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The Role of *Lipoteichoic acid* (LTA) *Lactobacillus plantarum* (*Lp*) on the number of tumor necrosis factor alpha (TNF-α) in the dental pulp inflammation (*in vivo* laboratory experimental research)

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Abstract

Background: Dental pulp inflammation caused by caries has higher prevalence than pulp inflammation caused by trauma. Lactobacillus is one of cariogenic and a gram-positive bacterium which is the largest lactic acid-producing bacteria. The species of Lactobacillus found in caries are Lactobacillus plantarum (Lp). Lp has the virulence factor Lipotechoic acid (LTA) which plays an important role in bacterial adhesion and induction of inflammatory mediators that cause tissue damage. Macrophage plays a role in the immune system and will increase during inflammation by producing proinflammatory cytokine, one of them is tumor necrosis factor alpha (TNF-α).

Objective: To know the role of LTA Lp on the number of tumor necrosis factor alpha (TNF-α) in dental pulp.

Methods: 48 wistar rats were divided into control and treatment groups. The tooth of samples in control group were perforated then filled with cention-N material, in other group, The tooth of samples in treatment group were perforated then added with LTA Lp 10 µg/ml and then filled with cention-N material. On day 1, 3, 5 rats were sacrificed and maxilla was removed and then stained with Hematoxylin Eosin (HE) on histopathological examination, the number of TNF-α was observed using microscope with 400x magnification. Statistical data analysis was carried out by using independent t-test.

Result: There is a significant difference in the number of TNF-α on day 1, 3, 5 and between the control and treatment groups.

Conclusion: Giving LTA Lp at a dose of 10 µg/ml can increase the number of TNF-α in the dental pulp.

Keywords: *lipoteichoic acid* (LTA), lactobacillus plantarum (LP), TNF-α, dental pulp

Introduction

Dental pulp inflammation caused by bacteria or trauma (injury). The first cause is due to bacteria exposure through the dentinal tubules in the dental crown has higher prevalence than the second cause is due to injury or fracture that causes a cut in the neurovascular supply to the pulp [1]. The prevalence of pulp inflammation due to caries is more common than pulp inflammation due to traumatic injury. This statement is in accordance with previous research that the prevalence of caries is 73.17% higher than traumatic injury to teeth, which is 20.9% [2]. This is also supported by research that about 55.6% of people have caries problems in their permanent teeth and 25.3% of people who experience trauma to their teeth [3].

Dental caries is a common infectious disease that affects 60-90% of children and most adults. Dental caries is an oral disease caused by cariogenic bacterial colonies, acid products from cariogenic bacteria, salivary influences, and genetics that cause damage to tooth structure. Dental caries is caused by an unbalanced process of demineralization and remineralization [4].

In the human oral cavity more than 700 species of bacteria have been detected. The largest species found in oral cavity are *Streptococcus* and *Lactobacillus. Lactobacillus* is the largest lactic acid-producing bacteria. In recent research consist of 93 samples of saliva with caries found 62,5% are *Lactobacillus* and 8,7% are *S.mutans* [5, 6]. *Lactobacillus* is the main bacterium that plays a role in the development of caries and deeper caries. *Lactobacillus* is an aciduric bacteria or has the ability as a pathogenic bacterium that is resistant to acidic conditions, which is up to pH 2, so it has a better survival than other genera of bacteria [9, 10] Isolation of *Lactobacillus* species obtained from saliva samples of patients with caries found that *Lactobacillus plantarum* (*Lp*) colonies were 15.08% and *Lactobacillus acidophilus* (*La*) colonies were 10.61% [11]. *Lp* has a dominant

amount than *La* because *Lp* is able to live at a more acidic pH than *La* [10, 12]. *Lp* is able to absorb a lot of sugar and form most of the amino so that it can associate with various surfaces and substrates for growth [7].

Species of *Lactobacillus* has virulence factor *Lipoteichoic acid* (LTA) which is derived from *glycolipid polymers* in cell membranes, which contain repeating *phosphodiester-linked plyols* called *Teichoic acids* (TAs) [13]. LTA plays an important role in bacterial adhesion and induction of inflammatory mediators that cause tissue damage [14]. LTA with a low dose of <0.5 µg/ml could not cause an inflammatory response so that pro-inflammatory cytokines were not produced, while LTA with a high dose of 0.5 µg/ml – 10 µg/ml was proven to be effective and optimum in causing a response inflammation. LTA *Lp* with a dose 10 µg/ml proven increase the number of neutrophil in dental pulp after 24, 47, and 72 hours [15, 16].

Under normal circumstances, the humoral response to acute inflammation occurs within the first 24 hours, is characterized by neutrophils as the predominant cell type. The peak of the acute inflammatory phase occurs when the number of monocytes that mature into macrophages increases [17]. The inflammatory response characterized by increasing the number of macrophage which able to recognize microbes through *patter recignition receptors* (PRRs) in the form of Toll-like receptors (TLR) [18].

LTA *Lp* is a signal of Pathogen-Associated Molecular Pattern (PAMP) which will bind to the TLR 2 receptor and produce pro inflammatory cytokines [17]. TLR 2 able to activates *nuclear factor kappa-light-chain-enhancer of activated B cell* (NF-κB) through *Myeloid Differentiation Primary-Response Protein 88* (MyD88). MyD88 will mediating IκB phosphorylation and production of proinflammatory cytokines will increase. TNF-α is the main proinflammatory cytokine which starts to produce on day 1, peaked on day 3 and began to decrease on day 5 [19]. Based on the above background, there is an opinion about the role of *Lp* in causing inflammation, so the researchers conducted a study on the role of LTA *Lp* on inflammation through the number of l TNF-α in the dental pulp of wistar rats.

Materials and Methods

Samples and Preparation of Animals

This type of research is an *in vivo* laboratory experiment using white male *R. novergicus* with the following criteria such as male, age 3-4 months, weight 270 to 300 g and still has the maxillary left first molar is fully developed and in good physical condition (no caries).

This study involved 48 specimens of *R. novergicus* with the number of each group at each observation time consisting of eight rats. This study was divided into two groups (first group with left maxillary molars were mechanically perforated, followed by 10 µg/mL LTA-*Lp* induction and then filled with fixed fillings and the other group. And the second group the left upper molar was mechanically perforated and then filled with a fixed filling. The observation times were divided into day one, two and three.

Extraction and Purification of *Lipoteichoic acid* (LTA) from *Lactobacillus plantarum* (*Lp*)

L. plantarum was cultured in Mueller-Hinton broth (Biokar Diagnostics, France) at 37°C and centrifuged at 150 rpm for 24 hours. After reaching the final *log phase*, 400 mL cultures were then centrifuged at 4,000 rpm for 15 minutes at 4°C. Cells were then washed three times withc0.1 M tris-HCL buffer, pH level 8 (Furobro, France) and then resuspended at 20 mL, 0.1 M acetate buffer, pH level 4.7 (Fisher Scientific, UK). Cells were mixed with N-Butanol and incubated for 30 minutes at 37°C with stirring at 300 rpm.

The extraction process started with the cells being centrifuged at 13,000 rpm for 15 minutes at 4°C. The aqueous phase was collected, resuspended in 20 mL of 0.1 M ammonium acetate buffer, pH level 4.7, and then sonicated three times; each for 1 minute to break the cell wall. After sonication, the cell suspension was mixed with N-Butanol for 30 min at 37°C and centrifuged at 300 rpm to produce crude lipoteichoic acid. The fractions in each extraction were placed at pH 8.5 with NH3, incubated overnight at 37°C, and monitored by *gas chromatography mass spectrometry*. *Lipoteichoic acid* castle fixation was achieved by adding 0.1M ammonium acetate buffer with 15% N-Propanol (pH = 4.7) and then filtered.

Lipoteichoic acid (LTA) of *Lactobacillus plantarum* (*Lp*) Dosages

The 0.4779 mg LTA-*Lp* were diluted with 1 mL of distilled water and shaken until homogeneous using a vibrator (Vortex). The LTA-Lp 10 µg/mL obtained from 20 µL of LTA-*Lp* solution was put into Eppendorf and then 780 µL of distilled water were added and shaken until homogeneous.

Lipoteichoic acid (LTA) of *Lactobacillus plantarum* (*Lp*) Induction Procedure

Each R. novergicus was anesthetized with Ketamine HCL (Ketalar, Warner Lambert, Ireland, 70 mg/kg BW) and Xylazine base (Xyla, PT Tekad Mandiri Citra, 7 mg/kg BW) dissolved in phosphate buffer saline according to applicable regulations. observation day group.cThe teeth of rats were treated with LTA *Lp* at a dose of 10 µg/ml. LTA was applied to the pulp surface using a micropipette as much as 1 µL, waited 5 seconds and then pressed with a paper point. The procedure for giving LTA was carried out 5 times in each rat. The cavity was cleaned with a cotton pellet and filled with filling material (Cention-N, Ivoclar Vivadent, Liechtenstein) using a probe, then irradiated with a light cure technique for 40 seconds, then irradiated with a light cure technique for 40 seconds. After days 1, 2, and 3 of this procedure, the left upper molar was extracted under anesthesia.

Analysis of the Number of TNF-α on Dental Pulp

Histological preparations were made using hematoxylins and eosin (HE) staining on dental specimens, and the number of TNF-α in each group was analyzed on the dental pulp. The histopathology of the pulp tissue was observed using a Nikon E100 light microscope with ×400 magnification in 10 fields of view to see the histological types of the dental pulp sections in the perforated area.

Data Analysis

The results of the study were calculated as mean and standard deviation. The normality test used the *Shapiro Wilk test*, followed by the homogeneity test using the *Levene test*. The parametric test was performed by using a *Independent t-test* with $p < 0.05$ considered as a significant difference.

Results

Table 1: Mean and standard deviation of TNF-α count for each group on day 1, 3, 5

Observation Time	Group	N	Mean	SD
Day 1	Control	8	3.13	0.515
	Treatment	8	4.5	0.378
Day 3	Control	8	5.13	0.515
	Treatment	8	5.50	0.423
Day 5	Control	8	3.00	0.378
	Treatment	8	6.88	0.515

Table 1 shows that the higher mean results were seen on treatment group than control group. Number of TNF-α continuously increase from day 1, 3 until 5.

Table 2: Test for normality, test for homogeneity, and test for differences in each research group

Observation Time	Group	Normality	Homogenity	Independent T-test
Day 1	Control	0.516	0.666	0.049*
	Treatment	0.120		
Day 3	Control	0.516	0.666	0.583
	Treatment	0.274		
Day 5	Control	0.120	0.666	0.000*
	Treatment	0.516		

Table 2. The homogeneity test column obtained $p > 0.05$ for all observation times and group, so it is said to have homogeneous data. The difference test column above showed a significant difference $p < 0.05$, for day 1and 5.

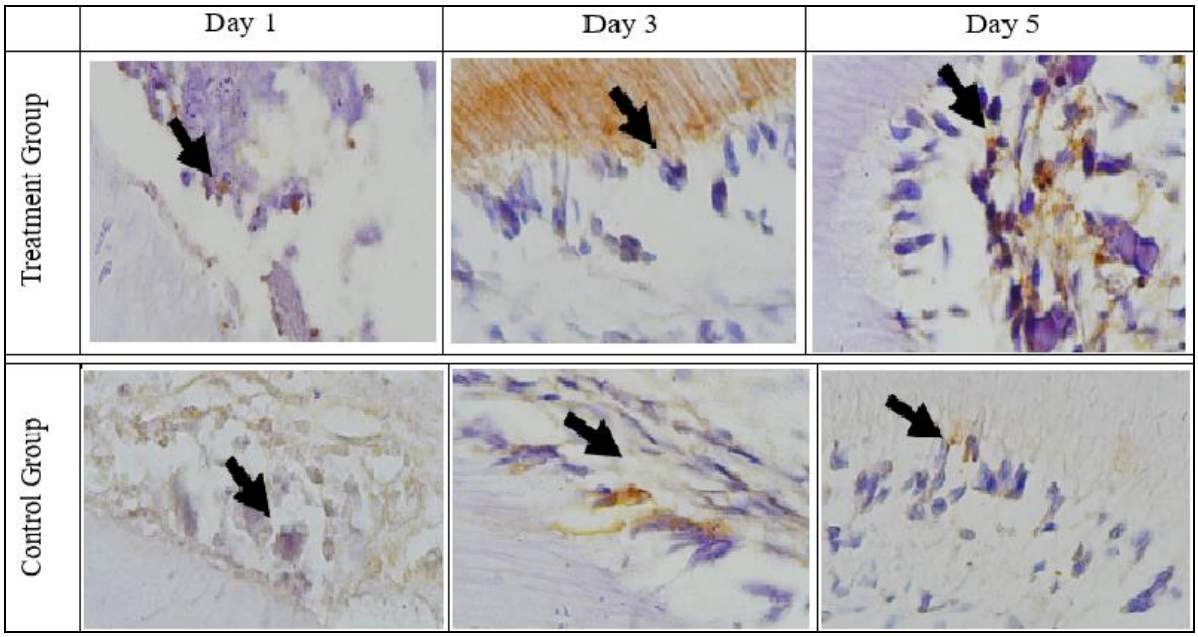

Fig 1: The number of TNF-α in the dental pulp (black arrows) used IHC staining with light microscope on ×400 magnification

Discussion

The mean results in table 1 on the control groups showed the number of TNF-α begin to start on day 1, then the highest number of TNF-α on day 3 and the lowest number of TNF-α on day 5. It is suspected that macrophage act as an immune response producing TNF-α as proinflammatory cytokine in the wound area since day 1. This is in accordance with the recent research which proved that macrophage was already in the wound area on day 1, then increased significantly on day 3, and decreased on day 5 and 7 [19, 20]. Therefore, the number of macrophage that producing TNF-α on day 1 was not much because phagocytic activity against injury was dominated by neutrophils. The number of TNF-α on day 5 decreased and start to replace by another anti-inflammatory cytokine such as IL-10 [19].

The mean results in table 5.1 on the treatment group continuously increase from day 1 to day 5. On the day 5, the number of TNF-α still increase compared to control group. *Lp* bacteria and mechanical injury are recognize as foreign antigens and will be captured by *antigen precenting cell* (APC) and then will bind to the *T cell receptor* (TCR) through the recognition of the *Major Histocompatibility Complex* (MHC) and protein antigen by the *T cell receptor* (TCR) [18]. APC will present antigens to T *helper* and differentiate into Th1 and Th2. Th1 will produces proinflammatory product, such as IFN-ɣ. Meanwhile Th2 will produce anti-inflammatory, such as IL-4 and IL-13. The activation of Thelper because of antigens will stimulate macrophage to the wound area. Macrophage have two different fenotypes, M1 produces proinflammatory cytokines begin on day 1, peaked on day 3 and decreased on day 5. M2 produces anti-inflammatory cytokines begin on day 3 and peaked on day 5. The imbalance of proinflammatory and anti-inflammatory cytokines on the inflammation process will affect the dental pulp tissue and wound healing process [18, 19, 20].

The results of the different test in table 2 of the treatment group showed a significantly greater increase in the number of TNF-α which was significantly different than the control group. It is suspected that in the treatment group there was exposure to LTA *Lp* 10 µg/ml, causing LTA as a virulence factor for gram-positive bacteria containing phosphate and glycolipid in cell walls that have an acidic pH so that TLR-2 can be recognized. LTA Lp has a 50% *D-alanine* structure that plays a role in *Lp* bacterial colonization which makes it easier for Lp bacteria to form biofilms compared to other *Lactobacillus* species so that inflammation occurs in the dental pulp [22, 23].

Lp has the ability to code for the absorption of many sugars and forms most of the amino acids. The large number of surface-linked proteins indicates that *Lp* can associate with various surfaces and substrates for growth, so that Lp becomes the dominant bacterium and can cause inflammation on the dental pulp [7]. Association of bacteria on the host cell surface through cross-linking between bacterial proteins and host cell *fibronectin*. *Fibronectin* is an important component in the extracellular matrix which helps in cell attachment. *Fibronectin* is a receptor for LTA on host cells [23].

LTA *Lp* can be recognized by TLR-2 because there is a third *acyl* chain in the *trihexosyl-diacyl glycolipid* structure. LTA has more *acyl* chains which makes it easier for *Lp* to attach to host cells so that *Lp* can enter host cells. The *acyl* chain plays an important role in TLR-2 stimulation. Because *Lp* has more *acyl* chains, more TLR-2 stimulation, so that more pro-inflammatory cytokines are produced [22, 24]. This is consistent with another study conducted by Kang *et al*., 2016 which has proven that LTA is a strong activator of TLR-2. When LTA enters the plasma, it is recognized by TLR-2 because TLR-2 is expressed on the cell surface, namely odontoblast cells, to recognize bacterial components such as LTA, and binding occurs between LTA and TLR-2 on the cell surface.[24]

The activation of TLR-2 will stimulate *Myeloid Differentiation Primary-Response Protein 88* (MyD88) and the inflammation transduction signal below. MyD88 will bind with TNF *Receptor-Associated Factor 6* (TRAF6) and mediates phosporilation of IκB, then the binding between IκB and NF-κB will release and cause transclocation of NF-κB to the nucleus. Translocation of NF-κB stimulates the producing of proinflammatory cytokines increase, such as TNF-α. The increase number of IFN-ɣ by Th1 also increase the producing of proinflammatory cytokines increase, such as TNF-α [18, 19, 20]

The significant difference in the number of TNF-α between the control and treatment groups, also thought to be due to the acidic pH produced by *Lp* bacteria. Lp can live well in very acidic environments. Lp can grow at pH below 3.5 and can survive at pH 2.[12]Acidic pH can cause environmental conditions outside the dental pulp cells to become hypertonic. Hypertonic causes the fluid in the cytoplasm to be pulled out and the cell becomes shriveled. This can trigger damage to odontoblast cells on the surface of the dental pulp, causing inflammatory reaction. In the inflammatory reaction, there will be an infiltration of inflammatory cells in the dental pulp to eliminate the injury [25].

Conclusion

Thus, from the results of the study in the treatment group, it was found that the number of TNF-α cells was significantly different than the control group. This shows that research on the role of LTA *Lp* at a dose of 10 µg/ml is able to induce pulp inflammation by increasing the number of l TNF-α in the dental pulp of rats.

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