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Research Report

# Effective dose of propolis extract combined with bovine bone graft on the number of osteoblasts and osteoclasts in tooth extraction socket preservation

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## *ABSTRACT*

***Background:*** *Maintaining a good ridge is required during prosthodontic treatment. Hence, adequate alveolar bone support is considered an important factor in pursuing successful dentures. Propolis extract combined with bovine bone graft is a recent and innovative material in the process of socket preservation, as the caffeic acid phenethyl ester (CAPE) it contains can suppress the inflammatory process.* ***Purpose:*** *This study aims to determine the effective dose of propolis extract combined with bovine bone graft on the number of osteoblasts and osteoclasts in socket preservation.* ***Methods:*** *Twenty-eight Cavia cobaya animals were divided into four groups of seven. Group I was given 25 grams of PEG, while Group II were given a propolis extract at a dose of 0.5% combined with bovine bone graft. Group III were given a propolis extract at a dose of 1% combined with bovine bone graft and Group IV were given a propolis extract at a dose of 2% combined with bovine bone graft. On day 30, the lower incisor of each subject was extracted and induced with PEG and propolis (dose 0.5%, 1%, 2%). Histopathological examinations of osteoblasts and osteoclasts were measured with a 400x magnification light microscope. One-way ANOVA and Tukey HSD tests were performed to analyse data statistically.* ***Results:*** *The propolis extract combined with bovine bone graft not only increased the number of osteoblasts but also reduced the number of osteoclasts. The most effective dose for the propolis extract combined with bovine bone graft was 2%.* ***Conclusion:*** *The propolis extract combined with bovine bone graft could be effective in tooth extraction socket preservation at a dose of 2%.*

***Keywords:*** *bovine bone graft; effective dose; osteoblasts; osteoclasts; Propolis extract*

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## INTRODUCTION

Dental and oral diseases are among the top-ten diseases in Indonesia. The significant number of dental disease cases also has an impact on tooth decay, which is a major cause of tooth-loss in the Indonesian population.[1] Moreover, this also leads to problems in subsequent dental treatment and has an impact on the tooth-supporting tissue and alveolar bone. Alveolar bone plays an important role in obtaining ideal prosthetic reconstruction.[2] Damage to bone tissue, as a result of tooth extraction, can cause atrophy of alveolar bone and the healing process can result in bone deformity. In other words, alveolar bone needs to be maintained during the healing process.[3]

Maintaining alveolar bone has been a focus of recent studies and one of the leading materials used to maintain alveolar bone is graft. Graft is a material used to support bone regeneration, reconstructing alveolar bone by filling the tooth extraction socket to maintain the height and width of the alveolar ridge.[4] The formation of new bone from graft material is very time-dependent, therefore, it requires a certain level of material innovation to stimulate the graft and accelerate bone formation.[5]

In addition to bone graft, there are also several other studies arguing that propolis can accelerate the bone remodelling process. Propolis possesses anti-inflammatory activity containing caffeic acid phenethyl ester (CAPE). CAPE inhibits receptor activator of nuclear factor kappa-B

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ligand (RANKL) induced through the activity of nuclear factor kappa beta (NF-κB) during the osteoclast formation process.[6] Hence, this study aims to reveal the effects of propolis extract combined with bovine bone graft on tooth extraction socket preservation, through experimental animal subjects (*Cavia cobaya*), to accelerate bone formation.

## MATERIALS AND METHODS

This study is experimental research with a randomized post-test control group. Research design has been approved by the Ethical Clearance Team, number: 595/HRECC. FODM/IX/2019. The research population of 28 male *Cavia cobaya*, aged 3-3.5 months and between 300-350 g in weight, were divided into four groups of seven subjects. The *Cavia cobaya* were anesthetized intravenously with ketamine, at a dose of 0.1 cc/300 g BB. Afterwards, their tooth was extracted with a needle holder and given a propolis extract combined with bovine bone graft, as much as 0.1 cc, according to the volume of the tooth extraction socket, and sewn.

Subsequently, they were divided into 4 groups.[7] In Group I, the extracted tooth sockets of those *Cavia cobaya* animals were given 24 g of PEG, as much as 0.1 cc (as control group). In Group II, the extracted tooth sockets were given 0.1 cc of the combination of 0.5 g of Propolis, 0.5 g of bovine bone graft, and 99 g of PEG, at a dose of 0.5%. In Group III, the extracted tooth sockets were given 0.1 cc of the combination of 0.5 g of Propolis, 0.5 g of bovine bone graft, and 49 g of PEG, at a dose of 1%. In the final group, Group IV, the extracted tooth sockets were given 0.1 cc of the combination of 0.5 g of Propolis, 0.5 g of bovine bone graft, and 24 g of PEG, at a dose of 2%.

After 30 days, the *Cavia cobaya* test subjects were sacrificed, and their jaw decalcified with EDTA for a month. Next, a paraffin block was prepared for each one, manufactured and cut to a thickness of 4μ with a rotary microtome, before being deparaffinized through dissolution in xylol for two intervals of 3 minutes. The residual xylol was respectively washed with 99%, 95%, 90%, 80%, and 70% absolute alcohol for two intervals of 1 minute. Any residual alcohol was removed with running water. At this point, haematoxylin eosin (HE) staining was performed for 30 seconds before rinsing with water. Staining with HE was conducted for 1–2 minutes prior to washing with 70%, 80%, 90%, 95%, and 99% absolute alcohol for two intervals of 1 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 on a worksheet with a mean value per FoV. At this point, the quantity of osteoblasts and osteoclasts was calculated.[7] The data obtained was statistically analysed using the one-way ANOVA test to observe differences in each group. If the data in each group proved significantly different, the data was then analysed using the HSD test.

## RESULTS

The results of this study showed an increase in the number of osteoblast cells in all the treatment groups (Figure 1 and 2). The results also showed a decrease in the number of osteoclast cells in all the treatment groups (Figure 1 and 3).

A Kolmogorov-Smirnov normality test was conducted on the results of the statistical analysis on the number of osteoblasts. In this research, all the research groups had a p value greater than 0.05, signifying that the data derived from all the test subjects (Table 1). Moreover, based on

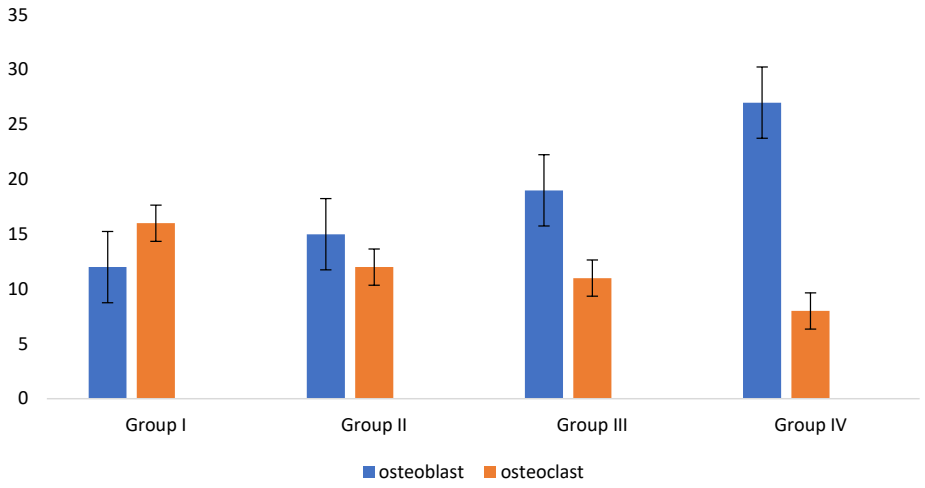


**Figure 1.** The diagram of the average number of osteoblasts and osteoclasts at a dose of 0.5%, 1%, and 2%.
**Note:** Group I: PEG only (control group); Group II: the combination of propolis extract and bovine bone graft at a dose of 0.5%; Group III: the combination of propolis extract and bovine bone graft at a dose of 1%; Group IV: the combination of propolis extract and bovine bone graft at a dose of 2%.

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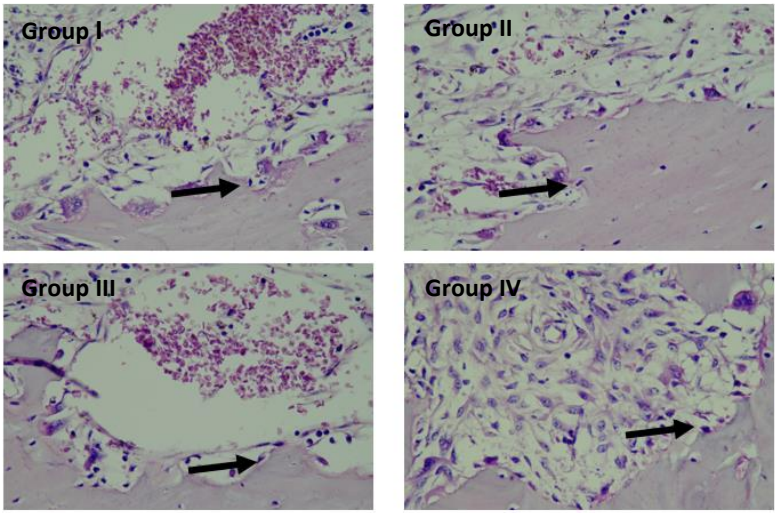


**Figure 2.** Black arrows indicate histopathological staining identifying osteoblasts (HE staining observed through a light microscope at a magnification of 400x). Group I: control group (PEG); Group II: the combination propolis extract and bovine bone graft at a dose of 0.5%; Group III: the combination propolis extract and bovine bone graft at a dose of 1%; Group IV: the combination propolis extract and bovine bone graft at a dose of 2%.

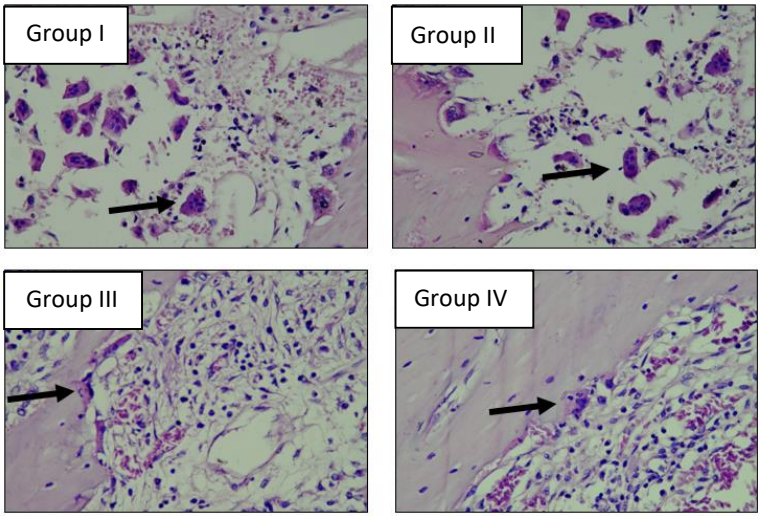


**Figure 3.** Black arrows indicate histopathological staining identifying osteoclasts (HE staining observed through a light microscope at a magnification of 400x). Group I: control group (PEG); Group II: the combination propolis extract and bovine bone graft at a dose of 0.5%; Group III: the combination propolis extract and bovine bone graft at a dose of 1%; Group IV: the combination propolis extract and bovine bone graft at a dose of 2%.

**Table 1.** Statistical analysis data (means, standard deviation, and normality) of the quantity of osteoblasts and osteoclasts in each group

| | Group | Mean | Standard deviation | Normality test |
|---|---|---|---|---|
| Osteoblast | I | 11.86 | 1.345 | 0.508 |
| | II | 15.03 | 1.254 | 0.674 |
| | III | 19.00 | 2.380 | 0.900 |
| | IV | 26.56 | 1.345 | 0.789 |
| Osteoclast | I | 15.71 | 1.799 | 0.680 |
| | II | 12.36 | 1.345 | 0.508 |
| | III | 11.14 | 1.952 | 0.738 |
| | IV | 7.18 | 1.799 | 0.680 |

Note: Normality test score of p>0.05 means the data follows normal distribution; Group I: PEG only (control group); Group II: the combination of propolis extract and bovine bone graft at a dose of 0.5%; Group III : the combination of propolis extract and bovine bone graft at a dose of 1%; Group IV: the combination of propolis extract and bovine bone graft at a dose of 2%.

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**Table 2.** Statistical analysis data Tukey HSD test relating to the quantity of osteoblasts

| | Group | Tukey HSD test | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| Osteoblast | I | - | * | * | * |
| | II | | - | * | * |
| | III | | | - | * |
| | IV | | | | - |

Note*: significant

**Table 3.** Statistical analysis data Tukey HSD test relating to the quantity of osteoclasts

| | Group | Tukey HSD test | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| Osteoclast | I | - | * | * | * |
| | II | | - | .868 | * |
| | III | | | - | * |
| | IV | | | | - |

Note *: significant

the results of the one-way ANOVA test, conducted on both the between groups as well as within the treatment groups, the data obtained was significant, with a value of 0.000 (α<0.05).

The results of the HSD test (Table 2) showed that there was a significant difference between Groups I and II, with α value of 0.017 (α<0.05). Similarly, there were also significant differences between Groups I and III, with α value of 0.000 (α<0.05); Groups I and IV, with α value of 0.000 (α<0.05); Group II and Group III, with α value of 0.000 (α <0.05); Groups II and IV, with α value of 0.000 (α <0.05); and Group III and Group IV with α value of 0.000 (α<0.05).

Next, the results of the statistical analysis on the number of osteoclasts. a Kolmogorov-Smirnov normality test was conducted. In this research, all the research groups had p value greater than 0.05 signifying that data derived from all (Table 1). Moreover, based on the results of the one-way ANOVA test conducted on both between groups as well as within the treatment groups, the data obtained were significant with value of 0.000 (α<0.05).

Based on the results of the HSD test (Table 3), there was a significant difference between Group I and Group II, with α value of 0.002 (α<0.05), and between Group I and Group III, with α value of 0.000 (α<0.05). There were also significant differences between Groups I and IV, with α value of 0.000 (α<0.05), as well as between Group II and Group IV, with α value of 0.000 (α<0.05), and between Group III and Group IV, with α value of 0.000 (α <0.05). However, unlike the previous results, there was no significant difference between Groups II and III, with α value of 0.868 (α<0.05), as the difference concentration was slightly than the others.

## DICUSSION

The purpose of this study is to determine the effective dose of propolis extract combined with bovine bone graft in socket preservation after 30 days. The results revealed that the combination of propolis extract (a dose of 0.5%, 1%, and 2%) and bovine bone graft can effectively affect the formation of alveolar bone during tooth extraction socket preservation after 30 days. Moreover, the results from this study also reveal that the combination of propolis extract (with dose of 5%, 1%, and 2%) and bovine bone graft also generates more osteoblasts than osteoclasts. We found the highest increased number of osteoblasts was in the treatment group treated with the propolis extract combined with bovine bone graft at a dose of 2% (group IV), compared to the other test groups (group I as the control, group II at a dose of 0.5%, and group III at a dose of 1%).

Meanwhile, the lowest number of osteoclasts was also found in the treatment group treated with the propolis extract combined with bovine bone graft at the dose of 2% (group IV), compared to the other test groups (group I as the control, group II at a dose of 0.5%, and group III at a dose of 1%). We discovered that the effective dose of propolis extract combined with bovine bone graft to increase the number of osteoblasts and decrease the number of osteoclasts in socket preservation is 2% (group IV). This endorses a previous study which argues that propolis combined with graft can potentially be used for alveolar bone regeneration during socket preservation.[8]

Propolis extract is known to contain antioxidants that can increase alveolar bone density, as well as accelerate the bone formation process. Moreover, CAPE contained in propolis extract is also known to have strong properties that support the growth and development of human bones, as well as activating osteoblast progenitor cells to increase collagen formation. Consequently, propolis extract can also inhibit the formation and maturation of osteoclasts.[6,9] CAPE is a natural NF-κB inhibitor derived from propolis; the inhibition of NF-κB and nuclear factor from T-cell activation (NFAT), triggered by CAPE, can result in weakening osteoclastogenesis. In other words, CAPE can suppress osteoclastogenesis and bone loss through the inhibition of mitogen-activated protein kinase (MAPK), induced by mitogen-RANKL.[10,11]

Moreover, the mechanism of inhibiting osteoclastogenesis through CAPE involves the inhibition of DNA binding activity and NF-κB transcription, therefore, CAPE can directly inhibit the binding of NF-κB DNA in osteoclast precursors by breaking down its mechanism. CAPE reduces the expression of NFAT and c-Fos after stimulation of RANKL, derived from osteoclast precursors.[12] In inhibiting the binding activity of NF-κB DNA, CAPE can not only reduce the transcription of c-Fos by NF-κB, but can also interfere with or damage the induction of NFAT by c-Fos during osteoclastogenesis. CAPE also inhibits M-CSF and RANKL, which induce osteoclast differentiation. Thus, CAPE is considered a potential therapeutic agent for the inhibition of bone resorption triggered by osteoclasts, as well as in the prevention of bone resorption.[13]



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CAPE, inhibits the release of inflammatory cytokines and increases stimulant production of anti-inflammatory cytokines, such as IL-10 and IL-4.[14,15] IL-10 stimulants have an anti-inflammatory function, which can reduce regulation of IL-5 production by T cells, while IL-5 plays a role in differentiation and activation of eosinophil function, by controlling eosinophil accumulation in inflamed tissue. IL-10 has main function, such as inhibiting TNF-α, IL-1, chemokines, and IL-12 produced by macrophages. IL-4 has an inhibitory effect on the expression and release of proinflammatory cytokines. These cytokines inhibit or suppress cytokines derived from monocytes, including IL-1 TNF-a, IL-6, IL-8, and the macrophage inflammatory protein (MIP). IL-4 is also known to suppress macrophage cytotoxic activity, kill parasites, and produce nitric oxide derived from macrophages, so that the role of IL-1, IL-6, and TNF-α in osteoclast mitogenesis is inhibited, causing osteoclastogenesis to be disrupted.[14]

Bovine bone graft is the main type of bone graft. The main function of bone graft is to stimulate osteogenesis. The biologic mechanisms that provide a rationale for bone graft are osteoconduction, osteoinduction, and osteogenesis. Osteoconduction is a graft resorption process, that replaces new bone from the margin. Graft material is used as a framework upon which to spread and generate new bone from the margin of defect. As in osteoinduction, it stimulates osteoprogenitor cells to differentiate into osteoblasts and begins the formation of new bone.[16,17] Osteoinduction is a process of attracting pluripotential cells from recipients around graft and bone, because the material of bone graft and bone contain osteoinduction mediators, such as bone morphogenic protein (BMP). Material from bovine bone graft is capable of supporting the attach and proliferate of osteoblast cells, which represents the first step in the process of osteogenesis.[16]

Consequently, since propolis extract reduces pro-inflammatory cytokines (IL-1, TNF-α), inhibits NF-κB, and increases certain osteoblast by TGF-β, it can be argued an inverse relationship exists between osteoblasts and osteoclasts. Bovine bone graft can stimulate the osteoblast on the biological mechanism to accelerate new bone growth. Similarly, the results of this research indicate that a combination of propolis extract (at a dose of 0.5%, 1% and 2%) and bovine bone graft increases the quantity of osteoblasts and reduces that of osteoclasts. However, the combination of propolis extract at a dose of 2% and bovine bone graft is more effective than the other doses. This research demonstrates that propolis extract combined with bovine bone graft at a dose of 2% effectively affects the number of osteoblasts and osteoclasts during tooth extraction socket preservation.

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