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Research Report

RANKL expressions in preservation of surgical tooh extraction treated with Moringa (*Moringa oleifera*) leaf extract and demineralized freeze-dried bovine bone xenograft

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ABSTRACT

Background: *Preservation of sockets is a procedure aimed to reduce bone resorption after tooth extraction. One of the most commonly used xenograft materials is demineralized freeze-dried bone bovine xenograft (DFDBBX). Meanwhile, one of the key regulations in osteoclast genesis process is RANKL bond. A decrease in the number of RANKL expressions can suppress the osteoclast genesis process so that bone resorption can be prevented. The combination of Moringa leaf extract and DFDBBX, as a result, is expected to decrease the number of RANKL.* ***Purpose:*** *This study aimed to measure RANKL expressions in tooth extraction socket treated with Moringa leaf extract combined with DFDBBX.* ***Methods:*** *Fifty six Cavia cobaya rats were divided into eight groups. The first group was a control group with PEG administration onto their extraction sockets. The second group was a treatment group with DFDBBX administration. The third group was a treatment group with Moringa leaf extract administration. The fourth group was a treatment group induced with a combination of DFDBBX and Moringa leaf extract. Examination then was performed on days 7 and 30. After 7 and 30 days, those Cavia cobaya rats were executed and tested with immunohistochemical techniques. Further research data collected then were tested with one-way ANOVA.* ***Results:*** *There were significant differences between the control group and the groups induced with the combination of Moringa leaf extract and DFDBBX. On days 7 and 30, the groups induced with the combination of Moringa leaf extract and DFDBBX had the lowest number of RANKL expressions.* ***Conclusion:*** *The combination of Moringa leaf extract and DFDBBX can decrease the number of RANKL expressions in Cavia cobaya rats on the day 7 and day 30 after tooth extraction.*

Keywords: *DFDBBX; Moringa leaf extract; socket preservation; RANKL; alveolar bone*

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INTRODUCTION

Tooth extraction is an act of removing a tooth from an alveolar bone socket. Tooth extraction may be performed due to caries, periodontal disease, impaction, cyst, tumor, and fracture. Tooth extraction can also be conducted on healthy teeth with the aim of improving malocclusion and esthetics.[1] Tooth extraction may trigger an inflammatory response and alveolar bone resorption in the bucolingual and apicocoronal dimensions of the edentulous ridge region.[2] Therefore, extraction sockets are necessary to maintain in order to keep their original forms, so the volume of alveolar bone can be maintained. Dental implants performed on poor alveolar bone conditions are at risk of poor osseointegration, thus increasing the risk of dental implant failure. The application of implant in edentulous ridge that has large resorption, as a result, requires intervention of augmentation procedure first.[3]

The process of alveolar bone resorption begins with a bond between the reactor activators of nuclear kapa-b ligand (RANKL) in the reactor activator of nuclear kapa-B (RANK) presented in preosteoclasts. RANKL/RANK is the key regulation in the osteoclastogenesis process.[4] The formation of osteoclasts, nevertheless, is also influenced

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by proinflammatory cytokines, such as tumor necrotizing factor-α (TNF-α), interleukin-1 (IL-1), and interleukin -6 (IL-6). The RANKL/RANK bond, consequently, will stimulate TNF receptor-associated factor 6 (TRAF6), NF-κB, c-Jun N-terminal kinase (JNK)/cJun/fos, and nuclear factor of activated T cells (NFAT) initiating differentiation of precursor osteoclasts into preosteclast cells.[5] The osteoclast mediation process for resorption usually takes about 1-4 weeks.[6]

One of procedures to prevent alveolar bone resorption is socket preservation. Socket preservation is an act of preserving the alveolar bone through a surgical procedure that aims to maintain the bone and soft tissue maximally after tooth extraction.[7] This action is essential for the preparation of dental implants. The most widely used material for regenerating bones for purposes of socket preservation is graft. The application of graft material can provide normal healing and bone-to-implant contact.[8] Autograft is a gold standard for bone regeneration. Unfortunately, it has limited amounts and high morbidity risk. Thus, it was abandoned. Allograft in the form of demineralized freeze-dried bovine bone xenograft (DFDBBX), on the other hand, is a substitute material that is biocompatible, osteoinductive, and osteoconductive. DFDBBX can be used for preserving alveolar bone sockets.[9]

Biomaterials that can decrease the post inflammatory response, therefore, are needed to prevent excessive resorption. One material that can be developed to reduce the inflammatory response after tooth extraction is *Moringa oleifera* leaf.[10] *Moringa oleifera* leaf is composed of amino acid, fatty acid, beta carotene, minerals, vitamin E, and flavonoids.[11] These flavonoids then can act as anti-inflammation, anticancer, antimicrobial, antiviral, immunomodulatory, antithrombotic, and osteoprotection.[10,12] Some previous researches have shown that Moringa leaf extract may inhibit the inflammatory pathway by inhibiting carrageenan in rats induced with edema. Barriers to the inflammatory pathway then will inhibit bone resorption.[13] Moringa leaf extract may also increase the proliferation and differentiation of osteoblast cells.[14,15]

An anti-inflammatory combination between Moringa leaves and osteoconductive and osteoinductive properties of DFDBBX, thus, is expected to provide a good response to the body in minimizing the formation of osteoclasts. A previous research even showed that 2% *Moringa oleifera* leaf extract and DFDBBX can generate osteoblasts, but decrease osteoclasts.[10] This study aimed to examine RANKL expressions in tooth extraction sockets treated with Moringa leaf extract and DFDBBX.

MATERIALS AND METHOD

This research was an experimental research with a randomized factorial design. This research was conducted in October-November 2016. Research subjects used were healthy and active male *Cavia cobaya* (n = 56) rats weighed 300-350 grams and aged 3-3.5 months old. Those rats also had to eat normally without any defects on their body, their skin, and their senses, so they could walk normally, not limping, as well as had normal body temperature. This research was also approved by the Ethics Committee of Faculty of Dental Medicine, Universitas Airlangga No. 026/HRECC.FODM/III/2017.

Moringa (*Moringa oleifera*) leaves were extracted at Balai Penelitian dan Konsultasi Industri Surabaya, while DFDBBX used was produced in Batan (Bonegraft®, size 10 mesh/2000 microns). The treatment of *Cavia cobaya* rats then was performed in Biochemistry Laboratory of Faculty of Medicine, Universitas Airlangga, Surabaya. *Cavia cobaya* rats were divided into eight groups, and then the left incisive tooth of the *Cavia cobaya* rats was extracted. The sockets of the tooth extraction in each research group were preserved differently. The sockets in group I (KI) and group V (KV) were filled with poly etyle glycol (PEG) as the control groups, while the sockets in group II (KII) and group VI (KVI) were filled with DFDBBX. Moreover, the sockets in group III (KIII) and group VII (KVII) were filled with Moringa leaf extract, while the sockets in group IV (KIV) and group VIII (KVIII) were filled with Moringa leaf extract and DFDBBX. Afterwards, the post-retrieval wounds and tooth extraction sockets were stitched. RANKL expressions on the extraction sockets in KI, KII, KIII, and KIV then were observed on day 7, while RANKL expressions in KV, KVI, KVII, and KVIII were observed on day 30.

On the observation days, the rats were sacrificed, and their mandible was taken for decalcification using EDTA for 30 days to make paraffin blocks. The process of making preparation and reading preparatory reading was conducted at Anatomical Pathology Laboratory of Dr. Soetomo Surabaya. RANKL expressions then were observed by imunohitochemical technique using anti RANKL monoclonal antibody (Biotech ®, Santacruz). The observations of the preparation and the measurement of RANKL expressions were performed by using a light microscope with a 1000x magnification. RANKL expressions were calculated by measuring the cells that emitted brown chromogenic. The data of RANKL expressions obtained were tested with one sample Kolmogorov Smirnov test to analyze the normality of the data. Afterwards, Levene's test was performed to analyze the homogeneity of the data. One-way ANOVA then was conducted to analyze differences between the research groups.

RESULTS

The expressions of RANKL on the day 7 can be seen in Figure 1. The IHC results indicated that the number of

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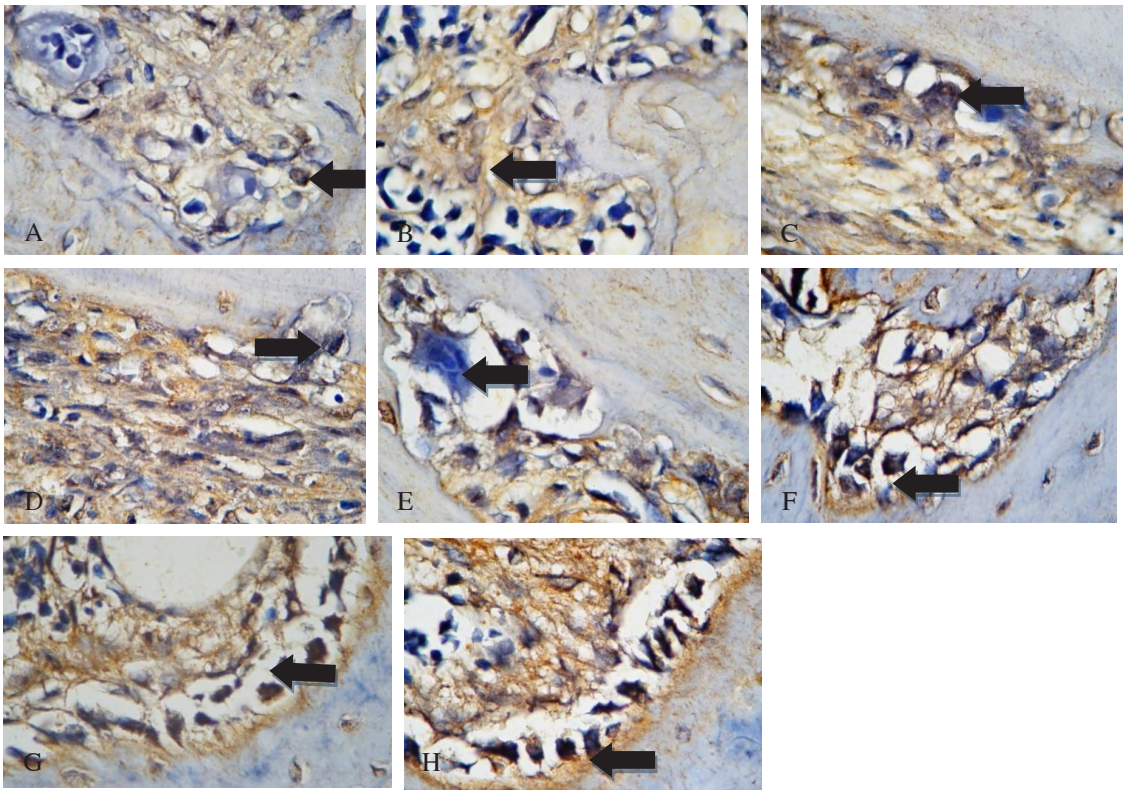

Figure 1. RANKL Expressions during IHC examination on day 7 in KI (A), KII (B), KIII (C), and KIV (D) and on day 30 in KV (E), KVI (F), KVII (G), and KVIII (H).

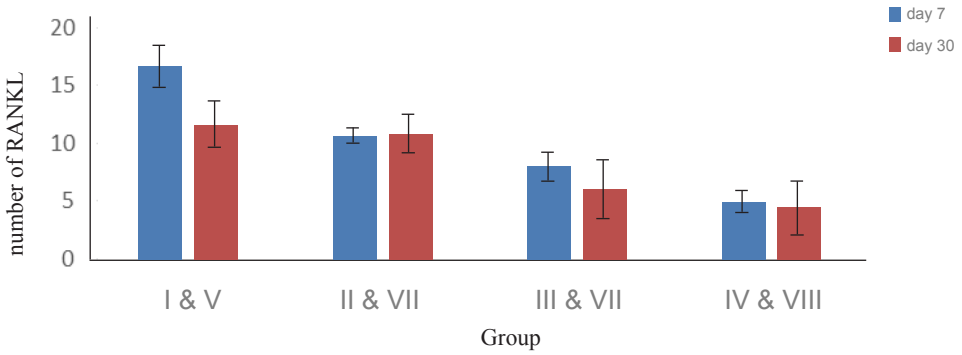

Figure 2. The mean number of RANKL expression on days 7 and day 30.

Table 1. Results of Tukey HSD test on the number of RANKL expressions on day 7

Groups	KI	KII	K III	KIV
KI		0.000*	0.000*	0.000*
KII			0.000*	0.002*
KIII				0.001*
KIV				

*: a significant difference (p<α=0.05)

Table 2. Results of Tukey HSD test on the number of RANKL expressions on day 30

Groups	Group V	Group VI	Group VII	Group VIII
Group V		0.881	0.000*	0.000*
Group VI			0.002*	0.000*
Group VII				0.540
Group VIII				

*: a significant difference (p<α=0.05)

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RANKL expressions on day 7 was higher than that on day 30 (Figure 2). However, the lowest number of RANKL expressions on days 7 and 30 was found in the treatment group induced with the combination of Moringa leaf extract and DFDBBX. The number of RANKL expressions in that treatment group even was lower than in the treatment groups only given Moringa leaf extract or DFDBBX. Meanwhile, the highest number of RANKL expressions was found in the control group.

The data of RANKL expressions on the day 7 were statistically analyzed with a normality test, one-sample Kolmogorov-Smirnov test. Results of the one-sample Kolmogorov-Smirnov test showed the data were normally distributed with a p value of 0.748 ($p>0.05$). The data then were analyzed with a homegeneous test, by using Levene's Test then done and got value $p = 0.123$ which shows research data is homogeneous data ($p>0.05$). Differences between groups were tested using Tukey HSD one-way ANOVA and can be seen in Table 1. There were statistically significant differences between treatment groups on day 7 ($p<0.05$).

The data of RANKL expressions observed on day 30 were tested for their normality by using Kolmogorov-Smirnov one-sample test. The results of the Kolmogorov-Smirnov one-sample test showed the data were normally distributed ($p>0.05$). Homogeneity test then was performed by using Levene's Test. The results of the Levene's Test revealed that the data were homogeneous ($p>0.05$). Next, Tukey HSD test and one-way ANOVA test were conducted to evaluate differences between research groups. The results can be seen in Table 2. On day 30, there were statistically significant differences between research groups ($p <0.05$), except between group VII and group VIII ($p>0.05$).

DISCUSSION

These experimental animals were selected since they have metabolism as well as immunological responses similar to humans.[16] DFDBBX is a type of xenograft derived from bovine. Xenograft has osteoconduction properties with porous internal surface allowing for revascularization and osteoblast migration from the socket base to support osteogenesis.[17] The structure and inorganic content of bone matrix from xenograft also have osteoconductive properties to facilitate bone formation.[18] Bone formation using bovine hydroxyapatite xenograft can lead to good results, namely an increase in osteoprotegrin (OPG) expressions and a decrease in RANKL expressions as indicators of bone formation.[19] Xenograft inserted into the extraction socket, as a result, can serve as a framework for new bone growth, derived from osteoblasts at the bottom of the socket.[20]

Moringa oleifera contains benzyl isothiocyanate compounds, and based on the results of phytochemical studies, also contains secondary metabolite compounds such as flavonoids, alkaloids, and phenols which can inhibit bacterial activity.[21] *Moringa oleifera* leaf extract may also decrease the production of nitrous oxide in LPS-induced macrophage cells (lipopolysaccharides).[22] In addition, moringa leaves can decrease pro-inflammatory mediators, such as prostaglandins, IL-1β, IL-6, and TNFα. This is due to the inhibition of serotonin and histamine release as well as prostaglandin synthesis.[23] Inflammatory mediators are osteoclast activating factors that play a role in bone resorption.[24] *Moringa oleifera* extract on tooth extraction wounds, is expected to inhibit the inflammatory process so that macrophage infiltration can be reduced. The decrease in TNF α, IL-1β, and IL-6 then leads to a decrease in RANKL production.[3] Moringa leaf extract is indirectly osteoinduced by suppressing NFκB activation.[22] Thus, NFκB products are reduced so that cytokines that act as inflammatory mediators, such as TNF α, IL- 1β, and IL-6 also serve to stimulate the formation of RANKL produced by osteoblast cells leading to a decrease in RANKL production.

The results of the treatments given in all research groups on day 30, indicated a trend of decreasing in the number of RANKL expressions when compared with the results on day 7. This suggests that on day 30, the number of osteoblasts was higher than that on day 7. Simialrly, a research conducted by Guskuma reveals that on day 7 bone defects are still in inflammation and enter the early stage of resorption, while on day 30, bone defects begin in the early stage of bone formation.[24] Like this previous research, a research conducted by Irinakis suggests that in the 4th week, bone deposition begins to occur in the socket retraction.[25] At that time, osteoblast and other osteogenic tissues begin to form with a significantly increased number. The results were also in line with a research conducted by Kresnoadi arguing that the number of osteoblasts on the 30th day increase, while osteoclasts decrease significantly compared to the previous day.[26]

In addition, the results of this research showed that post-extraction socket preservation, a procedure to reduce bone loss after tooth extraction to maintain dental alveoli/tooth socket in alveolar bone, can minimize alveolar bone resorption and accelerate bone formation in the area of damage. Besides, selection of materials used in preservation of socket retraction also has an important role in the process of bone formation. Like the results of this research, a research conducted by Grover demonstrates that DFDBBX has osteoconduction properties that serve as a scaffold for new bone growth, derived from osteoblasts at the bottom of the socket.[10]

Based on the results of this research, osteoconductive and osteoinductive properties of the two materials above can decrease the number of RANKL expressions significantly. This is likely to enhance the success of socket preservation further, so bone dimensions and volume after dental extraction will be maintained.[27] It can be concluded that the combination of Moringa leaf extract and DFDBBX can decrease the number of RANKL expressions in the tooth extraction sockets of the *Cavia cobaya* rats on days 7 and 30.

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