treatment, healthy pulp. Second, control group (+): was given mechanical injury then filled with cention. Mechanical injury is the preparation of cavity in the left maxillary molar of male Wistar rats using a round diamond bur until occurred pulp perforation. And third, Treatment group

of LTA 10µg/ml: was given mechanical injury and 10µg/ml LTA lesions then filled with cention. The LTA *L. plantarum* 10µg/ml.

In acute inflammatory process, neutrophil and macrophage plays very crucial and important role. The highest peak of neutrophil happened in the first 24 hours, and then decreased into the lowest peak in 48 hours, And then neutrophil substitute into macrophage in 24 - 48 hours. After 48 hours, in 72 hours, neutrophil and macrophage decreased and the acute inflammatory process will be ended.

Based on that data of exposure duration, each group which consisted of 15 rats will be divided into 3 groups, And each group have 5 rats that will be observed in 24 hours, 48 hours, and 72 hours.

LTA Lp

LTA Lp is extracted and purified from the Lactobacillus plantarum bacteria. This is certified by ULB Faculty of Science and Technology UNAIR. Then mixture of LTA and distilled water with a ratio of 20 µl of LTA and 780 µl of distilled water. Hence, concentration 10 µl/mL of LTA Lp is achieved.

Observations

Making histological preparations

The experimental animals from each group were subjected to necropsy according to the specified time at 24, 48, and 72 hours. Furthermore, the jawbone was taken and followed by cutting in the interdental area of the maxillary right first molar.

The process of making histopathological preparations consists of fixation, dehydration & infiltration, purification, paraffin infiltration, embedding, sectioning, and sticking to the object glass. Each paraffin block was cut, and each tissue sample was made into 4 µm thick slices. Furthermore, the preparation is used for immunohistochemical staining using dual phospho p38 MAPK antibody type rabbit polyclonal.

Interpretation

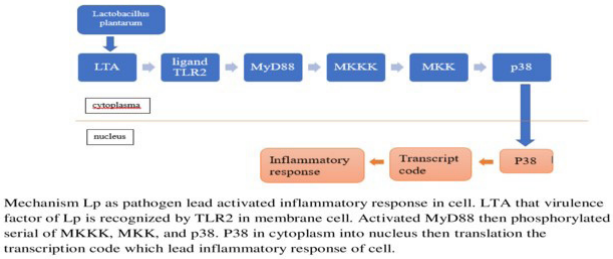
This study used perforated and treated rat tooth pulp tissue. Examination and calculation of the number of macrophage cells which indicating p38 MAPK expression were observed on each slide in the field of view with a magnification of 400x and as many as 10 fields of view.

Data analysis

This research is an experimental research. Randomized post test only control group design was the chosen research design, because initial measurements were not possible. To find out whether the data population of each sample group was normally distributed or not, the Shapiro-Wilk normality test was carried out. In this study, the results were normally distributed if the value ($p > 0.05$). Then the Levene homogeneity test was carried out and the results were homogeneous if the value ($p > 0.05$). Furthermore, a two way Anova test was carried out to determine whether or not there were differences in all experimental groups. If there are differences, continue with the Post Hoc test to find out the differences between groups. In this study, a statistical test was carried out at the 95% degree of confidence ($p \leq 0,05$).

RESULT

The inflammation response of the pulp was indicated by expression of p38 MAPK. The microscopic present p38 MAPK expression in macrophages in the pulp tissue using immunohistochemical staining on 24 h, 48 h and 72 h. Activated expression of p38 MAPK is described with Brown dots. P38 MAPK expression in negative control group of the pulp tissue at 24 h (Fig 2a), at 48 h (Fig 2b), and 72 h (Fig 2c). P38 MAPK expression in positive control group at 24 h (Fig 3a), 48 h (Fig 3b), and 72 h (Fig 3c). P38 MAPK expression in experimental group at 24 h (Fig 4a), 48 h (Fig 4b), and 72 h (Fig 4c). All figures with same magnification are 400x magnification.



Mechanism Lp as pathogen lead activated inflammatory response in cell. LTA that virulence factor of Lp is recognized by TLR2 in membrane cell. Activated MyD88 then phosphorylated serial of MKKK, MKK, and p38. P38 in cytoplasm into nucleus then translation the transcription code which lead inflammatory response of cell.

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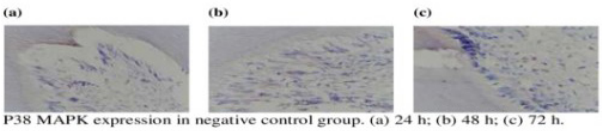
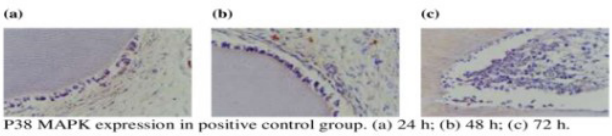


P38 MAPK expression in negative control group. (a) 24 h; (b) 48 h; (c) 72 h.

Fig. 2 : P38 MAPK expression in negative control group. (a) 24 h; **(b)** 48 h; **(c)** 72 h.



P38 MAPK expression in positive control group. (a) 24 h; (b) 48 h; (c) 72 h.

Fig. 3 : P38 MAPK expression in positive control group. (a) 24 h; **(b)** 48 h; **(c)** 72 h.

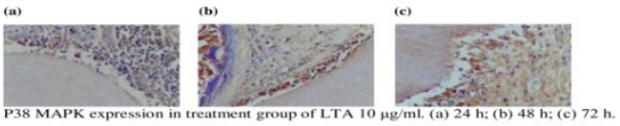


Fig. 3 : P38 MAPK expression in treatment group of LTA 10 µg/ml. (a) 24 h; **(b)** 48 h; **(c)** 72 h.

The induction of LTA affected on increasing the number of p38 MAPK expressions. Based on table I, it shows that p38 MAPK expression significantly increase in the control group (-) compared to the treatment group (p = 0.000), the control group (-) compared to the control group (+) (p = 0.001). Also shows p38 MAPK expression significantly increase in the control group (+) compared to to the treatment group (p = 0.000).

The duration of exposure to injury can increase the expression of p38 MAPK. Based on table 2, it showed that the amount of p38 MAPK expression was significantly increased in the 24 h group in the 72 h group (p = 0.003), while the amount of p38 expression was not significantly increased in the 24 h group comparison and the 48 h group comparison to the 72 h group.

DISCUSSION

In this study, LTA *L. plantarum* was used with consideration this bacterium is one of the main bacteria causing the development of dental caries and predominant bacteria in dental caries in children (1-2). *L. plantarum* had a great ability inducing pro inflammatory cytokine production from macrophages (3).

LTA is an important virulence factor of gram-positive bacteria which can lead inflammation and immune host response (4,5). LTA *L. plantarum* has a certain doses or concentration that can cause an inflammatory response. Based on the previous study that concentration of LTA *L. plantarum* which leads dental pulp inflammation is 10 µg / mL (6). Therefore, in this study we choose 10 µg / mL concentration of LTA L.plantarum to obseverd the p38 MAPK expression that cause inflammatory response in 24 h, 48 h, and 72 h.

Table I shows that there is a increasing significantly in p38 MAPK expression in the negative control group to the treatment group and in the positive control group to the treatment group. It is concluded that inducing LTA *L. plantarum* bacteria has an effect on increasing the amount of p38 MAPK expression. LTA *L. plantarum* had ability to induction cytokine by lead phosphoryation of p38 MAPK signalling pathway (7).

LTA is a bacterial component which recognized by receptors on host cells as a Pathogen Associated Molecular Pattern (PAMP). The cell receptors that recognize PAMP are called Pattern Recognation Receptors (PRR) which are located on the cell membrane. There are several types of PRR in cells, one of them is the Toll Like Receptor (TLR) (7,8). LTA gram-positive bacteria will be recognized through TLR2 on macrophage cells. After the ligand between LTA and TLR2 lead to activation of p38 MAPK through MyD88 activation. After p38 MAPK is activated, it will generate a transcription code that will increase various proinflammatory responses. Activation of the p38 MAPK signaling pathway will cause an inflammatory response in the form of cytokine production, migration and apoptosis in macrophages, monocytes, and neutrophils (6).

Table I also show that there is increase significantly in the control group (-) to the control group (+). It is concluded that mechanical injury which is perforated pulp because of preparation can lead activation of inflammation response characterized with increasing significantly the expression of p38 MAPK. Suitable with previous study that mechanical injury caused activation of body molecules released from damaged tissue, namely damage-associated molecular pattern (DAMP) one of them is heat shock protein (HSP70) (9). In normal condition, concentration of HSP70 is at a low level. There are various stimuli that make increasing HSP70 synthesis, there are environmental stimuli, pathological stimuli, or physiological stimuli (9,10). P38 MAPK expression is increased as a response of inflammation and stress stimuli, one of example is heat shock (11).

Table I : Comparison table of p38 MAPK expressions of each control group (-), control group (+), and treatment group

Group		P38 MAPK		Interpretation
		Mean ± SD	p	
Control (-)	Control (-)	3,13 ± 1,19		
	Control (+)	5,87 ± 2,03	0,001	Increasing significantly
	Treatment	11,80 ± 3,12	0,000	Increasing significantly
Control (+)	Treatment	11,80 ± 3,12	0,000	Increasing significantly

Table II : The expression for the amount of p38 MAPK in each time group

Group		P38 MAPK		Interpretation
		Mean ± SD	p	
24 h	24 h	5,60 ± 2,82		
	48 h	7,20 ± 4,78	0,070	Increasing not significantly
	72 h	8,00 ± 4,83	0,003	Increasing significantly
48 h	72 h	8,00 ± 4,83	0,732	Increasing not significantly

Table II shows that the duration of injury exposure can increase the amount of p38 MAPK expression. This can be due to increase the amount of p38 MAPK expression in acute inflammatory response. In inflammation process, there is increase amount of monosit that will differentiate into macrophage in tissue within 72 hours (12,13). Macrophages are one of the body's defense cells that are very important in the acute inflammatory process (14). Macrophages in tissue have an important role in the initiation, development and repair of inflammation (11). When bacteria invade, the macrophages on the tissue will be activated and phagocyte bacteria (14). Macrophages are providing signals to the body's defense cells in blood vessels, especially neutrophils and monocytes (15). Monocytes in blood vessels will infiltrate and differentiated into macrophages within 4 - 6 hours after the tissue is injured (15). Macrophage produce cytokine and mediator inflammatory by induce MAPK signalling pathways, that is activate p38 MAPK protein which the main play role in respon inflammation (11).

Based on previous research, inflammation in dental pulp induced p38 expression. Inflamed pulps significanlly increased p38 and pERK expression in neurons and other non neuronal cell types (6). A number of studies have indicated that p38 plays a significant role in inflammatory diseases mediated by macrophages, and, as a consequence, several p38 inhibitors have been developed to treat inflammatory diseases.

Activation p38 MAPK is stimulated with various factor, one of them are pathogen microbial and cytokine. the prolong exposure of injury lead increasing cytokine inflammatory level such as TNF-α (12). TNF-α is cytokine that can activate phosphorylation of p38 MAPK to produce inflammatory response (11). Once p38 MAPK is activated, phosphorilation process of p38 MAPK occurs cascade and continous (15). If the substance that causing inflammation which is in this study is LTA Lp is not eliminated, then phosphorylation of p38 MAPKwill continue as response to the presence of injury (12).

CONCLUSION

The highest increase of p38 MAPK expression happens in 24 and 72 hours after LTA exposure. So, it can be concluded that there is a relationship between the duration of LTA exposure to the inflammatory response in the pulp of the first molars of Wistar rats characterized by an increase in p38 MAPK.

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