ORIGINAL ARTICLE

Ethanol extract of propolis-bovine bone graft combination as a prospective candidate for socket preservation: Enhancing BMP7 and decreasing NFATc1

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Abstract

Objective: To analyse the expression of BMP7 in osteoblasts and NFATc1 in osteoclasts during the bone healing process in the extraction socket and the possible relationship between the expression of BMP7 and NFATc1.

Methodology: This study represented a post-test only control group design consisting of four groups, namely; a control group (polyethylene glycol), an ethanol extract of propolis (EEP) group, a bovine bone graft (BBG) group, and a EEP-BBG group. 56 Cavia cobaya were split randomly into four groups. The mandibula left incisors of the subjects were extracted, treated with certain materials according to their group, and sutured. The expression of BMP7 and NFATc1 was observed on day 7 and day 14 by means of immunohistochemical staining. Statistical analysis was performed using a combination of one-way ANOVA, Games-Howell post-hoc, and Pearson tests.

Results: The propolis-BBG combination group showed the highest BMP7 expression, on both day 7 and day 14. With regard to NFATc1 expression, the combination group experienced the lowest expression on day 7 and day 14. The combination group showed significant differences in all expressions compared to the control group. The BMP7 and NFATc1 expressions showed a strong relationship (r = -0.598, r > 0.5)

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1. Introduction

Xenograft material offers the advantages of being osteoconductive, readily available, and comparatively inexpensive. However, in several case reports, it was found that inserting BBGs alone could produce unfavorable results for patients, including foreign body reactions, chronic inflammation, and cysts (Bannister and Powell, 2008; Rodriguez and Nowzari, 2019). Propolis is widely used as an alternative medicine in the treatment of various diseases. Numerous studies also confirm the antibacterial activity of propolis in relation to gram-positive bacteria, the potent antioxidant activity, and its anti-inflammatory effects (Martinotti and Ranzato, 2015). Given the advantages of propolis, it is anticipated that the inflammatory reaction caused by individual BBG administration can be reduced or even completely eliminated.

The addition of 2% ethanol extract of propolis (EEP) to the tooth extraction socket of Cavia cobaya, combined with BBG through polyethylene glycol (PEG) as a carrier medium, is known to increase the proliferation of fibroblasts and osteoblasts and reduce the number of osteoclasts (Kresnoadi et al., 2020b; Lunardhi et al., 2019). A study by Meimandi-Parizi et al. (2018) also found that administering aqueous propolis extract combined with demineralized bone matrix percutaneously can increase bone formation in radius bone defects of rats. There were no significant differences between the autografted group and the group given demineralized bone matrix-propolis, indicating that this therapeutic strategy has high potential for the augmentation of autologous bone grafts (Meimandi-Parizi et al., 2018).

In this study, NFATc1 and BMP7 were used as markers of osteoclast differentiation and osteogenesis. Administering combination EEP-BBG is expected to reduce bone resorption and support bone formation. This study was intended to determine the expression of BMP7 and NFATc1 in post-tooth extraction sockets after the application of ethanol. In this study, the possible correlation between the expression of BMP7 and NFATc1 and whether this strengthens/weakens the expression of either is also analyzed.

2. Material and methods

This study was approved by Health Research Ethical Clearance Commission certificate number 374/HREC.FODM/VIII/2020.

2.1. Experimental animal preparations

56 male Cavia cobaya represented the subjects of this study being the number featured in Lemeshow’s formula according to a similar previous study conducted by Kresnoadi et al. (2020) (Kresnoadi et al., 2020a). The subjects had to satisfy certain criteria, namely; healthy, 3–3.5 months old, 300–350 g in weight, lesion-free and with full command of the five senses. The Cavia cobaya were acclimatized for one week, received standard pellet and water ad libitum, and were subjected to a 12-hour light/12-hour dark cycle before the experiment was conducted. They were randomly assigned to one of four groups, namely; a control group, an EEP group, a BBG group, and an EEP-BBG combination group.

2.2. Propolis preparation

Propolis was obtained from 4 to 6 week old Trigona sp. bees in an apiary located in Lawang, Jawa Timur (East Java). 1,000 g of propolis were roughly chopped to a length of 0.5–1 cm and inserted into the flask. The propolis was macerated by adding 2000 ml of 96% ethanol until it was completely submerged before being agitated at room temperature (±25°C) for 24 h at 150 rpm. The results were filtered and vaporized in a vacuum rotary evaporator at 50–60°C for 3–5 h to eliminate all the ethanol present, a process which produced a thick brown liquid.

2.3. BBG preparation

The BBG used in this study was obtained from the tissue bank of Dr. Soetomo General Hospital, Surabaya. Its particle size, ranging from 150 to 355μ/500 mg, underwent calcination at a temperature of 1000°C in order to eliminate organic matrix from the bone.

2.4. Material preparation

All materials were made into a gel. The gelling agent used in this study consisted of polyethylene glycol (PEG) 400 and 4000, combined at a ratio of 1:1. For the BBG group, the gelling agent, 24.5gms in weight, was mixed with 0.5gms of BBG group in order to obtain 2% active ingredient. For the EEP group, the gelling agent weighing 24.5gms was mixed with 0.5gms of the EEP group in order to obtain 2% active ingredient. For the combination of EEP-BBG, the gelling agent weighing 24 g was mixed with 0.5 g of the EEP group and 0.5gms of the BBG group in order to obtain 2% active ingredient. All of these percentages were obtained from the previous study that indicated the optimum healing process result (Kresnoadi et al., 2020b; Lunardhi et al., 2019; Nizar et al., 2020; Prabowo et al., 2020).

2.5. Experimental animal study

Cavia cobaya were injected intramuscularly with ketamine (KEPRO, ZA, Denmark) 20 mg/300 mg body weight in order to induce sedation and the anaesthetized stage (Kresnoadi et al., 2017). The left mandibular incisive tooth region was cleared of debris and carefully extracted using a sterile needle.
holder from a specific direction to prevent fracturing of the roots. After removal, the socket was irrigated with sterile distilled water, given 0.1 ml of materials according to the group: control group (PEG), EEP group (EEP gel), BBG group (BBG gel), and combination group (EEP-BBG gel). Simple suturing was subsequently performed with polyamide monofilament DS 12/3, 12 mm, 6/10 met, 0.7 (Braun VetCare SA, Rubi, Spain) (Kresnoadi, 2012).

2.6. Immunohistochemical staining

The Cavia cobaya in each group were sacrificed on day 7 and day 14 to enable observation of the expression of BMP7 and NFATc1. They were given 100 mg/cc 0.2 cc of ketamine (KEPRO, ZA, Denmark). The mandibles were cut median-sagittally with the mandibular samples being fixed with 10% formalin buffer for 24 h at -80 °C and decalcified with 2% nitric acid. Dehydration was then carried out with graded alcohol concentration, followed by clearing in xylol and embedding in paraffin. Finally, paraffin blocks were cut to a thickness of 4 μm and placed on object glass.

Tissue deparaffinization was completed using a solution of xylol, ethanol, and alcohol. The next step involved processing the tissues with a 3,3'-diaminobenzidine (DAB) staining kit (Pierce™ DAB Substrate Paint Kit 34002, Thermofisher™, Massachusetts, United States). The tissues were incubated with primary antibodies BMP7 (Abcam, ab93636) and NFATc1 (BioLegend, Cat number 649602) at room temperature. The antibody complex was observed after addition of the DAB buffer solution. Slides were observed under a light microscope (Nikon Eclips E 100, Japan). Area being observed was the apical third of the socket.

2.7. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences Software (SPSS) edition 24.0 (SPSS™, Chicago, United States). Results were expressed as mean and standard deviation. The data obtained was examined by one-way analysis of variance (ANOVA) followed by Games-Howell post-hoc and Pearson bivariate correlation tests.

3. Results

3.1. BMP7 expression

The results of statistical analysis showed a significant difference (p < 0.05) between the control and treatment groups. All treatment groups on day 7 had a significant difference in relation to the control group (p < 0.05), while on day 14 groups that demonstrated significant difference from the control group were the EEP and EEP-BBG groups (p < 0.05). The EEP group and the EEP-BBG group did not show significant difference on day 7 and day 14. The BBG group differed significantly from the EEP group and EEP-BBG group on day 14 (Fig. 1, Table 1). There was an increase in the number of BMP7 in the post-extraction socket in the majority of treatment groups from day 7 to day 14. The highest number of BMP7 expressions was observed in the EEP-BBG group on day 14, while the lowest number was in the control group on day 7. The expression of BMP7 as observed by means of a light microscope and immunohistochemical staining is indicated by the arrows in Fig. 2. Osteoblasts that expressed BMP7 were indicated by brown staining on their cells.

3.2. NFATc1 expression

NFATc1 expression of the propolis group and the propolis-BBG group differed significantly (p < 0.05) from the control group on each day. However, when the two groups were compared, there was no significant difference between the propolis group and the propolis-BBG combination on day 7 and day 14. In the control and BBG groups, there was an increase in expression from day 7 to day 14 (Fig. 1, Table 1). Osteoclasts that expressed most NFATc1 were those in the control group on day 7, while the cells that expressed the least were in the propolis-BBG combination group on day 14. Expression of NFATc1 in the histological field is shown by arrows (Fig. 3).

3.3. BMP7-NFATc1 correlation

The results of statistical analysis showed a relationship between the expressions of BMP7 and NFATc1. There was a significant linear relationship between the BMP7 expression and the NFATc1 expression, as evidenced by the significance value of 0.302 (p > 0.05). The conducting of a Pearson test
and 30 with an effective dose of 2% (Prabowo et al., 2020). This increase occurred according to the duration of the application of combination materials to the socket. In addition, heat shock protein (HSP) 70 which acts as an anti-inflammatory indicator and supports cell proliferation showed an increase after application of the combination of the two materials to the extraction socket, followed by an increase in osteocalcin, VEGF, and FGF2 expression (Kresnoadi et al., 2020b, 2020a). According to the results of both the previous and current studies, the number of osteoclast cells decreased with the result that bone resorption was avoided.

There was similar amount of BMP7 and NFATc1 expression between EEP and EEP-BBG group (Fig. 1), yet no significant differences found. This result showed that EEP is almost as effective as EEP-BBG in order to increase BMP7 and decrease NFATc1 expression. It is too early to say that propolis is a strong candidate for bone healing, yet a further and complete study is needed to support this result.

Based on the correlation analysis, a strong relationship was found between the expression of BMP7 and NFATc1. There was an increasing trend in BMP7 expression and a decreasing one in NFATc1 expression. This confirms the results of previous studies that BMP7 can inhibit the differentiation of monocytes into osteoclasts. The increase in BMP7 is thought to decrease the modulation of the osteoclastogenesis signaling pathway which includes NFkB, c-Fos, and NFATc1 and prevent NFATc1 synthesis against osteoclast formation. This result was confirmed by the reduction in transcription of cathepsin K which is controlled by NFATc1 and expressed specifically by osteoclasts (Maurer et al., 2012).

Phenolic compounds can be extracted effectively through the use of ethanol solvents, especially 70% ethanol. In this study, 96% ethanol was used in order that the phenolic compounds obtained were not excessively high. However, for the antioxidant content, there was no difference between the use of 70% ethanol and 96% ethanol, both of which demonstrate high levels of antioxidant activity (Wozniak et al., 2019).

The use of BBG as an individual material to prevent bone resorption can carry the risk of inducing bone pathology in the sinuses and maxilla (Bannister and Powell, 2008; Rodriguez and Nowzari, 2019). The addition of propolis which plays an anti-inflammatory role is known to reduce these risks. According to several studies, at certain doses, ethanolic extract of propolis has anti-inflammatory effects in experimental models of carrageenan-induced edema in vivo, Freund’s adjuvant-
Fig. 2  Expression of BMP7 in osteoblasts located in the third apical after tooth extraction on day 7 (A, B, C, D) and day 14 (E, F, G, H) under a 400x magnification light microscope is indicated by the yellow arrows. A, E: The control group was given PEG gel after extraction. B, F: Extraction followed by the application of BBG gel. C, G: Extraction followed by the application of EEP gel. D, H: Extraction followed by the application of EEP-BBG combination gel.
Fig. 3  Expression of NFATc1 in osteoblasts located in the third apical after tooth extraction on day 7 (A, B, C, D) and day 14 (E, F, G, H) using a 400x magnification light microscope is shown by the yellow arrows. Under a 400x magnification light microscope, the yellow arrow appears. A, E: The control group was given PEG gel after extraction. B, F: Extraction followed by administration of BBG gel. C, G: Extraction followed by administration of EEP gel. D, H: Extraction followed by administration of a combination of EEP-BBG gel.
induced arthritis, and foreign-body induced granuloma, and analgesic properties (Araujo et al., 2011; Park et al., 1996). The main anti-inflammatory mechanism of propolis results from its ability to act as a free radical scavenger which enables it to accelerate the healing process (Ichikawa et al., 2002).

The increase in RUNX2 is expected to occur through the large number of osteoblast cells formed in the extraction socket after administration of a combination of propolis and BBG (Lunardhi et al., 2019; Nizar et al., 2020). This statement shows that propolis and BBG have osteoconductive and osteoinductive properties which constitute a requirement for bone graft material in order to support bone regeneration and accelerate wound healing.

The research reported here could be further developed at a later date, given the highly significant role of propolis in wound healing. Moreover, this study was of only limited duration. In order to establish the benefits of this combination of materials, it is necessary to conduct long-term research involving completion of the bone regeneration.

5. Conclusion

The combination of EEP and BBG can increase the expression of BMP7 and reduce the expression of NFATc1 in tooth extraction sockets. BMP7 expression has a relationship to NFATc1 expression where an increase in the BMP7 expression can reduce that of NFATc1. This study indicated the potential role of a combination of ethanol extract of propolis and bovine bone graft as a means of socket preservation.

CRediT authorship contribution statement

Utari Kresnoadi: Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration. Jennifer Widjaja: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Funding acquisition. Harry Laksono: Methodology, Validation, Writing - review & editing, Supervision, Project administration.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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