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Systemic IL-1β and TNF-α Productions of *E. coli* Lipopolysaccharide-Induced Periodontitis Model on Rats

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Abstract

Periodontal disease, a common inflammatory oral disease involved periodontal tissues, has been linked with the evidence of some systemic disorders. Recently, periodontal disease has been suspected as a trigger of systemic disorders. Penetration of bacterial products, such as lipopolysaccharide (LPS) may reach into deeper periodontal tissues. Therefore there may affect systemic blood and cytokines production. Interleukin-1β (IL-1β) and Tumour Nuclear Factor-α (TNF-α) are known as pro-inflammatory cytokines. The production of systemic IL-1β and TNF-α of *E. coli* lipopolysaccharide-induced periodontitis model on rats was investigated in this research. Fifteen male Wistar rats, aged 6-8 weeks used for this study were divided into 3 groups. For group 1 and 2, silk ligature 3/0 were inserted in interdental area between upper right molar 1 and 2. First and second group received solution containing 10μg/ml and 1mg/ml *E. coli* lipopolysaccharide, respectively, mixed with 2% carboxymethylcellulose (CMC) diluted in 100μl of phosphate buffer saline (PBS). The solution was topically applied on gingival tissues around the gingival sulcus, a single topical application of solution once per 2 days for 14 days. Untreated subjects were used as negative control. On day 15, the blood was collected from vena orbitalis, and rats were sacrificed. The blood serum of each group was divided into 2 groups and cultured for 4 hours with or without 20μl of 100ng/ml of *E. coli* LPS. ELISA techniques were used to measure the cytokine productions of the supernatant. The data was analysed using Repeated Measure ANOVA. This study showed that there was a significant increase of IL-1β production on low dose of LPS compared to control and high dose of LPS groups ($p<0.05$). Whereas TNF-α not significantly showed increasing trend. The increasing trend of pro-inflammatory cytokine productions, such as IL-1β and TNF-α, on LPS-induced periodontitis model in this experiment supports the previous studies about the contribution of periodontal disease in the pathogenesis of systemic diseases.

Keywords: IL-1β, TNF-α , E. coli LPS, rat's periodontitis model

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1. Introduction

Periodontitis is a chronic inflammatory disease affecting periodontal tissues[1]. The progress of this disease is multifactorial and often caused by the interaction between specific bacterial infections and the host immune responses. At early periodontitis stages, the lesions are characterized by the migration of leucocytes into the gingival sulcus, loss of the collagen fiber network and proliferation of basal cells in the junctional epithelium. At advance stages, bacteria and bacterial products act on inflammatory cells inducing production of various inflammatory mediators and cytokines leading to destruction of the organ supporting the teeth, including soft tissues and bone, either directly or indirectly [2,3].

A major part of the outer membrane of gram-negative bacteria (lipopolysaccharide / LPS) is the most potent imunostimulator in mamals that induces the synthesis of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) from macrophages, fibroblasts, and endothelial cells [4]. It is widely accepted that LPS penetrates deeper in the periodontal tissues, recruits and activates immune cells[5]. The elevation of IL-1, and TNF productions has been detected in the gingival crevicular fluid and inflamed gingiva of patients with periodontal disease[2,6]. These cytokines appear to play a central role in the pathogenesis of inflammatory periodontal diseases. The application of *E. coli* LPS into the rat gingival sulcus induces the production of pro-inflammatory cytokines such as TNF-α, IL-1α and IL-1β[7].

The relationship between poor oral health condition, particularly periodontal disease and systemic disorders has been extensively investigated. Recent studies suggest that this relationship may exist, which periodontal disease may trigger the initiation of several systemic diseases or conditions, such as diabetes mellitus, atherosclerosis and pulmonary disease, lung disease, osteoporosis and preterm low birth weight infants[8]. Munford[9] has shown that the trace of [^{3}H]LPS has been found in spleen, liver, adrenal gland, and lung, 10 minutes after an intravenous injection of LPS on rat. Therefore, it is predicted that the local LPS induction on bone jaw may also affect systemically including some internal organs and blood system. However, at this stage, the exact mechanism remains unclear.

Cytokines are small polypeptides with a wide spectrum of inflammatory, hemopoietic, metabolic and immunomodulatory properties. Comparable to bacterial infection, bacterial lipopolysaccharide is the potent immunostimulator that can induce acute inflammatory responses[10]. They are produced by a variety of cells, including blood cells. As a consequence, cytokines and their receptors form a network of high complexity that is under tight but complex biological control, including positive and negative feedback by the cytokines themselves.

LPS-stimulated blood cell have been used to investigate potential differences in immune capacity[11,12]. Blood monocytes become activated by the LPS and release pro-inflammatory cytokines and prostaglandins. IL-1β plays a major role in acute and chronic inflammation and is secreted mainly by blood macrophages[13]. Another important inflammation marker in blood serum is TNF-α[10]. LPS from gram-negative bacteria are potent TNF-α inducers. The peak of circulating TNF occurs rapidly within 90 min after LPS or bacteria injection and The TNF-α peak occurs before of IL-1, IL-6, and IL-8. Other study revealed that TNF-α mRNA levels peak *in vivo* at 15 min after LPS injection and at 1 hr in *in vitro* assays[14]. This study is to investigate the level of IL-1β and TNF-α from *ex vivo* whole blood culture of *E. coli* lipopolysaccharide-induced periodontitis model on rats.

2. Materials and Methods

Male wistar rats (6-8 weeks old, 200-250 grams) were purchased from LPPT Universitas Gadjah Mada were used in all studies. Purified *E coli* serotype O55:B5 lipopolysaccharide (phenol-extract) was purchased from Sigma Chemical Co. Ten mg/ml or 1mg/ml of *E. coli* LPS were mixed with 2% carboxymethylcellulose sodium salt high viscosity (MP Biomedicals, Inc) and diluted in 1 ml of phosphate buffer saline (PBS) to make a thick solution. Rat ELISA Kit for TNF-α and IL-1β were purchased from Bender MedSystem.

2.1. Periodontitis model

The model of periodontitis done in this research was the modification of Hasturk's method[15]. Briefly, a total of 15 male wistar rats were divided into three groups: two treated groups and control group. Under general anaesthesia (50-75mg/kg of Zolethyl, Virbac Lab., France; IM injection) rats were fixed on his back. Ten rats were treated by inserting silk ligature 3/0 in the interdental area between upper right molar 1 and 2. Fifty ml of LPS slurry solution was then topically applied to the gingival tissues around the gingival sulcus, a single topical application of solution once per 2 days for 14 days. The ligatures were checked at every application and lost ligatures were replaced. Untreated subjects were used as negative control. On day 15, under the anaesthesia, the blood was withdrawn from vena orbitalis into 3ml EDTA containing tube (Becton Dickinson Vacutainer System, Franklin Lakes, NJ, USA) and rats were sacrificed. The experimental protocol has been approved by Ethics Committee.

2.2. Ex-vivo culture

Whole blood (990ml) was diluted 1:1 with RPMI 1640 (Sigma Chemical Co.) containing 25mM HEPES, 100 U/ml benzylpenicillin, dan 100 μg/ml of streptomycin. The mixtures were set up in 24 flat-bottomed plates (Iwaki, Japan). Cultures were incubated with or without 20μl of 100ng/ml E. coli LPS for 4 hours at 37°C in a 5% CO_2. After centrifugation, supernatants were collected and stored at -80°C until cytokine determination. ELISA techniques were used to measure the cytokine productions of the supernatant according to the manufacture's instructions. The data was analysed using Repeated Measure ANOVA.

3. Results and Discussion

In vivo periodontitis model reflected initial LPS challenge which was done by inserting silk ligature 3/0 in the molar interdental area followed by topical application of 10μg/ml or 1mg/ml LPS solution around the gingival sulcus. The production of the inflammatory mediators IL-1β and TNF-α was measured in the supernatants of the whole blood of the periodontitis model after 4 hours ex vivo culture with or without LPS secondary challenge.

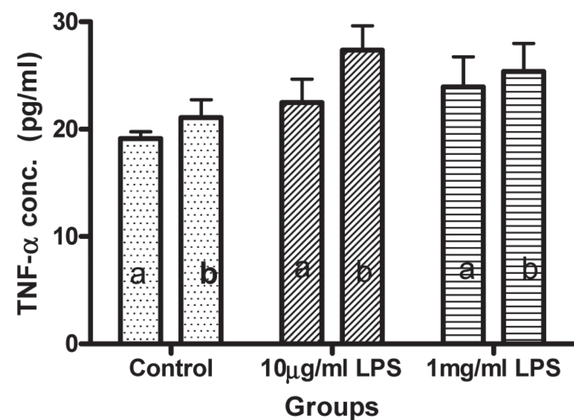

Figure 1. TNF-α level of supernatant from *ex vivo* whole blood culture of periodontitis model. a) without and b) with 20ml of 100ng/ml LPS secondary challenge.

Figure 1 showed TNF-α level between control and the two treatment groups. Results showed no significant difference between the study groups (p=0.1045). However, TNF-α level measured in supernatant of the 10μg/ml LPS group with second LPS challenge showed the highest TNF-α level compared to other groups. The high dose of LPS was comparable to the low dose. Repeated measures ANOVA also showed that pairing were not effectively significant. Therefore, there was no difference of TNF-α level from the supernatant between culture with and without LPS secondary challenge in all study groups (p=1.223). This finding was similar to the research done by Fokkema, *et al*. [11]. That study showed that there was no difference on TNF-α level in whole blood culture between periodontitis patients and control indicating that the peripheral blood cells of patients with periodontitis showed a similar competence as control subjects to produce these latter cytokines. The relationship between clinical parameters and concentrations of the pro-

inflammatory cytokines is important in the initiation and progression of periodontal disease, within inflamed gingival tissues and serum samples from patients with severe chronic periodontitis[16].

Preliminary study done by Queiroz et al. showed that the concentrations of IL-1β, TNF-α, IL-2, IFN-γ were, on average, significantly higher in serum samples and gingival tissue biopsies from periodontitis patients than in healthy controls. However, there was no association between cytokine concentrations and clinical parameters of periodontitis was found since serum samples from both groups showed high individual variability of cytokine profiles[17].

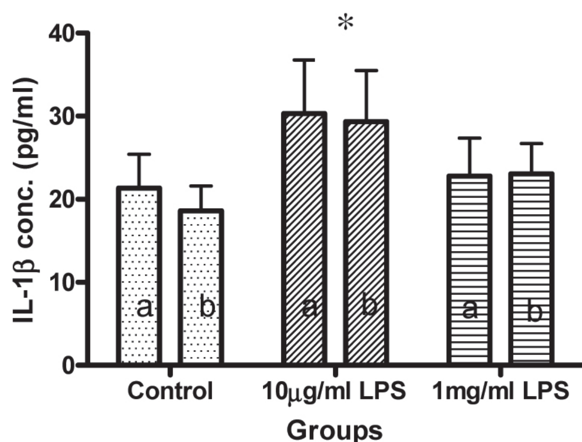

Figure 2. IL-1β level of supernatant from *ex vivo* whole blood culture of periodontitis model. a) without and b) with 20μl of 100ng/ml LPS secondary challenge.

*** significant difference ($p<0.05$) compared to control and 1mg/ml LPS groups.**

IL-1β showed similar trend with the previous cytokine with no significant matching between supernatant with and without LPS secondary challenge. However, low dose of LPS topical application resulted in significant higher cytokine production ($p<0.05$) (Figure 2). There was no significant difference between control and high dose of of LPS. High dose of LPS can be lethal, however, some studies have shown that repeated application of endotoxin can reduce immune response. This phenomenon is firstly investigated by Beeson and referred as endotoxin tolerance[18].

Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor alpha (TNF-α) are involved in Th1 immune responses and induce mainly cell-mediated immunity[16]. LPS-induced cytokine expression is via a pathogen ligand called toll-like receptors (TLRs). TLRs trigger a cascade of signaling pathways leading to the activation of NF-κB, which in turn activates the expression of cytokine genes, including IL-1β, TNF-α, and IL-6. TLR4 is a predominant signaling receptor in human whole blood. TLR4, but not TLR2, contributes in mediating cellular activation in response to *E. coli* LPS[19]. However, Faure, et al. shown that *E. coli* LPS also induces TLR2 expression in human endothelial cells[20].

Secondary challenge of LPS in ex vivo whole blood culture showed no differences compared to culture without LPS challenge. This findings contrasts to some studies that showed the reduction of serum level of TNF-α in the animal groups receiving repeated injection of LPS compared with the groups that only received a single dose of LPS[10]. The *ex vivo* challenge of 1μg/ml *E. coli* LPS into the whole blood collected from mice, twenty four hour after *i.v.* injection 100μg of *E. coli,* have resulted in a significant decrease of TNF-α level from cells supernatants[21]. This discrepancy may due to the application method of *in vivo* LPS initial challenge in periodontitis model. In this experiment, LPS was topically applied onto the periodontal tissues, whereas some previous studies applied the LPS by directly injected into the body system. Only some of thick slurry LPS applied onto the gingival tissue was able to penetrete deeper tissues and this took more time than injection method. The time taken for mimicking periodontitis model were 2 weeks, that might not sufficient to induce or impair immune response. These difference methods affected the immune response thus failed to prove the existence of endotoxin tolerance phenomenon.

4. Conclusion

Periodontitis model in rats done by the combination of ligature insertion and LPS topical application change the trend of IL-1β and TNF-α production. This experiment, once again, proved the contribution of periodontal disease in the pathogenesis of systemic diseases. Further study needs to be done to investigate close relationship between periodontal and systemic disease.

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