The effect of combined *Moringa oleifera* and demineralized freeze-dried bovine bone xenograft on the amount of osteoblast and osteoclast in the healing of tooth extraction socket of *Cavia cobaya*

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ABSTRACT

**Background:** Alveolar bone has an important role in providing support to teeth and dentures. Loss of support caused by alveolar resorption will cause functional and aesthetic problems. Preservation socket using bone graft is one way to maintain the dimensions of the alveolar bone. *Moringa oleifera* leaf can increase the activity of bone graft in the formation of new bone. **Purpose:** This study was aimed to evaluate the effect of combined *Moringa oleifera* leaf extract and demineralized freeze-dried bovine bone xenograft (DFDBBX) towards the formation of osteoblasts and osteoclasts in the tooth extraction sockets of *cavia cobaya*. **Method:** This study used 28 cavia cobayas divided into four groups. The combination of *Moringa oleifera* leaf extract and DFDBBX was inducted into the sockets of lower incisor tooth with certain dose in each group, ointment 1 containing PEG (a mixture of PEG 400 and PEG 4000) for control group, ointment 2 containing *Moringa oleifera* leaf extract and DFDBBX and PEG (at active substance concentration of 0.5%) for group 1, ointment 3 containing *Moringa oleifera* leaf extract and DFDBBX and PEG (at active substance concentration of 1%) for group 2, and Ointment 4 containing *Moringa oleifera* leaf extract and DFDBBX and PEG (at active substance concentration of 2%) for group 3. Paraffin block preparations were made for histopathology examination using hematoxylin eosin staining. **Result:** The results showed that there were significant differences of the number of osteoblasts and osteoclasts in each treatment group (p < 0.05). **Conclusion:** It can be concluded that the combination of *Moringa oleifera* leaf extract and DFDBBX at 2% concentration can increase the number of osteoblasts and decrease osteoclasts in the healing of tooth extraction sockets of *cavia cobaya*.

Keywords: demineralized freeze-dried bovine bone xenograft; *Moringa oleifera* leaf; osteoblast; osteoclast

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INTRODUCTION

Alveolar bone has an important role in providing support to teeth and dentures. Making dentures require sufficient structure and volume of alveolar bone as a buffer for removable dentures, fixed dentures and implant placement. Loss of the structure and volume of alveolar bone will affect stability, retention and comfort in the use of dentures in patients. This condition is usually caused by resorption process of alveolar bone due to mechanical factors, pathological processes, trauma on face and resorption process triggered by tooth extraction. Therefore, it is essential to maintain alveolar ridge after tooth extraction. One of techniques aimed to maintain alveolar ridge after tooth extractions by using graft materials either with or without membrane.
Graft materials are natural or synthetic substances used to correct defect or deficiency in tissue by providing extra cellular matrix in the form of scaffold during bone regeneration process. However, the use of graft materials in post-extraction sockets is still questionable because it can interfere with healing process in the socket when the graft will be used for the placement of implant. The formation of new bone derived from graft materials moreover also depends on time, therefore a substance that can simulate the activity of the graft materials is needed to accelerate new bone formation process.

Moringa oleifera, is a plant that has many benefits because it contains many nutrients, including vitamin C, α and β tocoferol, α and β-carotene, and 12 kinds of flavonoids including kaempferol and quercetin with high concentration. Flavonoid compound serves as an anti-inflammatory, anti oxidants and osteoprotection. Several other researchers even claim that Moringa oleifera plant can inhibit cyclooxygenase enzyme, prostaglandin synthesis and nitrous oxide production in macrophages cells induced by lipopolysacaride (LPS).

Tooth extraction can cause trauma triggering inflammation. Inflammatory reaction is a sign of first activated defense cells. This process occurs as a result of prostaglandin synthesis. This prostaglandin synthesis then will increase prostaglandin E2 (PGE2). PGE2 will directly lead to the increasing of osteoclast activity. In addition, macrophages infiltration as protection against infection will also be increased in the area of trauma and will induce nuclear factor kappa β (NF-κB). Consequently, NF-κB will trigger the secretion of pro-inflammatory mediators, namely interleukin-1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) to strengthen immune response and also accelerate metabolic process. Those pro-inflammatory mediators then can regulate the receptor activators of nuclear kappa β ligand (RANKL) to bind to the receptor activators of nuclear kappa (RANK) causing the increasing of differentiation of pre-osteoclasts into osteoclasts, later accelerating bone resorption process. In the healing process of the sockets after tooth extraction, osteoblast cells have been formed, derived from pluri-potential cells of periodontal ligament. This process indicates osteogenic function occurred in the third week. In this week, cortical bone in the sockets will also remodel, and the crest of the alveolar bone will be rounded by the activity of osteoclastic resorption. Based on the description above, therefore, this research is aimed to evaluate the effect of combine bonegraft and moringa leaf extract on the formation of osteoblasts and osteoclasts in the tooth extraction.

MATERIALS AND METHOD

This was an experimental laboratory research using experimental animals with true experimental design (Post only control group design). Subjects in this research were healthy male Cavia cobayas weighed 300-350 grams, aged 3-3.5 months obtained from Biochemistry Laboratory unit of experimental animal, Faculty of Medicine, Universitas Airlangga. PEG is a compound serves as carrier for extract of Moringa oleifera leaf and DFDBBX, making them readily absorbed by human’s body.

Twenty-eight Cavia cobaya were divided into four groups, each consisting of seven samples. Mixture of Moringa oleifera leaf extract, DFDBBX and PEG were made. Ointment 1 containing 25 grams of PEG (a mixture of PEG 400 and PEG 4000), ointment 2 containing 0.5 grams of Moringa oleifera leaf extract, 0.5 grams of DFDBBX and 99 grams of PEG (at active substance concentration of 0.5%), ointment 3 containing 0.5 grams of Moringa oleifera leaf extract 0.5 grams of DFDBBX and 49 grams of PEG (at active substance concentration of 1%), and ointment 4 containing with 0.5 grams of Moringa oleifera leaf extract, 0.5 grams of DFDBBX and 24 grams of PEG (at active substance concentration of 2%). Those ointments were applied on the tooth extraction sockets of lower left incisors of Cavia cobayas previously got anesthesia injection with ketamine. Ointment 1 was applied on the sockets in the control group, ointment 2, 3 and 4 were applied to the sockets in group 1, 2 and 3.

Histological examination was conducted by dissolving them in xylol for 2 x 3 minutes. Residual xylol was washed with absolute alcohol 99%, 95%, 90%, 80%, and 70%, respectively for 2 x 1 minutes. The residual alcohol was washed with running water. HE staining was conducted for 30 seconds and then rinsed with water. Eosin staining was conducted for 1-2 minutes, and then washed with absolute alcohol 70%, 80%, 90%, 95%, and 99% for 2 x 3 minutes and with xylol for 2 x 2 minutes. Each preparation was closed with a cover glass previously dropped with Canadian balsam. Histology examination of osteoblasts and osteoclasts were done by using a light microscope at 400x magnification. The data collected from the observation was analysed statistically. Kolmogorov Smirnov test, Levene test and one way Anova were done, followed with multifactorial comparison test using Tukey-HSD.

RESULTS

It can be seen that the highest mean number of osteoblast cells was in the group treated with the active substance concentration of 2%, while the lowest in the control group. Furthermore, the highest mean number of osteoclasts was in the control group, while the lowest was in group 3 treated with the active substance concentration of 2% (Figure 1). The increased number of osteoblasts and the decreased of osteoclasts in the control group and the treatment groups can be seen in Figure 2 and 3.

In addition, the results of normality test showed that the
distribution of data in all groups of osteoblasts was normal (p = 0.160). Based on the results of homogenity test, it is also known that the variance of data obtained from those groups was homogeneous (p = 0.975). It was shown that all data in the groups of osteoclasts was normally distributed (p = 0.395) with homogeneous variance (p = 0.083). The results of Anova test showed that there were significant differences among the treatment groups (p < 0.05). The results of multiple comparison using, Tukey HSD test (Table 1).

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Table 1. Results of Tukey HSD test on the number of osteoblasts and osteoclasts in each treatment group

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*= No significant difference (p<0.05)

Note: Control: Control group; group 1: at active substance concentration of 0.5%; group 2: at active substance concentration of 1%; Group 3: at active substance concentration of 2%.
number of osteoblasts between control group and group 1. While there were significant differences in the number of osteoblasts between control group and both group 2 and group 3. There were also significant differences of the number of osteoblasts between group 1 and both group 2 and group 3. Similarly, there were significant differences in the number of osteoblasts between group 2 and control group, and also between group 1 and group 3. There were significant differences of the number of osteoblasts between group 3 and the other treatment groups.

The result of Tukey HSD showed that there was a significant difference in the number of osteoclasts between the control group and all of those treatment groups. There were significant differences of the number of osteoclasts between group 1 and the control group, and also between group 2 and group 3. It is also known, furthermore, that there were significant differences between group 2 and both the control group and group 1. Nevertheless, there was no significant difference between group 2 and group 3. It also shows that there were significant differences between group 3 and both the control group and group 1. There was no significant difference between group 3 and group 2.

**DISCUSSION**

Based on data analysis, the mean number of osteoblast cells was getting increased from the control group to group 3. It is also known that based on the results of one way Anova test, there was a significant difference of the number of osteoblast cells among the treatment groups. It indicates that the combination of Moringa oleifera leaf extract and both PEG and DFDBBX can influence the formation of osteoblasts in the tooth extraction sockets. Similarly, a previous research also claims that Moringa oleifera leaf extract has osteogenic effects. A compound playing a role in this condition is flavonoid, especially kaempferol and quercetin. Osteogenic effects derived from flavonoid can be detected from the increasing of alkaline phosphatase (ALP) enzym in cultured osteoblast cells. In addition to the can be detected from the increasing of alkaline phosphatase and quercetin. Osteogenic effects derived from flavonoid activities during bone formation. In addition to osteogenic effects, kaempferol also has estrogenic effects. Kaempferol and quercetin contained in Moringa oleifera leaf extracts playing an important role as anti-inflammation. In inflammatory reaction triggered by tooth extraction, pro inflammatory mediators are released from macrophages in the form of cytokines, including IL-1, IL-6, TNFα and prostaglandin E2 (PGE2). These components then will stimulate the formation of osteoclasts either directly or indirectly. PGE2 can directly induce vascular changes in inflammation process, and also induce bone resorption in the absence of inflammatory cells with a few of multinucleated osteoclasts when attached directly to the surface of bone. Cytokine then is produced by macrophages as a process of bone destruction, namely IL-1 that can improve the regulation of RANKL in osteoclasts. RANKL binds to RANK as the initial process of bone destruction. The increasing of osteoclast differentiation process is influenced by the increasing of RANK fusion on the precursor of the surface of osteoclasts, resulting in bone resorption.

Moringa oleifera leaf, contains the large amount of flavonoids, namely kaempferol and quercetin. Flavonoids can be used in all inflammatory conditions because they can inhibit cyclooxygenase2 enzyme playing a role in the formation of inflammatory mediators derived from arachidonic acid. It can also inhibit the release of histamine by mast cells in basophils that have anti-inflammatory effects. The effects of this enzyme then will reduce a number of pro-inflammatory mediators, such as IL-1, IL-6 and TNFα. Kemferol and quercetin activities can also directly inhibit TNFα activity and RANKL expression. Both of these flavonoids have a tendency to bind atoms or act as scavenging for free radical atoms, so reactive oxygen species (ROS) can not be formed redundantly. ROS can stimulate phosphorylation process of inhibitor kappa β (IKβ), which serves to bind NFκβ, so it can be inactive in cytoplasm. If IKβ is phosphorylated, the bond of the bond IKβ and NFκβ will be separated, so NFκβ becomes active and moves to cell nucleus. This process is called activation process of NFκβ. Therefore, with the barriers of extract. A graft material used in this study is DFDBBX, a kind of xenograft produced by Batan. Xenograft is useful to stimulate the proliferation of osteoblasts, fibroblasts and endothelial cells. Xenograft also has an ability to regenerate tissue osteoconductively because it has inner surface properties, porosity, calcium ratio, and mineral factors, such as hydroxyapatite owned by bovine similar to human bone minerals. DFDBBX plays a role as osteo conductor in osteoinduction. But, inorganic materials derived from this graft can make the attachment and proliferation of osteoblast cells as the first step for the formation of osteoblasts to form bone.
flavonoids in forming ROS, the activation of NFκB will also be inhibited.34 NFκB is known as gene transcription that will induce the formation of pro-inflammatory cytokines, such as TNFα. As mentioned before, TNFα and RANKL are precursors of osteoclast cell maturation playing a role in bone resorption. In addition, quercetin is also able to inhibit differentiation and activation as well as to induce apoptosis of osteoclast cells.35,36 Thus, in this research, it is known that Moringa oleifera leaf extract can reduce the number of osteoclasts, but on the other hand it can increase the number of osteoblasts. This is caused by the suppression phase of differentiation terminal of osteoclasts, so the formation of pre-osteoblast cells become more accelerated.

The combination extract of Moringa and DFDBBX accelerates bone formation due to its good compatibility and osteoconductivity. Acceleration of bone regeneration mechanically might be caused by combination substance which acts as scaffold, stabilizing blood clot and preventing epithelium from growing inward the socket, while biologically the substances provide extra minerals.37 Combination of substances could be caused resorption and gradation in formation of new bone so that enabling osteocompetent penetration and endothelial cells and vascularization in order to accelerate new bone formation.38 It can be concluded that the combination of Moringa oleifera leaf extract and DFDBBX at 2% concentration can increase the number of osteoblasts and decrease osteoclasts in the healing of tooth extraction sockets of cavia cobaya.

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