

# Increased Expression of RUNX2 and ALP Resulting from a Combination of Propolis Extract and Bovine Bone Graft in Socket Preservation Material

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The aim of this study was to determine the inductive effect of a combination of propolis and BBG extract on RUNX2 and ALP expression in the tooth extraction sockets of *Cavia cobaya*. Fifty-six *Cavia cobaya* were divided into four groups: polyethylene glycol (PEG), propolis extract + PEG, BBG + PEG, and propolis extract + BBG + PEG. The lower left incisor was extracted, and the socket subsequently filled with material according to the specific group of which the subject was a member. The subjects were sacrificed on the 14<sup>th</sup> and 30<sup>th</sup> days. Immunohistochemical staining was carried out under a light microscope at 400x magnification. Statistical analysis was then carried out by means of One-Way ANOVA and Tukey HSD tests. The mean number of RUNX2 and ALP expressions in each group was significantly different. The highest number of RUNX2 and ALP expressions occurred in the propolis + BBG + PEG group on the 30<sup>th</sup> day, while the lowest expressions were observed in the control group on the 14<sup>th</sup> day. A combination of propolis and BBG extract at a concentration of 2% of active substance effectively increases the expression of RUNX2 and ALP in preserving the tooth extraction sockets of *Cavia cobaya*.

**Keywords:** Propolis extract. Bovine bone graft. RUNX2. ALP. Socket preservation.

## INTRODUCTION

The bone resorption process resulting from tooth extraction trauma can result in serious problems with regard to implant dentures and restorative dentistry. One means of maintaining the dimensions of alveolar bone after tooth extraction is to immediately perform bone grafting on the socket. Bovine bone xenograft constitutes a commonly employed contemporary grafting material. Bovine Bone Graft (BBG), frequently used because of its osteoconductive qualities, is a component of an inorganic matrix that provides a skeleton / scaffold during bone regeneration, although its results are far less stable than expected. (Pagni *et al.*, 2012; Kresnoadi, Hadisoesto, Prabowo, 2016). This graft was also showed increasing bone fraction area from the crestal to the apical region

and decreasing connective tissue in post extraction socket (Artzi, Tal, Dayan, 2000b). This graft was acceptable for edentulous ridge preservation at sites prepared to receive endosseous implants (Artzi, Tal, Dayan, 2000a).

It is a minimum requirement that the graft material possesses osteoconductive and osteoinductive properties. Osteoconduction occurs when grafting material serves as a scaffold for new bone growth, while osteoinduction results from osteoprogenitor cells differentiating into osteoblast cells and initiating the formation of new bone cells. Grafting materials demonstrating osteoconductive and osteoinductive properties also trigger the formation of new osteoblast cells and induce more rapid integration. (Kumar, Vinitha, Fathima, 2013)

Several researchers have argued that propolis extract possesses a range of properties, including; anti-bacterial, anti-fungal, anti-viral, anti-oxidant, anti-cancer and anti-inflammatory, in addition to the ability to promote more rapid wound healing. (Khurshid *et al.*, 2017) Propolis contains caffeic acid phenethyl ester (CAPE), one of the

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major active components, which induces antioxidant and anti-inflammatory activity, while also increasing growth factor, extra cellular matrix remodeling and reepithelialization, thereby accelerating post-extraction socket healing.(Ernawati, Puspa, 2018) Through CAPE, propolis can affect the migration and proliferation of cells and also increasing the expressions of VEGF and TGF- $\beta$  in healing process.(Rahayu *et al.*, 2020) Tooth extraction causes macrophage cells to release proinflammatory cytokines and TGF $\beta$ . Application of propolis extract and bovine bone graft combination in tooth extraction socket is expected to cause TGF $\beta$  to regulate osteoblast cell, therefore induce stem cells signalling to become progenitor osteoblast cells.

During bone growth, bone morphogenetic protein (BMP)-2 triggers the osteoblastogenetic process through stimulation of osteoblastic specific factor 2 / core binding factor 1 / RUNX2 which induces the production of osteoblast cells and, by extension, alveolar bone growth. (Kresnoadi *et al.*, 2017) Potential osteoinduction during the bone grafting process can be evaluated by means of RUNX2 and alkaline phosphatase (ALP) protein markers. RUNX2, a protein first detected in pre-osteoblast cells, constitutes the first transcription factor required to induce the differentiation of mesenchymal stem cells into osteoblast cells. RUNX2 expression increases in immature osteoblast cells but decreases in mature osteoblasts, while also triggering expression of major bone matrix genes during the early stages of osteoblast cell differentiation and precipitating immature bone formation.(Komori, 2010; Brunetti *et al.*, 2013)

ALP is a membrane-bound tetrameric enzyme present in various cells, including the plasma membrane of osteoblast cells. ALP plays an important role in the formation and mineralization of osteoid by degrading mineralization inhibitor enzymes, pyrophosphates under alkaline pH conditions. This enzyme induces an alkaline atmosphere in the osteoid tissue formed, with the result that calcium can be easily deposited in the tissue. In addition, this enzyme causes an increase in phosphate concentrations within the bone with the result that calcium-phosphate bonds are created in the form of hydroxyapatite crystals.(Yudaniayanti 2005; Linder, 2016; Shetty *et al.*, 2016)

The purpose of this study was to determine the inductive effect of a combination of propolis and BBG on RUNX2 and ALP expression in the tooth extraction sockets of *Cavia cobaya*.

## MATERIAL AND METHODS

This research was conducted using combination of ethanol extract of propolis and bovine bone graft. The propolis extract was obtained from Lawang, East Java, meanwhile the bovine bone graft was obtained from tissue bank of Dr. Soetomo General Hospital. The bovine bone graft size varies from 150 to 355 $\mu$ /500 mg, obtained from calcination process (1000°C) to eliminate organic matrix of the bone.

### Ethical approval

This research was conducted following approval by the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (number 587/HRE.CCFODM/IX/2019).

### Materials

The materials being used of this study divided into four groups: control group (only polyethylene gel), propolis extract group, bovine bone graft group, and combination of propolis extract and bovine bone graft group. The active ingredient being used in each group was 2%. This dosage were chosen according to previous study that showed the best results in bone healing (Prabowo, Kresnoadi, Hidayati, 2020).

### Research procedure

The experimental subjects consisted of healthy, active, 3-3.5 month-old male *Cavia cobaya* weighing approximately 300-350 grams. Fifty-six subjects were divided into four groups of seven members. The groups consisted of PEG, BBG + PEG, Propolis extracts + PEG, and a combination of propolis + BBG + PEG. The *Cavia cobaya* were intravenously administered 0.2 cc ketamine / 300 gr BB, prior to extraction of the lower left incisors

with a needle holder. The extraction sockets were then filled with 0.1 cc material and subsequently closed with sterile DS 12 3/8c, 12 mm, 6/10 met, 0.7 polyamide monofilament yarn (Braun Aesculap).

### Immunohistochemical observation

After 14 and 30 days, the subjects were sacrificed, the tooth extraction area on the lower jaw being removed and softened, prior to production of a paraffin block preparation. The paraffin block was cut using a rotary microtome to a thickness of 4  $\mu\text{m}$  and then placed on a microscope slide glass. Deparaffinization was performed by dissolving the specimen in xylol solution for 2 $\times$ 3 minutes and washed with 99%, 95%, 90%, 80%, and 70%, absolute alcohol for 2 $\times$ 1 minutes respectively. Immunohistochemical staining of RUNX2 and ALP (Santa Cruz) monoclonal antibodies was then carried out<sup>7</sup> with calculation of RUNX2 and ALP expressions being completed under a light microscope. Clone for Runx2 was IgG1 (kappa light chain), for ALP was IgG2a (kappa light chain). The dilution factor of each antibody was 1:200. Each slide was examined at 400x magnification in nine visual fields.

The measurements of RUNX2 and ALP immunoexpression were accounted at osteoblast cells with positive immuno-area in the 2/3 apical area (apical-cervical) of socket. The measurement of each immunoexpressions were done using manual observation under light microscope with 400x magnification. The cells that had brownish color in their cytoplasm (DAF) and identified as osteoblast cells based on its morphology were counted as positive cells that express RUNX2 and ALP. The calculations of average results per field of view were recorded on a worksheet and tabulated for the purposes of statistical analysis.

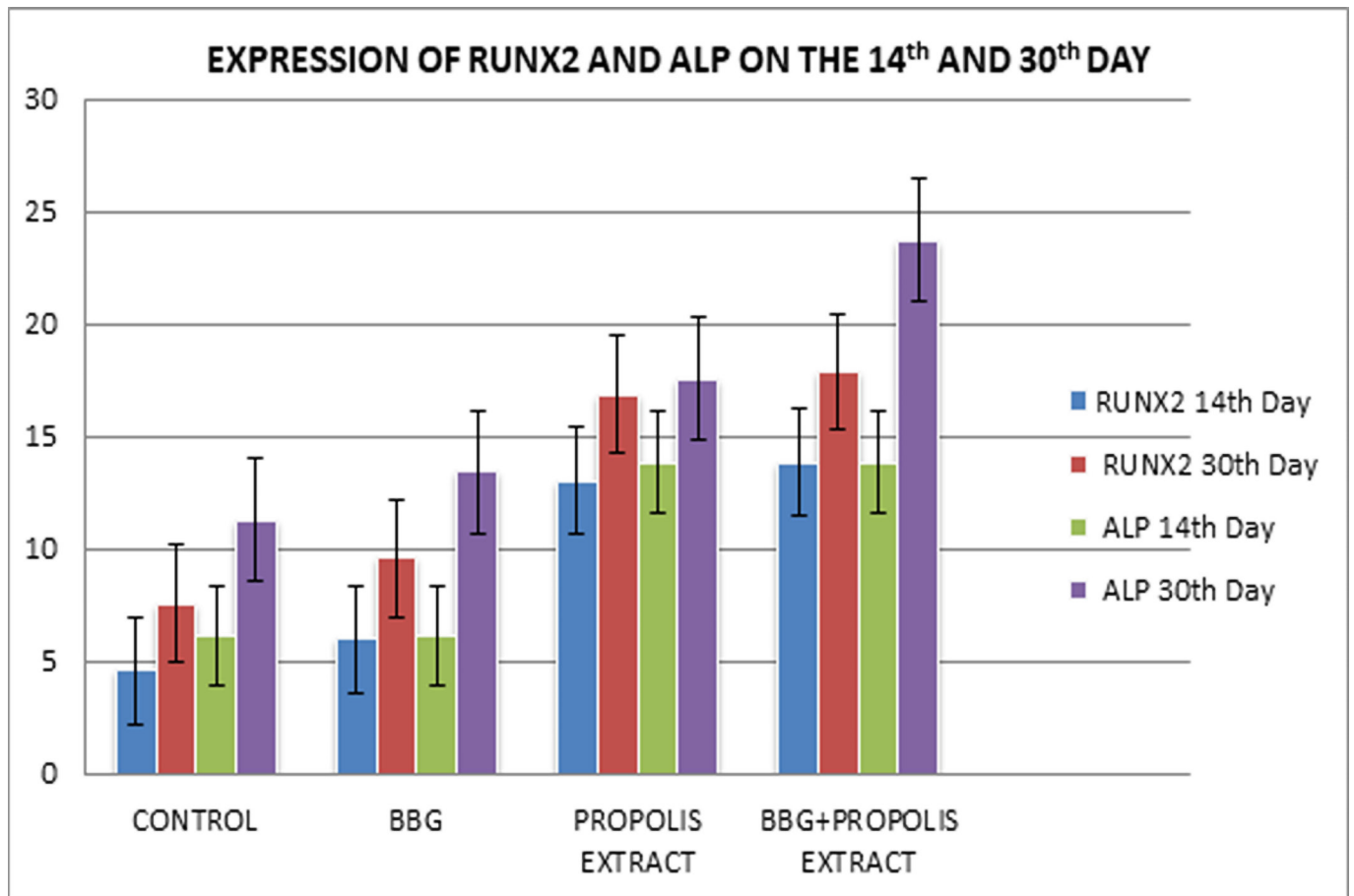
### Statistical analysis

Data normality was established by means of a Kolmogorov-Smirnov test, while the homogeneity of the data was quantified by the conducting of a Levene's test. A One-Way ANOVA test was conducted in order to identify any potential differences between groups, followed by completion of a Tukey test (HSD) to determine the differences between each treatment group.

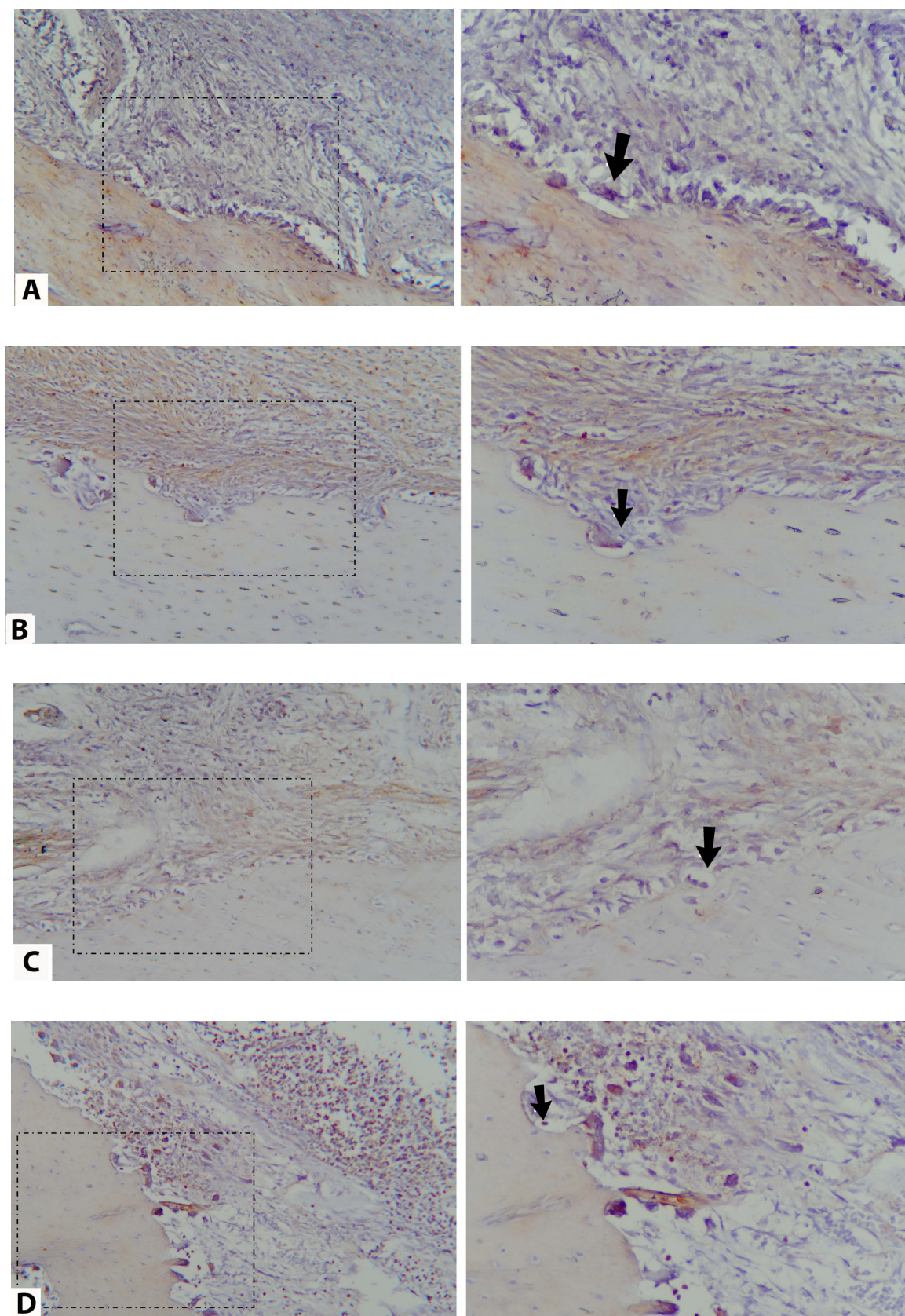
### RESULTS AND DISCUSSION

From the calculation results, it was evident that the highest RUNX2 expression (18.43 + 3.5) occurred in the propolis + BBG + PEG group on the 30th day, while the lowest (4.57 + 1.2) was recorded by the PEG group on the 14th day. The highest ALP expression (23.71 + 2.0) also occurred in the propolis + BBG + PEG group on the 30th day, with the lowest (6.14 + 2.1) registered by the PEG group on the 14th day. The differences between the expression of RUNX2 and ALP in each treatment group on the 14th and 30th days can be seen in Figure 1 below. There was bovine bone graft and propolis particles left on immunohistochemical observation as seen on Figure 6. There can be seen those remnants seems enclosed to proliferating cells.

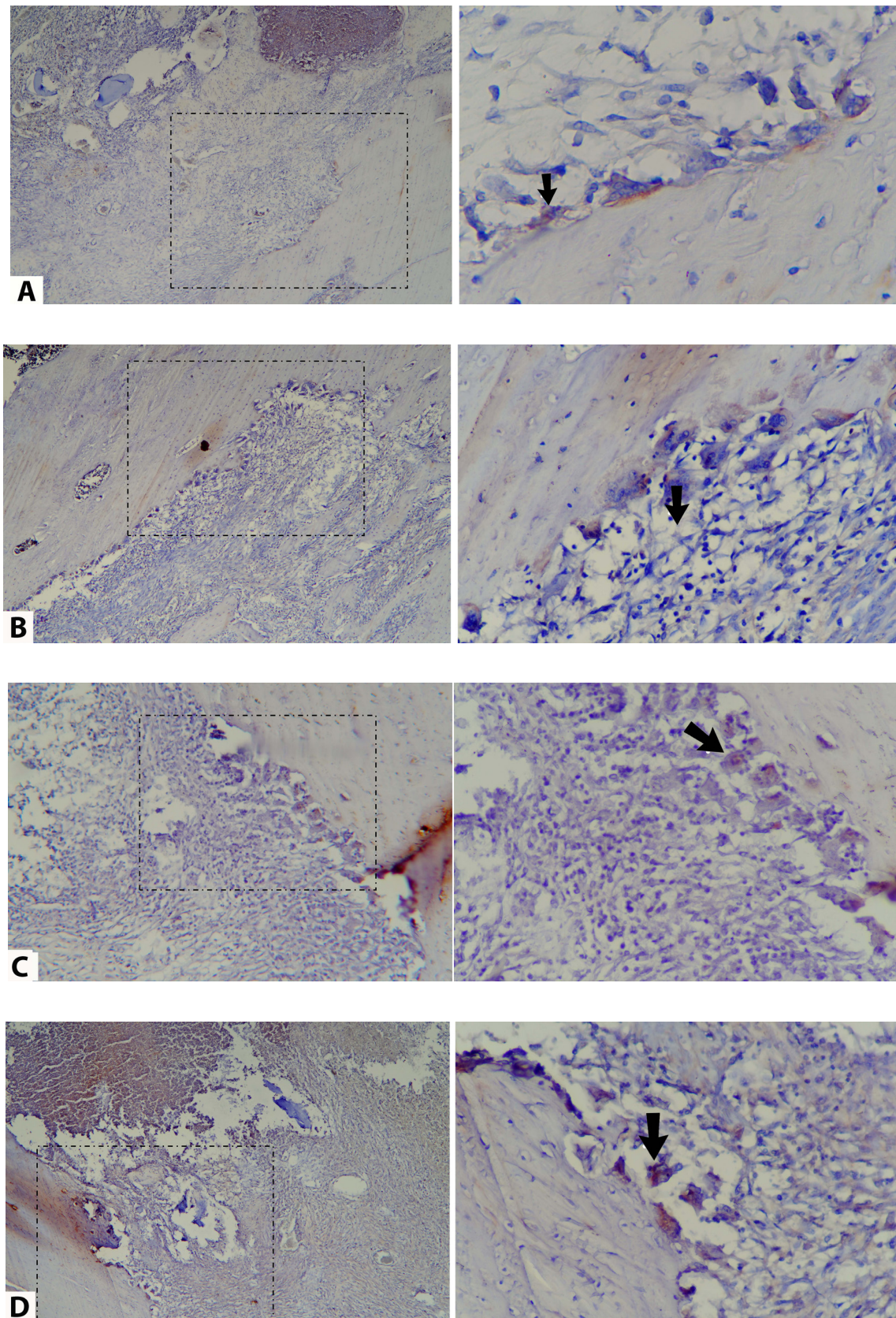
The data produced by the Kolmogorov-Smirnov test was normally distributed ( $p > 0.05$ ), while it was evident that the Levene's test data was homogeneous ( $p > 0.05$ ) across all groups. From the One-Way ANOVA test conducted, a value of  $p = 0,000$  ( $p < 0.05$ ) was obtained from all data groups. Significant differences existed between the treatment groups in relation to the expression of RUNX2 and ALP on the 14th and 30th days. Microscopic images of RUNX2 and ALP expression on the 14<sup>th</sup> and 30<sup>th</sup> day are contained the image below (Figure 2,3,4,5).



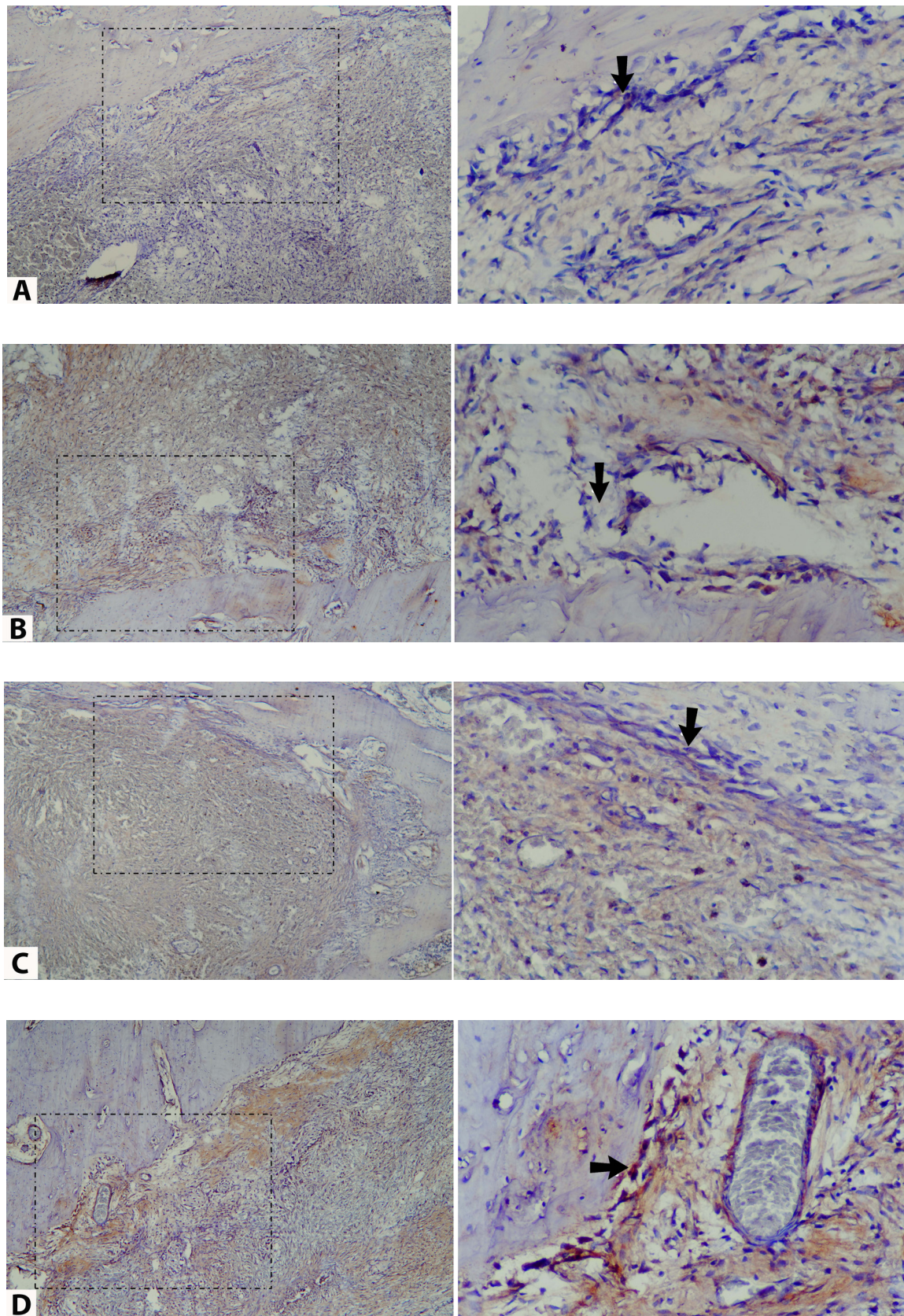
**FIGURE 1** - RUNX2 and ALP expressions in each group on the 14th and 30th days.



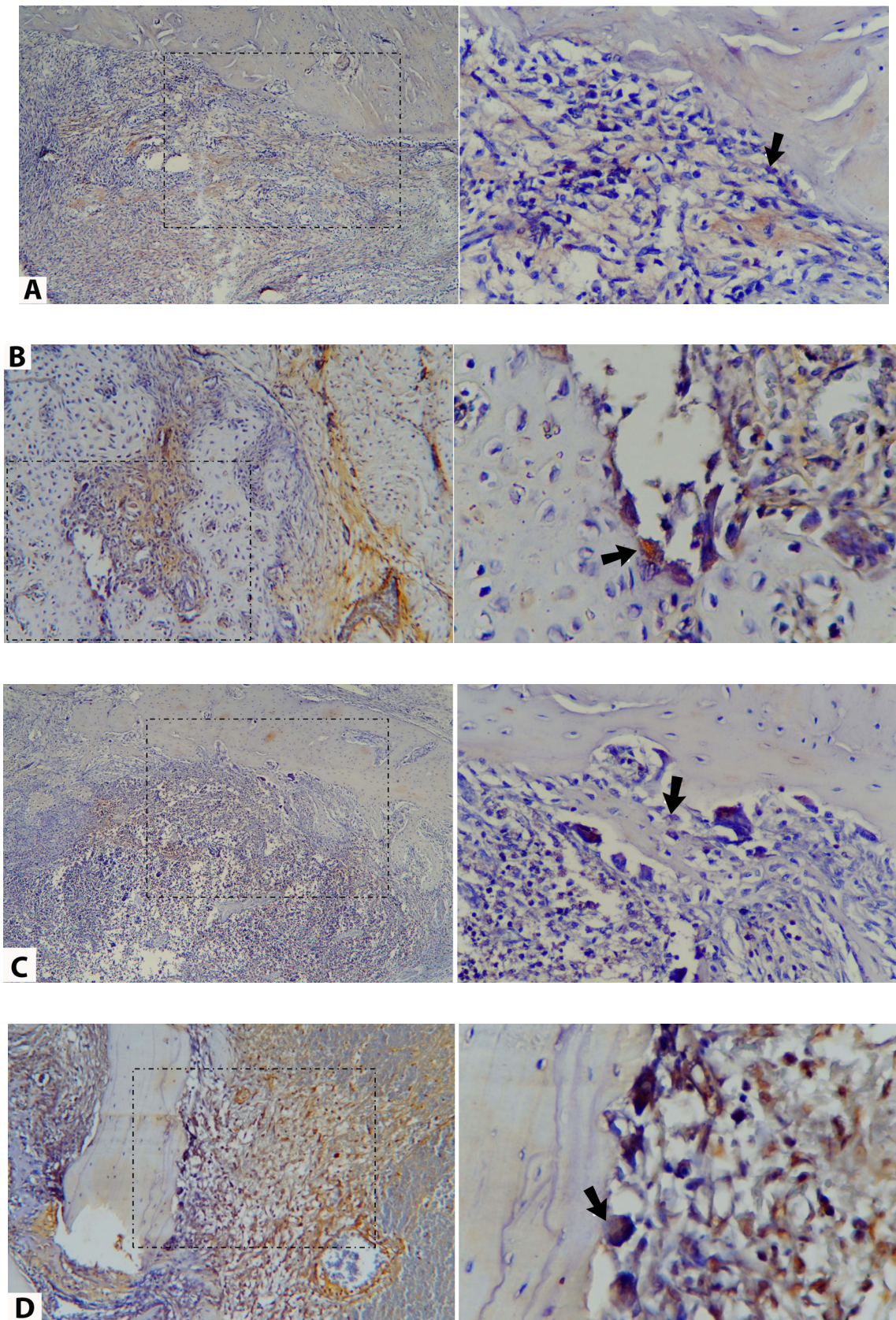
**FIGURE 2** - RUNX2 expression on day 14. A. Control / PEG Group, B. Propolis extract + PEG group, C. BBG + PEG group, D. Propolis extract + BBG + PEG group. Each figures consist of 100x and 400x magnification. The arrows showed cell with positive expression of RUNX2.



**FIGURE 3** - RUNX2 expression on day 30. A. Control / PEG Group, B. Propolis extract + PEG group, C. BBG + PEG group, D. Propolis extract + BBG + PEG group. Each figures consist of 100x and 400x magnification. The arrows showed cell with positive expression of RUNX2.

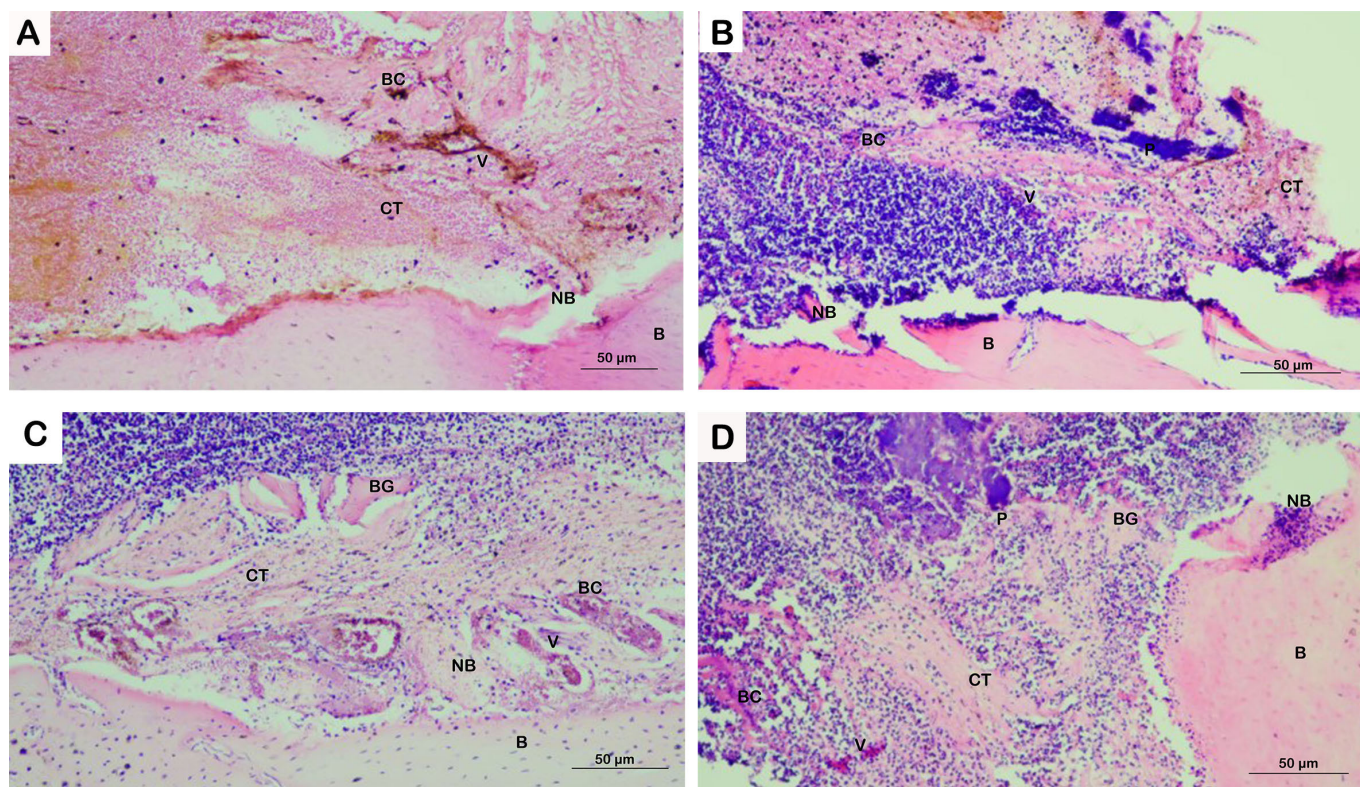


**FIGURE 4** - ALP expression on day 14. A. Control / PEG Group, B. Propolis extract + PEG group, C. BBG + PEG group, D. Propolis extract + BBG + PEG group. Each figures consist of 100x and 400x magnification. The arrows showed cell with positive expression of ALP.



**FIGURE 5** - ALP expression on day 30. A. Control / PEG Group, B. Propolis extract + PEG group, C. BBG + PEG group, D. Propolis extract + BBG + PEG group. Each figures consist of 100x and 400x magnification. The arrows showed cell with positive expression of ALP.





**FIGURE 6** - Hematoxylin-eosin staining with 100x magnification on day 30 of all groups. A. Control / PEG Group, B. Propolis extract + PEG group, C. BBG + PEG group, D. Propolis extract + BBG + PEG group. B: bone, NB: new bone, CT: connective tissue, V: vessels, BG: bovine bone graft, BC: blood clot, P: propolis residual.

Post-extraction alveolar bone resorption is inevitable and can be prejudicial to future dental treatment. It is important to preserve the socket by performing bone grafting immediately after tooth extraction in order to maintain the dimensions of the alveolar bone. (Pagni *et al.*, 2012; Kresnoadi, Hadisoento, Prabowo, 2016) The bone grafting material used in post-extraction socket preservation can accelerate bone cell regeneration due to its osteoinductive and osteoconductive properties. (Kumar, Vinitha, Fathima, 2013)

The results of the Surabaya Industrial Research and Consultation Institute investigation indicated that the bee propolis ethanol extract from Lawang-Malang utilised for the purposes of this research was found to contain: 2.5% caffeic acid phenethyl ester (CAPE), 1.05% apigenin, 1.28% flavonoids, 0.82% saponins, 1.03% quercetin, and 1.15% terpenoids. In this study, up to 0.1 cc of a combination of propolis extract with BBG at an active substance concentration of 2% was inserted into

the socket as a preservation grafting material after the subjects had undergone tooth extraction.

The bioactive ingredients present in propolis extract has antioxidant effects that can suppress osteoclastic activity while, in contrast, increasing osteoblastic activity.<sup>6</sup> Greater osteoblastic activity will increase the expression of RUNX2 and ALP as a result of BMP induction. (Yamaguchi, Komori, Suda, 2000; Saraç, Saygılı, 2007)

From Figure 1 and the results of the statistical analysis, a significant difference in the expression of RUNX2 and ALP between the control group (PEG), BBG + PEG, Propolis extract + PEG, and the combination of BBG + propolis extract + PEG is evident. The results of this study indicate that filling the post-extraction sockets of *Cavia cobaya* with a combination of BBG + propolis extract + PEG effectively increased the expression of RUNX2 and ALP on the 14th and 30th days, thereby reducing the number of both osteoclast and osteoblast cells and accelerating the bone repair and regeneration

processes. The results of this study are in line with those of the research of Wang *et al.*, (2014) and Ke *et al.*, (2016) which contended that the content of bioactive substances in propolis, such as saponins and quercetin, can enhance the activity of RUNX2, ALP, and osterix which stimulate osteoblast cell differentiation and suppress LPS, resulting in an increase in mineralization and bone formation. (Wang *et al.*, 2014; Ke *et al.*, 2016)

Osteoblast cell development is regulated by activation of the Wnt /  $\beta$ -catenin signal and the expression of several major transcription factors, including RUNX2, which are necessary to determine the expression of osteogenic genes such as collagen I, osteopontin, alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin. RUNX2 functions by binding to the regulatory site promoter of genes in order to activate the transcription of genetic codes. The potential of osteoinduction as an early indicator of cellular activity and differentiation of osteoblast cells within the bone grafting process can be evaluated with RUNX2 and ALP protein markers. RUNX2 constitutes the first transcription factor required to induce differentiation of mesenchymal stem cells (MSC) into osteoblast cells. This protein was first detected in pre-osteoblast cells. RUNX2 expression increases in immature osteoblast cells and decreases in mature cells. RUNX2 triggers expression of major bone matrix genes during the early stages of osteoblast cell differentiation, induces ALP activity and leads to immature bone formation. (Yamaguchi, Komori, Suda, 2000; Golub, Boesze-Battaglia, 2007; Komori, 2010; Brunetti *et al.*, 2013; Ling *et al.*, 2017) ALP is a membrane-bound tetrameric enzyme present in various cells, including osteoblast cell membrane plasma, which at alkaline pH plays an important role in the formation and mineralization of osteoid by degrading pyrophosphate, a mineralization inhibitor enzyme. This enzyme produces an alkaline atmosphere in the osteoid tissue formed with the result that calcium can be easily deposited within the tissue. This enzyme also causes an increase in phosphate concentrations, precipitating the developing of calcium-phosphate bonds in the form of hydroxyapatite crystals. (Yudaniayanti, 2005; Linder, 2016; Shetty *et al.*, 2016) Kirkham and Cartmell (2007) revealed that the ALP mRNA level increases at least two days after stimulation

with a steady rise during the osteoblast cell differentiation process lasting up to 14 days.

These results were in accordance with previous study by Nizar, Kresnoadi and Soekobagiono (2020). Their study was to analyzed the number of osteoblast and osteoclast after 14 and 30 days. The results showed that the most osteoblast numbers were in propolis-bovine bone graft group day 30 (Nizar, Kresnoadi, Soekobagiono, 2020). This findings were in line with the normal process of osteoblastogenesis where the role of RUNX2 were high in earlier stage (differentiation from mesenchymal cells to preosteoblast cells), whether ALP expression were high in later stage (differentiation from preosteoblast to osteoblast cell) (Zhang, 2010).

The results of this study indicate an increase in RUNX2 expression followed by one of ALP. These findings are in line with those of the studies by Zhang *et al.*, (2012) and Pudyani *et al.*, (2014) which state that the stimulation of osteogenic differentiation by RUNX2 is followed by increased ALP activity and mRNA expression of genes associated with osteogenesis (ALP, type I collagen and osteocalcin) and maturation of the osteoblast phenotype. RUNX2 is the main regulator that plays an important role in determining the transcription of many genes involved in osteogenic differentiation by controlling its promoter activity, including that of ALP, collagen type I, osteocalcin and osteopontin. (Zhang *et al.*, 2012; Pudyani *et al.*, 2014) Jo *et al.*, (2019) suspect the existence of a reciprocal control relationship between RUNX2 and ALP in the process of osteoblast differentiation. (Jo *et al.*, 2019)

Saponins and quercetin in propolis can promote an increase in BMP activity that plays a role in the expression of RUNX2, ALP, and osterix, in stimulating osteoblast cell differentiation, in suppressing LPS, and in promoting bone formation and mineralization. (Wang *et al.*, 2014; Ke *et al.*, 2016)

## CONCLUSION

The combination of propolis and BBG extract effectively increases the expression of RUNX2 and ALP during attempts to preserve post-extraction tooth socket of *Cavia cobaya* at an active substance concentration of 2%.

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