Secretory Leukocyte Protease Inhibitor (SLPI) Decreased the Cellular Expression of NF-Kβ and IL-1β on Wound Macrophages of Rattus novergicus Post Tooth Extraction

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ABSTRACT

Prevalence of tooth extraction or dental surgery was 48.5% of all dental care in Indonesia. Tooth extraction carries potential health risks and side effects such as pain, swelling, trismus and dysfunction of the oral cavity during recovery. Secretory leukocyte protease inhibitor (SLPI) is one of innate immunity proteins that can inhibit the activation of macrophages. We are expecting the provision of SLPI can decrease excessive inflammatory response in healing after tooth extraction. This study was to investigate the administration of SLPI on cellular expression of NF-Kβ and IL-1β on wound macrophages of *Rattus novergicus* post tooth extraction. The research design is in vivo experimental study. In total, 20 rats were randomly divided into four groups (each group n=5) and underwent tooth extraction on left incisor teeth of mandible. One of the groups did not receive SLPI administration (control group) and the socket was stitched after tooth extraction. Meanwhile, the remaining three groups (experimental groups) were given SLPI administration after tooth extraction with three different doses (0.1 μM, 0.5 μM and 2.5 μM, respectively). After SLPI administration, the socket of experimental groups was stitched. The effects of SLPI administration were evaluated by counting at the percentage of NF-Kβ translocation and the expression of expression of IL-1 in macrophages cells of the rat socket using immunohistochemistry analysis. The cellular expression of NF-Kβ and IL-1β were significantly decreased ($p < 0.05$) on groups with SLPI may decrease cellular expression of NF-Kβ and IL-1β on wound macrophages cells of rats post tooth extraction in a dose-dependent manner.

Keywords: *Tooth extraction, Inflammation, SLPI, Macrophages, NF-Kβ, IL-1β*

INTRODUCTION

Tooth extraction is the removal of teeth from the dental alveolus (socket) in the bone which is the most widely performed by dentist. According to survey by RISKESDAS 2007 [1], the prevalence of tooth extraction or dental surgery was 48.5% of all dental care in Indonesia. Tooth extraction carries potential health risks and side effects such as pain, swelling, trismus and a general dysfunction of the oral cavity during recovery.

Post tooth extraction, the wound induces platelet aggregation and produces chemokine to attract neutrophils in the lesion. After 48 hours, neutrophils undergo apoptosis and replaced by macrophages. Macrophages will be actively induced by LPS bacteria residing around the lesion in the mouth and stimulate the activation of macrophages into the lesion. Activated macrophages secrete proinflamatory cytokines, such as Tumor Necrosis Factors α (TNF-α), interleukin-1 (IL1), interleukin-6 (IL6), interleukin-8 (IL8) and interleukin-12 (IL12) [2]. In the prolonged inflammation and delayed wound healing, activated macro-phages may secrete excessive cytokines such as TNF-α and IL1. Thus, it may lead to excessive inflammation.

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Secretory leukocyte protease inhibitor (SLPI) is a cationic protein with 11.7 kDa and one of innate immunity proteins. SLPI is a non-glycosylated protein, acid-stable, cysteine-rich, 107-amino acid and single chain polypeptide [3]. Some studies showed that mice deficient SLPI or SLPI gene deletions may cause impair or delay wound healing [4, 5]. It has been known that SLPI may down regulated macrophage response to bacterial lipopolysaccharide (LPS) [3]. In vitro study by Sano *et al.* (2003) [6] found that SLPI may inhibit the activation of NF-Kβ and TNF- α in macrophages that was stimulated by LPS. Other studies also performed that administration of SLPI may inhibit the secretion of TNF-α inflammatory mediators in spinal cord injury and arthritis [7, 8]. Decreasing expression of IL1 by macrophages may accelerate the lesion entering proliferative phase of healing through increased the synthesis of fibroblasts, collagen and ECM.

The aim of this study was to investigate the effect of SLPI administration on pro-inflammatory cytokines post tooth extraction using in vivo models of *Rattus novergicus* by counting at the precentage of NF-Kβ and IL1 on activated macrophage cells.

MATERIALS AND METHODS

Ethics

This research was approved by Health Research Ethics Commission from Faculty of Medicine, Brawijaya University, Indonesia with ethical number 355/EC/KEPK-S2/06/2015

Chemicals

SLPI was used in this experiment is a Human Recombinant SLPI (R&D system, 1274-PI). Antibodies anti-NF-kB/p65 Ab-1 (LOT: 1638P8031, Neomarker) and anti-IL-1β antibody (7884, Santa Cruz).

Research design

The research design is in vivo experimental study that using Randomized Post Test Only Control Group Design. In total, 20 rats were randomly divided into four groups (each group n=5) and underwent tooth extraction on left incisor teeth of mandible. One of the groups did not receive SLPI administration (control group) and the socket was stitched after tooth extraction. Meanwhile, the remaining three groups (experimental groups) were given SLPI administration after tooth extraction with three different doses (0.1 μM, 0.5 μM, and 2.5 μM, respectively). After SLPI administration, the socket of experimental groups was stitched. The effects of SLPI administration were evaluated by

counting at the percentage of expression NF-Kβ and IL-1 in macrophages cells of the rat socket.

Tooth extraction of rats

R. norvegicus were anesthetized intraperitoneally by mixing Ketalar (60 mg/kg) and Diazepam (10 mg/kg) using syringe (1 mL). Then, the rats underwent aseptic on their extraction site. Mandibular incisor tooth extraction is done by using clamp that is specifically modified for rat tooth extraction. After luxation the teeth using Lecron that is modified as being, the teeth was clamped with clamp and extracted.

SLPI administration

SLPI was given post tooth extraction on the rat socket. The experimental groups received SLPI 0.1 μM, 0.5 μM, and 2.5 μM, respectively using specific pipette after the socket is cleaned up by gauze. The wound was stitched with silk, half moon needle (Mani 1.8) and needle holder. Meanwhile, the control group underwent stitched socket after tooth extraction without SLPI administration.

Sampling

After 5 days of tooth extraction, the rats were sacrificed, the mandible of rats were put into a tube containing 10% of formalin for tissue fixation, then soaked in 14% EDTA for 30 days and labeled.

IHC staining

After HPA preparation, IHC staining was done by IHC kit, NF-Kβ (anti-NF-Kβ/p65 Ab-1 LOT:1638P8031, Neomarker) and IL-1β antibody (IL-1β anti-7884, Santa Cruz) on the slide. The results were observed under the light microscope with 400× magnification. Cellular expression of NF-Kβ p65 subunit was obtained by counting the number of macrophages that was detected by kromagen DAB (brown) in the cytoplasm and nucleus, in the form of the average percentage in 10 visual field (Figure 1). Cellular expression of IL-1β was obtained by the number of macrophages that was detected by kromagen DAB (brown) in the cytoplasm and around cells, in the form of the average percentage in 10 visual field (Figure 3).

Statistical analysis

All data are presented as mean ± SD. The statistical analysis was performed using the *IBM SPSS Statistic 20 Windows* (IBM Corp., Armonk, NY, USA). Differences between groups were determine by One Way
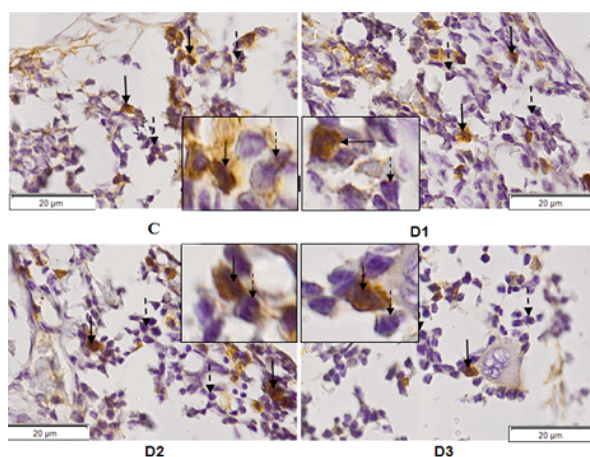
Figure 1. IHC staining: Cellular expression of NF-Kβ (C = Control; D1 = Treated with dose 1 (0.1μM); D2 = Treated with dose 1 (0.5μM); and D3 = Treated with dose 1 (2,5μM))

Figure 2. Percentage of macrophage cells as cellular expression of NF-Kβ (C = Control; D1 = Treated with dose 1 (0.1μM); D2 = Treated with dose 1 (0.5μM); and D3 = Treated with dose 1 (2,5μM))

ANOVA test, followed by Post-Hoc test. Correlation analyses between variables were evaluated using Corelation-Regresion test. Group differences at the level of p < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The effects of SLPI on NF-Kβ cellular expression

The highest percentage (38.86%) of macrophage cells that expressed NF-Kβ was detected in control group. Inversely, the lowest percentage (18.77%) of macrophage cells that expressed NF-Kβ was found in the experimental group with highest dose of SLPI (2.5 μM) (Figure 2). Both of groups (control group vs experimental group with SLPI 2.5 μM shows significant difference (p = 0.00). Pearson correlation shows that there was significantly strong relationship between SLPI concentration and the percentage of macrophage cells that expressed NF-Kβ. This finding suggested that SLPI concentration was negatively associated with cellular expression of NF-Kβ.

The effects of SLPI on IL-1β cellular expression

The highest percentage (51.10%) of macrophage cells that expressed IL-β was observed in control group. However, the lowest percentage (23.52%) of macrophage cells that expressed IL-β was detected in the experimental group with highest dose of SLPI (2.5 μM) (Figure 4). Both of groups (control group vs experimental group with SLPI 2.5 μM shows significant difference (p = 0.00). Pearson correlation shows that there was significantly strong relationship between SLPI concentration and the percentage of macrophage cells that expressed IL-1β. This finding suggested that
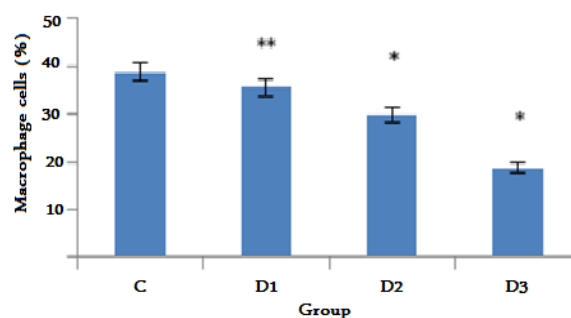
SLPI concentration was negatively associated with cellular expression of IL-β (p = -0.906).

Pearson correlation demonstrates that there was significantly strong relationship between the expression of cellular NF-Kβ and IL-β (p = 0.954). The positive association was also observed between the expression of NF-Kβ and IL-β.

Macrophages are the cells that normally involved in wound healing. Initially, macrophages produce proinflammatory cytokines and modulate inflammatory response by recruiting and activating leukocytes. LPS stimulate macrophages and activate transcription factor. Activation of NF-Kβ has been associated with the production of TNF-α, IL-1, IL-6, IL-8, COX2, intracellular adhesion molecules (ICAM)-1 and kolagenase [9].

Our study showed that the highest cellular expression of NF-Kβ and IL-1β was found in the control group that did not receive SLPI post tooth extraction. Physical trauma and tissue injury after tooth extraction may activate transcription factor NF-Kβ. Lipopolysaccharide (LPS) of bacteria, the endotoxin produced by gram-negative bacteria which are located around the wound can be recognized by the TLR4 and stimulate pro-inflammatory by macrophages and activate signaling pathways for transcription factor NF-Kβ [10]. Increased activation of NF-Kβ through the canonical pathway caused by tooth extraction in rats may induce the secretion of pro inflammatory cytokines (IL-1β).

In the prolonged inflammation and delayed wound healing, activated macrophage will secrete excessive IL-1β that lead to excessive inflammation and may interfere the balance of inflammation. A small amount of IL-1 is necessary for the body's defense and wound healing, but excessive IL-1 may inhibit the early phase of wound healing [11, 12].

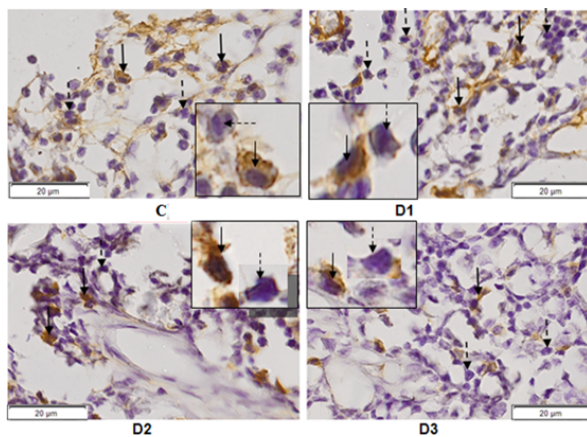Our study also observed that there was significantly

Figure 3. IHC staining: Cellular expression of IL-1β (C = Control; D1 = Treated with dose 1 (0.1μM); D2 = Treated with dose 1 (0.5μM); and D3 = Treated with dose 1 (2,5μM))

decreased in cellular expression of NF-Kβ and IL-1β by administration of SLPI. The amount of cellular expression of NF-β and IL-1β were negatively associated with the doses of SLPI. The most significant decreased in cellular expression of NF-Kβ and IL-1β was found in experimental group with SLPI of 2.5 μM. In line with previous study by Murata *et al.* (2003) [13] found that SLPI administration topically inhibit the development allergies conjungtivitis.

Previous research demonstrated that SLPI may down regulate the activation of macrophage to bacterial lipopolysaccharide (LPS). SLPI bind to annexin II receptors which located on the cell surface of macrophages and may inhibit the activation and downstream of NF-Kβ through protection inhibitor of kappa (I-Kβ) from degradation by the ubiquitin proteosom pathway [14, 15, 16]. Additionally, SLPI may compete with NF-Kβ (p65) to bind with the binding site of NF-Kβ in nukleus [17]. Thus, SLPI may inhibit macrophage activation and prevent the secretion of proinflammatory cytokines such as TNF-α, IL1 and nitric oxide (NO). In vitro study by Sano *et al.* (2003) [6] showed that SLPI inhibit activation of NF-Kβ and production of IL-1 in macrophages stimulated by LPS. Several other studies also observed that administration of SLPI inhibits the secretion of inflammatory mediators TNF-α in spinal cord injury and arthritis [7, 8].

Recently, NF-Kβ has been widely known as a key target of anti-inflammatory drug. However, genetic studies in rats have demonstrated that targeting NF-Kβ was problematic in inflammatory diseases. NF-Kβ pathways may regulate the production of pro-inflammatory cytokines, leukocyte recruitment, or survival of cells, which play an important role in the inflammatory
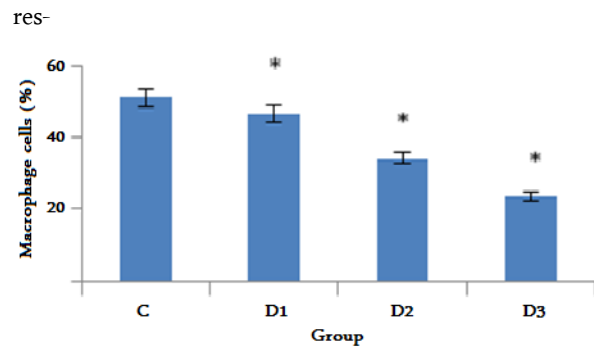
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Figure 4. Percentage of macrophage cells as cellular expression of IL-1β (C = Control; D1 = Treated with dose 1 (0.1μM); D2 = Treated with dose 1 (0.5μM); and D3 = Treated with dose 1 (2,5μM))

ponse [10]. However, the apoptotic function of NF-Kβ also protect against inflammation in the case of epithelial cell survival and mucosal barrier. The apoptotic functions of NF-Kβ keep the inflammatory response through activation of leukocytes persistenly. In contrast, NF-Kβ may promote apoptosis of leukocytes in a particular context that may contribute to the resolution of inflammation. It is clear that NF-Kβ controlling inflammation through a variety of mechanisms [10]. Thus, further studies are required to evaluate the activation of NF-Kβ pathway as a therapeutic target.

CONCLUSIONS

In conclusion, the present data confirm that SLPI may decrease cellular expression of NF-Kβ and IL-1β on wound macrophages cells of rats post tooth extraction in a dose-dependent manner.

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