The effect of a combination of propolis extract and bovine bone graft on the quantity of fibroblasts, osteoblasts and osteoclasts in tooth extraction sockets

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ABSTRACT

Background: Tooth extraction is a procedure frequently performed in the field of dentistry that can cause alveolar bone resorption during the healing process. Therefore, preservation of sockets is necessary to maintain alveolar bone which represents one of the important factors in the successful manufacture of dentures. A combination of propolis extract and bovine bone graft (BBG) can accelerate bone regeneration. Purpose: The purpose of this study was to determine the effect of a combination of propolis extract and BBG on the quantity of fibroblasts, osteoblasts, and osteoclasts in the tooth extraction socket. Methods: 56 Cavia cobaya were divided into eight groups. The lower left incisor of each subject was extracted and induced with polyethylene glycol (PEG), propolis extract+PEG, BBG + PEG, combination of propolis extract + BBG + PEG at a concentration of 2% active substance. Experimental subjects were sacrificed on days 3 and 7. Histopathological examination with a microscope at 400x magnification was conducted to calculate the quantity of fibroblasts, osteoblasts, and osteoclasts. Statistical analysis was performed by one-way ANOVA and Tukey HSD tests. Results: The highest average quantity of fibroblasts and osteoblasts and the lowest average quantity of osteoclasts occurred in the group to which a combination of propolis extract and BBG had been administered on both days 3 and 7. According to the statistical analysis results, all the treatment groups recorded a significant difference in the quantity of fibroblasts, osteoblasts, and osteoclasts with a p value: 0.000 (p<0.05). Conclusion: A combination of propolis extract and BBG can increase the quantity of fibroblast and osteoblast cells, while reducing the number of osteoclast cells in tooth extraction sockets treated with 2% concentration of the active substance. Keywords: bovine bone graft (BBG); fibroblasts; osteoblasts; osteoclasts; propolis extract; socket preservation

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INTRODUCTION

Tooth extraction constitutes a commonly performed dental procedure that invariably precedes bone resorption and regeneration. Tooth extraction followed by the socket healing process usually leads to alveolar bone deformities, including a reduction in residual ridge height and width. After extraction, bleeding in the tooth socket precedes initiation of the inflammatory mediator and produces a blood clot that seals the exposed socket. Inflammation causes an increase in pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) and osteoclasts, with the result that receptor activators of both nuclear factor kappa-B (RANK) and nuclear factor kappa-B ligand (RANKL) proliferate. If osteoclasts increase in number, alveolar bone resorption ensues.

Three to five days post-extraction, fibrin degeneration will occur together with the formation of granulation tissue containing blood vessels, fibroblasts, and chronic inflammatory cells. During the process of inflammation, fibroblasts stimulate osteoprogenitor (OPG) to inhibit RANKL binding to RANK and trigger an increase in fibroblasts growth factor-2 (FGF-2) which plays a role in the process of osteoblast cells proliferation and differentiation.
Within a period of seven days post-extraction, immature bone has formed which differs from osteoblasts and bone matrix. Alveolar bone resorption is known to occur rapidly for one year after tooth extraction and will continue during the subsequent 12 months. A 25% reduction in bone width in the cervical region and a 40% reduction of width in the labial plate of the bone occurs in the first and third years after extraction.

Large resorption which is not offset by rapid bone regeneration can change the structure of the maxilla and mandible bones, thereby rendering the use of dentures problematic. Consequently, it is important to perform socket preservation in order to maintain the alveolar bone after tooth extraction and minimize alveolar bone resorption.

A biomaterial often used in socket preservation procedures is bone graft, one form of which is bovine bone graft (BBG). BBG, produced from cow bone which has been demineralized to remove any organic minerals present, has an osteoconductive effect in forming scaffold for bone regeneration.

Natural ingredients, one of which is propolis (a resin material found in beehives), are extensively employed in traditional medicine. Propolis, which had previously been obtained from Lawang, East Java, possesses certain bioactive components such as artepyline, apigenin, flavonoid, cinnamic acid, saponin, quercetin, terpenoid and caffeic acid-phenetyl ester (CAPE) which have many beneficial effects, including; antibacterial, antifungal, anti-inflammatory, antiviral, antioxidant, immunostimulative, and anticancer. Propolis extract is also known to contain polyphenolic compounds such as flavonoids and CAPE which can increase the number of osteoblasts and are anti-inflammatory. Therefore, this study aims to prove the effects of the combination of propolis extract and BBG on the quantity of fibroblasts, osteoblasts and osteoclasts in the tooth extraction socket on days 3 and 7.

MATERIALS AND METHODS

This investigation constituted experimental laboratory research which received ethical approval from the Ethics Committee, Faculty of Dental Medicine, Universitas Airlangga (number 316/HRECC.FODM/XII.2018). The research population of 56 male Cavia cobaya (C. cobaya) aged 3-3.5 months and 300-350mg in weight were divided into eight groups of seven subjects. These were subsequently designated either 3-day groups or 7-day groups each of which received four treatments. The C. cobaya were taken from their treatment facility and anesthetized intravenously with 0.2cc/300 g ketamine.

Extraction of the lower left incisor was effected using anesthetized intravenously with 0.2cc/300 g ketamine.

The groups were classified as follows. Groups I and II: tooth extraction sockets were filled with 25g of PEG only. Groups III and IV: tooth extraction sockets were filled with 0.5g of propolis extract and 24.5g of PEG. Groups V and VI: tooth extraction sockets were filled with 0.5g of BBG and 24.5g of PEG. Groups VII and VIII: tooth extraction sockets were filled with a combination of 0.5g of propolis extract, 0.5g of BBG and 24g of PEG. Groups I, III, V and VII were examined after three days and groups II, IV, VI and VIII after seven days.

In preparation for histopathologic (HPA) examination, after three days and seven days of the treatment the C. cobaya were euthanized and their jaws removed by means of a surgical incision. Their mandibles were cut to be decalcified with ethylene diamine tetra acetate (EDTA) for a period of three months. Paraffin blocks were manufactured and cut to a thickness of 4µ with a rotary microtome before being deparaffinized by being dissolved in xylol for 2x3 minutes. The residual xylol was respectively washed with 99%, 95%, 90%, 80%, and 70% absolute alcohol for 2x1 minutes. Any residual alcohol was removed with running water. At that point, hematoxylin eosin (HE) staining was subsequently performed for 30 seconds before rinsing with water.

Staining with HE was subsequently conducted for 1-2 minutes prior to washing with 70%, 80%, 90%, 95%, 99% absolute alcohol for 2x1 minutes. Fibroblast, osteoblast, and osteoclast cells were washed out with 99%, 95%, 90%, 80%, 70% absolute alcohol for 2x1 minutes. Observation was subsequently carried out under a light microscope, each slide being examined at 400x magnification and a maximum of 8 fields of view (FoV). The calculation results were recorded in a worksheet with a mean value per field of view. At this point, the quantity of fibroblasts, osteoblasts, and osteoclasts was calculated.

For statistical analysis, a Kolmogorov Smirnov statistical test was performed on the data obtained. In order to identify differences between the groups, a one-way ANOVA test was conducted, followed by a multifactorial comparison test involving a Tukey honest significant difference (HSD) test. A Levene’s homogeneity statistical test was also performed.

RESULTS

A HPA examination identified the presence of fibroblast, osteoblast and osteoclast cells. Fibroblast cells appeared as large cells with multiple nuclei (Figure 1). Osteoblast cells could be observed as circular cells located on the edge of the trabecular bone (Figure 2), while osteoclast cells appeared as large cells with multiple nuclei (Figure 3).
Figure 1. Black arrows indicate histopathological staining identifying fibroblasts on day 7 (HE staining observed through a light microscope at a magnification of 400x). (A) control group (PEG); (B) propolis extract + PEG; (C) BBG + PEG; (D) a combination of propolis extract + BBG + PEG.

Figure 2. Black arrows indicate histopathological staining identifying osteoblasts on day 7 (HE staining observed through a light microscope at a magnification of 400x). (A) control group (PEG); (B) propolis extract + PEG; (C) BBG + PEG; (D) combination of propolis extract + BBG + PEG.

Figure 3. Black arrows indicate histopathological staining identifying osteoclasts on day 7 (HE staining observed through a light microscope at a magnification of 400x). (A) control group (PEG); (B) propolis extract + PEG; (C) BBG + PEG; (D) combination of propolis extract + BBG + PEG.
Figure 4. The bar chart displays the average and standard deviations of the quantity of fibroblasts after 3 and 7 days of treatment. The Y-axis shows the average quantity of fibroblasts. The X-axis shows treatment groups which the socket filled with PEG, propolis+PEG, BBG+PEG, propolis+BBG+PEG.

Figure 5. The bar chart displays the average and standard deviations of the quantity of osteoblasts after 3 and 7 days of treatment. The Y-axis shows the average quantity of osteoblasts. The X-axis shows treatment groups which the socket filled with PEG, propolis+PEG, BBG+PEG, propolis+BBG+PEG.

Figure 6. The bar chart displays the average and standard deviations of the quantity of osteoclasts after 3 and 7 days of treatment. The Y-axis shows the average quantity of osteoclasts. The X-axis shows treatment groups which the socket filled with PEG, propolis+PEG, BBG+PEG, propolis+BBG+PEG.
The calculated averages and standard deviations of the fibroblasts, osteoblasts, and osteoclasts in each treatment group and the control group are contained in Figures 4-6. A histopathologic (HPA) examination indicated that the quantity of fibroblasts and osteoblasts was higher in group VIII whose members had been administered a combination of propolis extract, BBG, and PEG on day 7, than in the group control which only received PEG on day 3. The quantity of fibroblasts and osteoblasts on day 7 was higher than that on day 3.

On the other hand, the highest concentration of osteoclasts in the tooth extraction socket was found in the control group (group I) on day 3, while the lowest was in group VII which had received a combination of propolis extract, BBG, and PEG on day 7. The quantity of osteoclasts on day 3 was higher than that on day 7.

Prior to an analysis of the test results for each group, a Kolmogorov-Smirnov normality test was conducted. In this research, all the research groups had a p value greater than 0.05 signifying that data derived from all the research groups were normally distributed. A one-way ANOVA test score of p<0.05 means there is significant difference; a Kolmogorov-Smirnov normality test score of p>0.05 signifies that the data follows normal distribution; a homogeneity test result of p>0.05 indicates that data derived from all the research groups are contained in Figures 4-6.

### Table 1. Statistical analysis data relating to the quantity of fibroblasts, osteoblasts, osteoclasts in each treatment group on day 3

| Group | Mean ± SD | Normality test | Homogeneity test | One-way ANOVA test | Tuckey HSD test |
|-------|-----------|----------------|------------------|-------------------|----------------|
|       |           |                |                  |                   | I              |
| Fibroblasts |         |                |                  |                   | III            |
| I     | 12.86 ± 1.77 | 0.789          |                  |                   | *              |
| III   | 15.29 ± 2.63 | 0.494          |                  |                   | -              |
| V     | 18.57 ± 2.23 | 0.595          | 0.546            | 0.000             | *              |
| VII   | 22.29 ± 2.29 | 0.999          |                  |                   | *              |
| Osteoblasts |         |                |                  |                   |               |
| I     | 10.29 ± 1.11 | 0.985          |                  |                   | -              |
| III   | 12.00 ± 1.41 | 0.964          |                  |                   | *              |
| V     | 13.00 ± 1.15 | 0.833          | 0.219            | 0.000             | *              |
| VII   | 18.86 ± 2.61 | 0.858          |                  |                   | *              |
| Osteoclasts |        |                |                  |                   |               |
| I     | 15.71 ± 1.80 | 0.960          |                  |                   | -              |
| III   | 13.57 ± 1.72 | 0.988          |                  |                   | *              |
| V     | 11.86 ± 1.35 | 0.986          | 0.492            | 0.000             | *              |
| VII   | 9.71 ± 1.11  | 0.985          |                  |                   | *              |

* = There is a significant difference (p<0.05)

**Note:** a normality test score of p>0.05 signifies that the data follows normal distribution; a homogeneity test result of p>0.05 indicates that the data demonstrates homogenous distribution; a one-way ANOVA test score of p<0.05 means that significant difference exists; group I: 25 grams of PEG on day 3; group II: 25 grams of PEG on day 7; group IV: propolis extract+PEG on day 3; group VI: BBG+PEG on day 3; group VII: propolis extract+BBG+PEG at the active substance concentration of 2% on day 3.

### Table 2. Statistical analysis data on the quantity of fibroblasts, osteoblasts, osteoclasts in each treatment group on day 7

| Group | Mean ± SD | Normality test | Homogeneity test | One-way ANOVA test | Tuckey HSD test |
|-------|-----------|----------------|------------------|-------------------|----------------|
|       |           |                |                  |                   | I              |
| Fibroblasts |         |                |                  |                   | III            |
| II    | 15.71 ± 2.75 | 0.985          |                  |                   | *              |
| IV    | 17.86 ± 3.13 | 0.997          |                  |                   | -              |
| VI    | 23.00 ± 3.42 | 1.000          | 0.942            | 0.000             | *              |
| VIII  | 27.57 ± 2.88 | 0.997          |                  |                   | *              |
| Osteoblasts |        |                |                  |                   |               |
| II    | 11.86 ± 1.35 | 0.986          |                  |                   | -              |
| IV    | 14.71 ± 1.25 | 0.701          |                  |                   | *              |
| VI    | 19.00 ± 2.38 | 0.905          | 0.829            | 0.000             | *              |
| VIII  | 27.86 ± 1.35 | 0.986          |                  |                   | *              |
| Osteoclasts |        |                |                  |                   |               |
| II    | 12.86 ± 1.46 | 0.761          |                  |                   | -              |
| IV    | 10.29 ± 0.95 | 0.422          | 0.602            | 0.000             | *              |
| VI    | 8.00 ± 0.82  | 0.905          |                  |                   | -              |
| VIII  | 5.71 ± 1.25  | 0.701          |                  |                   | *              |

* = There is a significant difference (p<0.05)

**Note:** a normality test score of p>0.05 signifies that the data follows normal distribution; a homogeneity test result of p>0.05 indicates that the data demonstrates homogenous distribution; a one-way ANOVA test score of p<0.05 means there is significant difference; group II: 25 grams of PEG on day 7; group IV: propolis extract+PEG on day 7; group VI: BBG+PEG on day 7; group VII: propolis extract+BBG+PEG at the active substance concentration of 2% on day 7.
of them demonstrated normal distribution. Based on the homogeneity test results, all research groups had a significance value greater than 0.05. This indicates that all groups had homogeneous variance. Given the prerequisite normal and homogeneous distribution, a one-way ANOVA test was conducted to quantify the significance among the various groups. Based on the results of the one-way ANOVA test, a significant value of 0.000 was obtained, which was lower than 0.05. This indicates that there was a significant difference in the quantity of fibroblasts, osteoblasts, and osteoclasts between the control and treatment groups.

The results of a Tukey HSD test confirmed a significant difference in the quantity of fibroblasts, osteoblasts, and osteoclasts between the control group (PEG) and the treatment group (propolis extract, BBG, and PEG) on both day 3 and day 7, with a significance of \( p = <0.05 \) (see Table 1 and 2).

**DISCUSSION**

Based on the results outlined above, it is evident that the quantity of fibroblasts increased between days 3 and 7 in both the control and treatment groups. The quantity of fibroblasts on day 7 was higher than that on day 3. This may have occurred because the inflammatory phase enters the healing stage on day 7 and the combination of propolis extract and BBG affects the ROS produced by macrophages.

Propolis was obtained from the Research and Consultation Laboratory Lawang, East Java, Indonesia. It contained cinnamic acid (2.56%), apigenin (1.05%), flavonoid (1.28%), saponin (0.82%), quercetin (1.03%) and terpenoid (1.15%) which produced several effects, including; anti-inflammatory, antibacterial, antiviral, immunostimulatory, antifungal, and anticancer.

The flavonoids present in propolis promote wound healing by increasing the formation of fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor A (VEGFA). FGF2 constitutes a pleiotropic growth factor capable of stimulating fibroblast cells and progenitor osteoblasts. This assertion concurs with the findings of research conducted by Puspasari *et al.* which showed that the expression of FGF2 in the group whose members received an application of propolis extract was significantly higher than that of the control group.

Caffeic acid phenethyl ester (CAPE) represents a group of flavonoids in propolis that has an antioxidant effect which can include excessive oxidative reactions as a result of inflammatory reactions and metabolic processes followed by cell injury. As an anti-inflammatory, CAPE acts to inhibit phospholipase in the arachidonic acid cascade. Consequently, it does not release prostaglandins and leukotriens. CAPE can also inhibit lipoxygenase (LOX) and cylooxygenase (COX) which play a role in the metabolic pathway. COX is inhibited by flavonoids which suppress the stimulation and synthesis of prostaglandins and thromboxanes. LOX is inhibited by a propolis component, quercetin, which impede leukotrin and lipoxin stimulation. CAPE is lipophilic and facilitates cell infiltration, releases anti-inflammatory cytokines (TGF-\( \beta \), IL-10, IL-4), inhibits both pro-inflammatory cytokine (TNF-\( \alpha \), IL-6, IL-1\( \beta \)) and the activity of nuclear factor-kB (NF-kB), while increasing FGF-2 proliferation. In this research, the anti-inflammatory effect of propolis extract was employed to reduce inflammation resulting from tooth extraction trauma by stimulating fibroblast growth. BBG produces an osteoconductive effect that can promote the wound healing process and enhance bone formation.

Based on the foregoing results, it is evident that the osteoblasts increased in number between days 3 and 7, while the quantity of osteoclasts in both the control and treatment groups declined significantly. The quantity of osteoblasts was higher on day 7 than on day 3. The quantity of osteoclasts was initially high in the control group, but decreased on day 7. This could be due to propolis extract and BBG impeding the inflammatory process with the result that both the wound healing and bone regeneration processes were accelerated.

Research conducted by Yuanita, *et al.* shows that the administration of propolis extract can inhibit RANKL binding on RANK receptor, resulting in inhibited activation of NF-kB and a subsequent decrease in the number of osteoclasts. Propolis has an antioxidant effect and will inhibit reactive oxygen species (ROS), a free radical that plays a role in bone resorption, and the process of osteoclastogenesis by activating osteoclast cells. ROS can stimulate the phosphorylation process involving kappa \( \beta \) (IK\( \beta \)) inhibitors which function to bind NF-kB, causing it to remain inactive in the cytoplasm. When IK\( \beta \) is phosphorylated, IK\( \beta \) and NF-kB bonds are released and NF-kB becomes active in moving to the cell nucleus. This process is called NF-kB activation. The presence of flavonoid in propolis acts against the formation of ROS thereby inhibiting the activation of NF-kB. This may induce a reduction in bone resorption.

Propolis extract can accelerate osteoblast cell maturation and bone remodelling activity. Osteoblast cells are regulated by several growth factors, such as bone morphogenetic protein (BMP), runt-related transcription factor 2 (Runx2) and osterix. Propolis extract containing flavonoid plays a role in increasing osterix and Runx2 with the result that osteoblasts mature more rapidly, thereby promoting bone remodelling. Propolis extract containing flavonoid can reduce bone resorption and increase bone remodelling.

Saponins in propolis play an active role in intensifying alkaline phosphate (ALP) activity, increasing mineralization, and promoting expression of the osteogenic ALP, RUNX2 gene. RUNX2 is a transcription factor involved in osteoblast differentiation and bone formation. Saponins also increase...
the activity of bone morphogenetic protein (BMP) which serves as a signal of bone formation, while BMP-2 promotes RUNX2 gene expression.12

In addition, quercetin in propolis promotes an increase in the formation of osterix and RUNX2 which plays a role in stimulating osteoblast differentiation and bone formation by suppressing lipopolysaccharide (LPS).13 Cinnamic acid in propolis acts as an immunomodulator. It can also increase ALP activity and calcium that can stimulate bone formation, while also inhibiting the production of NF-KB and TNF-α.14

Consequently, since propolis extract reduces pro-inflammatory cytokines (IL-1, TNF-α), inhibits NF-KB and increases certain osteoblast progenitors (BMP-2, osterix, RUNX2), it can be argued an inverse relationship exists between osteoblasts and osteoclasts. The reduction in the number of osteoclast cells will occur when their growth is stimulated by homeostasis in bone cells.15 Similarly, the results of this research indicate that a combination of propolis extract and BBG increases the quantity of osteoblasts, but reduces that of osteoclasts. Another cause influencing the increase in osteoblast cells in this research is the administration of graft material (BBG) combined with propolis extract.

BBG constitutes a graft material in the form of xenograft employed in this study. Xenograft is used to stimulate the proliferation of fibroblasts, osteoblasts, and endothelial cells. BBG is the most commonly used graft because it contains hydroxyapatite which is almost identical to human bone and enables the graft to revascularize and be replaced with new bone. Inorganic material from BBG is osteoconductive and acts as a scaffold in a medium for stem cells and osteoblast cells. BBG is capable of supporting the attachment and proliferation of osteoblast cells which represents the first step in the process of osteogenesis. The material supports the bone matrix for regulation through three mechanisms, namely: formation of a strong filling space, establishment of osteoblast adhesion and proliferation and stimulation of bone formation.16

BBG produces an osteoconductive effect which acts as a scaffold in a medium for stem cells and osteoblast cells to attach to, live within, and develop properly in bone defects. In addition, scaffold promotes the development of blood vessels during the formation of new bone. The osteoconductive effect in graft can stimulate bone growth and cause bone apposition in existing bone.16 It can be concluded, therefore, that a combination of propolis extract and BBG with an active substance concentration of 2% can increase the quantity of both fibroblasts and osteoblasts, while decreasing the quantity of osteoclasts on days 3 and 7 after tooth extraction. This, in turn, can accelerate wound healing and bone regeneration.

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