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The effect of propolis-xenograft on the expression of SOX2, SOX9, and woven bone in alveolar bone remodeling

Utari Kresnoadi *, Jennifer Widjaja, Primanda Nur Rahmania, Muhammad Dimas Aditya Ari and Imam Safari Azhar

Department of Prosthodontic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

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Abstract

Objective: This study was intended to analyze the expression of SRT-box transcription factor (SOX)2, SOX9 and woven bone in the preservation of tooth extraction sockets due to induction with propolis extract and bone graft.

Materials and methods: 56 Cavia cobaya was divided into four groups according to the socket filling material used: control group, a propolis extract group, a bovine xenograft group, and a propolis extract-bovine xenograft group. An incisor tooth was extracted from each subject and the resulting socket filled with specific materials based on the group of which it was a member. After 3 days and 7 days, the Cavia cobaya were sacrificed in order to obtain their mandibles. Histopathological samples were made by means of hematoxylin-eosin and immunohistochemical staining under a light microscope at 400x magnification. Statistical analysis: The results were analyzed using one-way ANOVA.

Results: A combination of propolis extract and bovine xenograft produced the highest expressions of SOX2, SOX9, and woven bone on day 3 and day 7, followed by the propolis group. The combination group experienced a significant difference with the control group on day 3 and day 7 ($p<0.001$). Even though the combination group presented the highest expressions, the results of a Tukey HSD test indicated no significant difference between the propolis and combination groups on day 3 and day 7.

Conclusion: A combination of propolis and bovine xenograft increased the expressions of SOX2, SOX9, and woven bone. Further research is required to validate the bone remodeling acceleration hypothesis with regards to propolis.

Keywords: SOX2; SOX9; Woven Bone; Propolis; Alveolar Bone Remodeling; Medicine

1. Introduction

Tooth extraction, one of the most common practices in dentistry, is performed on teeth with a poor prognosis due to dental caries, periodontitis, trauma or, occasionally, as a prelude to orthodontic treatment. The process of wound healing and post-extraction alveolar socket involves the complex roles of various body cells. During the healing period, several complications related to the post-extraction area may occur, such as nerve injury, a dry socket, bleeding, hematoma, severe pain, infection, slow healing, swelling or trismus [1,2].

Wound healing constitutes one step in restoring barrier function to prevent further damage and infection [3]. The wound healing process involves a variety of complex biological processes including homeostasis and inflammation. Natural tooth extraction usually produces trauma resulting in inflammation which, in turn, triggers the growth of

* Corresponding author: Utari Kresnoadi
Department of Prosthodontic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

osteoclasts and causes alveolar bone resorption. It is necessary to preserve the extraction sockets of natural teeth in patients in order to reduce the occurrence of alveolar bone resorption, and identify ways of optimizing this reduction in order to facilitate successful implant therapy.

The post-extraction socket restoration has a similar pattern involving the bone healing process [4,5]. Moreover, it is necessary to understand the vascular changes that occur during the inflammatory transition period for the development of new therapeutic approaches related to the management of the inflammatory process [6]. In the fields of medicine and dentistry, the employing of grafts in repairing bone defects and augmentation has become established practice but the use of a stable graft has not produced the anticipated results [7–11]. The materials commonly used for socket preservation include autograft, allograft, alloplast, xenograft and growth factor [12,13]. Materials ideally used for alveolar bone rehabilitation are ones possessing osteogenic, osteoinductive, osteoconductive properties which are able to stimulate neo-angiogenesis. They do not have antigenic, teratogenic or carcinogenic properties, are sufficient in number, hydrophilic, easy to handle and affordable [14].

Propolis contains resins and numerous bioactives including bioflavonoids, artepilin, apigenin, Caffeic Acid Phenethyl Ester (CAPE) which have anti-inflammatory, anti-oxidant, antibacterial, antiviral and immunomodulatory properties. Moreover, they stimulate tissue healing [15]. Consequently, it was anticipated that this material would accelerate the wound healing and bone formation processes.

The development and maintenance of most tissues and organs requires the presence of multipotent and unipotent stem cells capable of self-renewal and differentiating into various cell types. The transcription factor Sox2 is essential for embryonic development and maintaining plurality of and self-renewal in embryonic stem cells. It is expressed in immature osteoblasts/osteoprogenitors in vitro and in vivo and is induced by FGF signaling which stimulates osteoblast proliferation and inhibits differentiation. Sox2 overexpression, by itself, can inhibit osteoblast differentiation [16]. The SOX9 gene provides instructions for making proteins that play an important role during embryonic development, while the SOX9 protein is essential for skeletal development and, as a result, referred to as a transcription factor [17].

Woven bone is the earliest form of bone in the embryo and during growth consists of a network of irregularly shaped collagen. During adulthood, woven bone is replaced by stratified bone consisting of cortical and trabecular bone.

This study was conducted to determine the effect of induction of a combination of propolis extract and bovine bone graft on post-extraction sockets by increasing the expression of SOX2, SOX9, and woven bone in the post-extraction alveolar bone remodeling process.

2. Material and methods

The ethical feasibility of this study was submitted to the Health Research Ethics Committee (KKEPK) of the faculty prior to conduct of the research which followed the issuing of an ethical certification by the Research Ethics Commission (014/HRECCFODM/III/2018). This study incorporated research that was experimental in nature with a randomized post test only control group design incorporating the use of a sample selected completely at random.

2.1. Research preparation

Propolis extract was extracted from raw propolis produced from Apis mellifera bees found in Lawang, East Java, Indonesia by soaking it in ethanol solvent. The Cavia cobaya were acclimatized for a week under 12-hour day/night conditions with unlimited access to food and water.

2.2. Research procedure

The research subjects underwent general anesthesia through the intra venous administration of 0.2cc ketamine / 300g body weight before extraction of the left mandible incisor with special pliers. Depending to their group, the extraction socket was administered with up to 0.1cc of materials and then sutured. After 3 and 7 days, the subjects were sacrificed in order to allow removal of the jaw which was decalcified with Ethylenediaminetetraacetic acid (EDTA) for a month.

The paraffin block was produced after a decalcification process and immunohistochemical staining had been carried out. The slides were observed under a light microscope at 400x magnification of the third apical of the roots. The calculation results were recorded and tabulated with statistical analysis being carried out by means of one-way ANOVA. Multiple comparisons were carried out through a Tukey HSD test. All statistical analyses were processed using a

Statistical Package for the Social Sciences Software (SPSS) edition 24.0 (SPSSTM, Chicago, United States). P<0.05 was considered statistically significant.

3. Results

3.1. SOX2 expressions

The bar chart below contains the average values of SOX2, SOX9 expressions, and woven bone on the third day of analysis. The highest result of all expressions was that of the propolis-bovine bone graft group with p value < 0.05 compared to the control group, followed by the propolis group (Figure 1). Significantly, only the propolis-bovine bone graft group demonstrated a significant value of woven bone amount compared to the control group (p<0.001). All groups showed increasing numbers from day 3 to day 7.

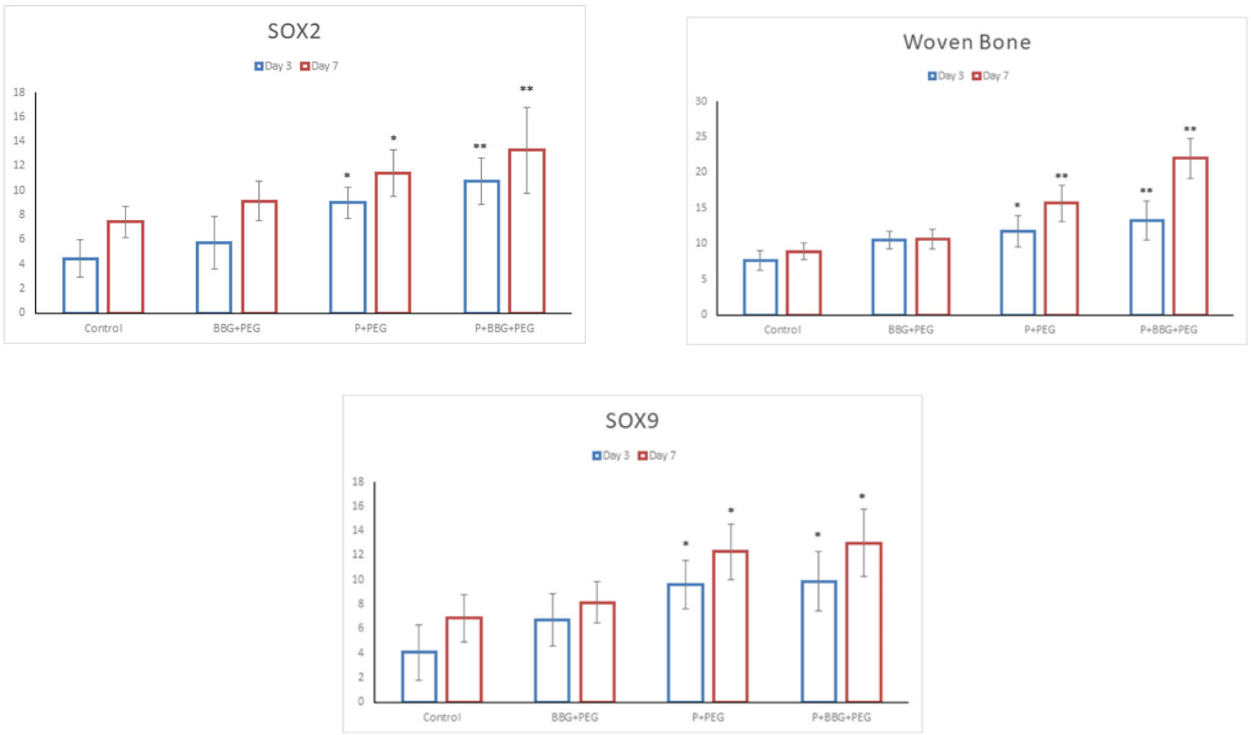

Figure 1 The graph above contains the SOX2, SOX9, and woven bone expressions of all groups on days 3 and 7. The control group was filled with polyethylene glycol (PEG). PEG was used in this study as a carrier of the materials. BBG: bovine bone graft, P: propolis. An asterisk (*) represents the group with a significant difference compared to the control group on the same day (p<0.05), while a double asterisk (**) represents p value < 0.001.

Microscopic description of SOX2 expression during a 3-day and 7-day examination can be seen in the image below (Figure 2)

One-way ANOVA test from the average value of SOX2 expressions on day 3 and day 7 showed a significant difference with p value < 0.001. Multiple comparison with Tukey HSD was performed all details of which can be seen in the table below.

3.2. SOX9 expressions

The propolis-bovine bone graft group showed the highest of all expressions which was significantly different to that of either the control group or the propolis group (p<0.001). The propolis group had the second highest of all expressions. All groups experienced an increasing number of expressions between day 3 and day 7 (Figure 1).

Microscopic description of SOX9 expression during a 3-day and 7-day examination can be seen in the image below (Figure 3).

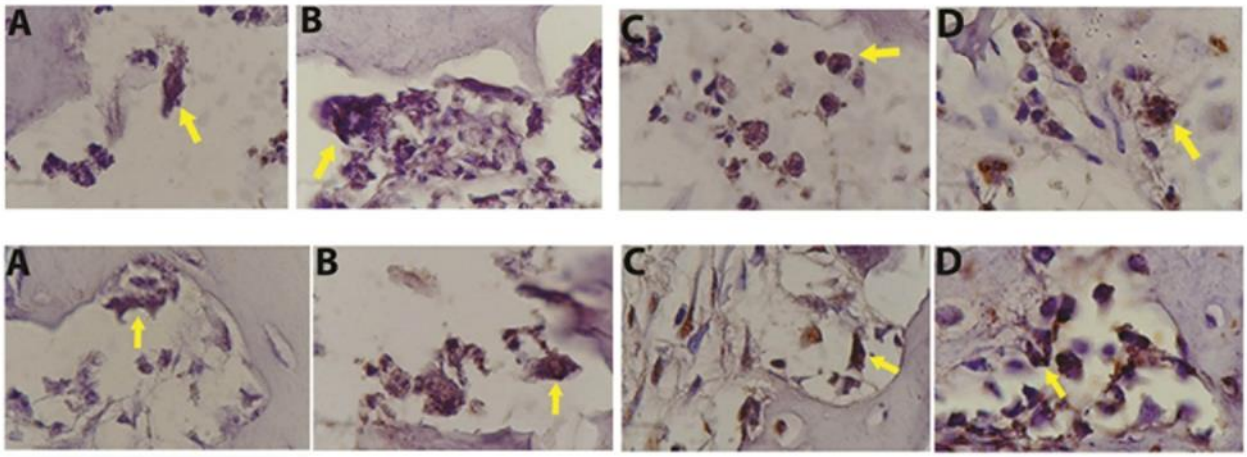

Figure 2 SOX2 expressions in all groups after 3 and 7 days. This location was obtained from the third apical of the roots under 400x magnification. The yellow arrow indicates osteoblast cells that expressed SOX2. A: control group (PEG only),B: bovine bone graft, C: propolis extract, D: propolis and bovine bone graft.

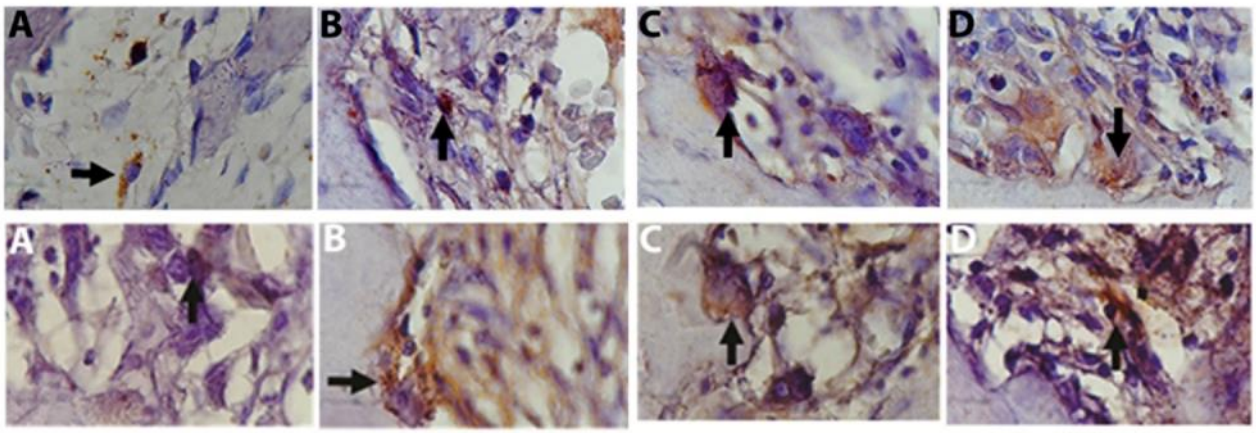

Figure 3 SOX9 expressions in all groups after 3 and 7 days. This location was obtained from the third apical of the roots under 400x magnification. The yellow arrow indicates osteoblast cells that expressed SOX9. A: control group (PEG only),B: bovine bone graft, C: propolis extract, D: propolis and bovine bone graft.

There were significant differences between all groups. An ANOVA test showed a significance value of less than 0.05. A Multiple comparison test Tukey HSD was subsequently conducted, the results of which can be seen in the table below:

3.3. Woven bone

The propolis-bovine bone graft group experienced the highest expression of woven bone. This group showed a significant difference compared to the control group with p value < 0.001 on day 3 and day 7 (Figure 7). The propolis group showed a significant difference compared to the control group with p value <0.05 and <0.01 on day 3 and day 7 respectively. The examination was continued with an ANOVA test to determine the differential between each group. The p value result was less than 0.05 indicating that all groups differed significantly. A Tukey HSD test was performed to analyze the p value between each group. Further details can be seen in the attachment below. In the propolis-bovine bone graft group, there was a significant difference between day 3 and day 7 ($p<0.001$).In the propolis-bovine bone graft group, there was a significant difference between day 3 and day 7 ($p<0.001$).

The picture of the woven bone microscope on day 3 and 7 can be seen in the image below (Figure 4).

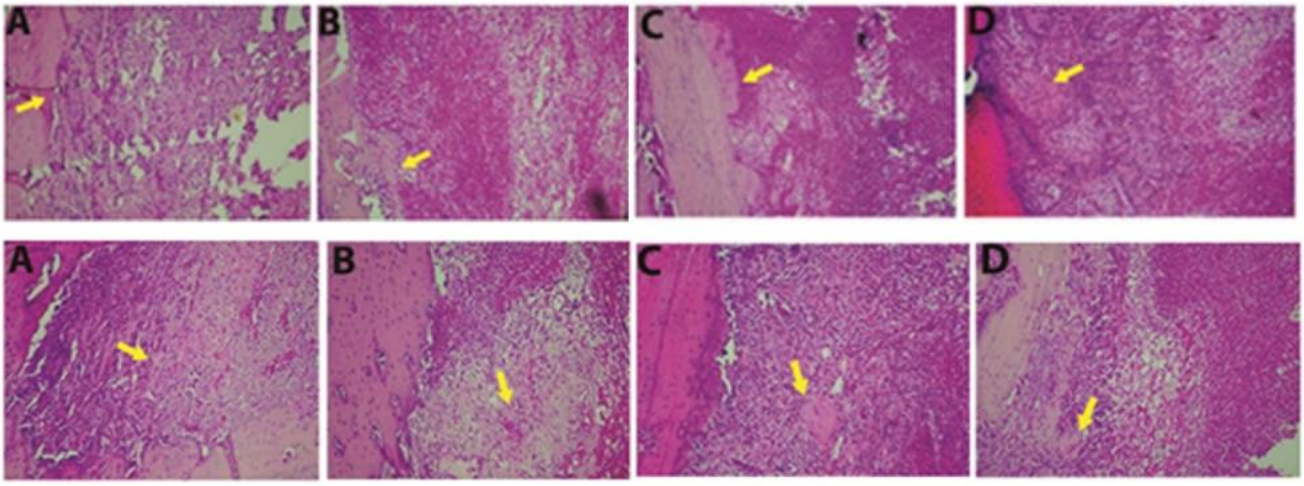

Figure 4 Woven bone expressions in all groups after 3 and 7 days. This location was obtained from the third apical of the roots under 400x magnification. The yellow arrow indicates osteoblast cells that expressed woven bone. A: control group (PEG only),B: bovine bone graft, C: propolis extract, D: propolis and bovine bone graft.

Table 1 Multiple Comparison differences in the expression of SOX2 on treatment days 3 and 7

	Control 3	**BBG 3**	**P 3**	**P+BBG 3**	**Control 7**	**BBG 7**	**P 7**	**P+BBG 7**
Control 3	----	0.927	0.002*	0.000**	0.118	0.001*	0.000**	0.000**
BBG 3	0.927	--	0.064	0.001*	0.747	0.046*	0.000**	0.000**
P 3	0.002*	0.064	------	0.747	0.820	1.000	0.331	0.005*
P+BBG 3	0.000**	0.001*	0.747	----	0.064	0.820	0.997	0.263
Control 7	0.118	0.747	0.820	0.064	------	0.747	0.011*	0.000**
BBG 7	0.001*	0.046*	1.000	0.820	0.747	----	0.407	0.007*
P 7	0.000**	0.000**	0.331	0.997	0.011*	0.407	-----	0.664
P+BBG 7	0.000**	0.000**	0.005*	0.263	0.000**	0.007*	0.664	------

Note: BBG: bovine bone graft, P: propolis, P+BBG: combination of propolis and bovine bone graft. The number after the acronym indicates the examination day. An asterisk (*) shows the significant value ($p<0.05$), while a double asterisk (**) indicates significant value with $p<0.001$

Table 2 Multiple Comparison differences in the expression of SOX9 on treatment days 3 and 7

	Control3	**BBG3**	**P3**	**P+BBG 3**	**Control 7**	**BBG 7**	**P 7**	**P+BBG 7**
Control3	--------	0.763	0.005*	0.002*	0.691	0.123	0.000**	0.000**
BBG 3	0.763	--------	0.255	0.159	1.000	0.925	0.001*	0.000**
P 3	0.005*	0.255	--------	1.000	0.315	0.925	0.315	0.094
P+BBG 3	0.002*	0.159	1.000	--------	0.203	0.828	0.456	0.159
Control 7	0.691	1.000	0.315	0.203	--------	0.956	0.020*	0.004*
BBG 7	0.123	0.925	0.925	0.828	0.956	--------	0.020*	0.004*
P 7	0.000**	0.001*	0.315	0.456	0.020*	0.020*	---------	0.999
P+BBG 7	0.000**	0.000**	0.094	0.159	0.004*	0.004*	0.999	--------

Note: BBG: bovine bone graft, P: propolis, P+BBG: combination of propolis and bovine bone graft. The number after the acronym indicates the examination day. An asterisk (*) shows the significant value ($p<0.05$), while a double asterisk (**) indicates significant value with $p<0.001$

Table 3 Multiple Comparison using Tukey HSD test to determine differentiation between each groups of woven bone expression on day 3 and 7

	Control 3	**BBG3**	**P3**	**P+BBG 3**	**Control 7**	**BBG 7**	**P 7**	**P+BBG 7**
Control 3	--------	0.226	0.017	0.000**	0.951	0.165	0.000**	0.000**
BBG 3	0.226	----------	0.956	0.256	0.864	1.000	0.001*	0.000**
P 3	0.017*	0.956	----------	0.883	0.236	0.982	0.025*	0.000**
P+BBG 3	0.000**	0.256	0.883	---------	0.009*	0.337	0.431	0.000**
Control 7	0.951	0.864	0.236	0.009*	--------	0.785	0.000**	0.000**
BBG 7	0.165	1.000	0.982	0.337	0.785	----------	0.002*	0.000**
P 7	0.000**	0.001*	0.025	0.431	0.000**	0.002*	---------	0.000**
P+BBG 7	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	======

Note: BBG: bovine bone graft, P: propolis, P+BBG: combination of propolis and bovine bone graft. The number after the acronym indicates the examination day. An asterisk (*) shows the significant value ($p<0.05$), while a double asterisk (**) indicates significant value with $p<0.001$

4. Discussion

Propolis-bovine bone graft expressions reached their most remarkable levels in on SOX2, SOX9, and woven bone expressions of day 3 and day 7. This group differed significantly to the control group, followed by propolis group. Propolis is a very popular herbal medicine with a variety of uses. In this study, one of the main functions of propolis extract, which is thought to support the process of osteogenesis, is that of an anti-inflammatory [18]. Anti-inflammatory properties support the increase in bone stem cells during osteogenesis [19]. This study combines bovine bone graft, which constitutes supporting material for accelerating bone formation, with propolis extract which has an anti-inflammatory function through the evaluation of SOX2, SOX9, and woven bone markers.

The transcription factor Sox2 is essential for embryonic development and maintaining plurality and self-renewal in embryonic stem cells. It is expressed in immature osteoblasts and osteoprogenitors in vitro and in vivo, being induced by fibroblast growth factor (FGF) signaling which stimulates osteoblast proliferation [20]. In this study, SOX2 experienced an increasing trend from day 3 to day 7. The propolis-bovine bone graft group showed the highest SOX2 expression, followed by the propolis group. Propolis itself showed enormous capacity to enhance bone formation, probably because of polyphenolic compounds in the form of flavonoids and CAPE which can reduce the number of osteoclasts and increase that of osteoblasts, while also playing an anti-inflammatory role by decreasing proinflammatory cytokines [21]. Similarly, in the research-based opinion of Iqbal et al. (2017), 40% propolis extract can reduce the number of osteoclasts in guinea pigs on day 14 post-extraction [22]. This is due to the high content of flavonoids in 40% propolis extract gel which can inhibit the process of osteoclastogenesis and accelerate the maturation of osteoblast cells and bone remodeling activity.

Sox9, the main transcription factor that regulates various chondrogenesis events, plays an important role in determining the type of cell differentiation, especially in embryonic development. Sox9 plays an important role in the process of osteogenesis, especially at its onset and end. An in vitro study by Loebel et al, found that the differentiation of osteogenesis can be established through the ratio of Runx2 and Sox9 [23]. Sox9 is known to regulate bone marrow mesenchymal stem cells (BMSC) proliferation and osteogenic differentiation via the Wntβ/Catenin signaling pathway [24]. According to the results of the study, there was an increasing trend in the amount of SOX9 expression on day 3 to day 7 in all groups.

At this point, it is thought that an early stage of chondrogenesis occurs where SOX9 is required to maintain chondrocytes during the incipient differentiation, where chondrocytes proliferate in the growth plate column and maintain permanent cartilage homeostasis. The propolis-bovine bone graft combination group had the highest level of expression on day 3 and day 7, followed by the propolis group. Propolis has benefits as an anti-inflammatory and anti-oxidant agent, with the result that it can indirectly support an increase in SOX9 expression. SOX9 is important to study because it is required in certain amounts in order to inhibit inflammation and immune responses [25]. With the addition of SOX9, propolis as a potent anti-inflammatory can inhibit the inflammatory process.

In this study, to prove an increase in osteoblast growth, histopathological examination with hematoxylin-eosin (HE) woven bone is necessary. It was seen that woven bone numbers in all groups started to increase from day 3 to day 7. This observation is in line with the increase expression of SOX 2 and SOX9. The healing of extraction socket involving some sequence markers, such as coagulum, provisional matrix, woven bone, and lamellar bone and bone matrix. The results of propolis-bovine bone graft group showed abundance numbers of woven bone and it is differed significantly compared to control group, means that the process of healing from tooth extraction can be fastened using this material. In the future, this study needs longer time to observe until the extraction socket healing completely.

5. Conclusion

Increased expression of SOX2 and SOX9 and woven bone due to the induction of the combination of propolis and Bovan Bone graft can increase the growth of osteoblast cells. Sox2 is required to maintain the survival of osteoblasts, while Sox9 has an important role in the process of osteogenesis, especially at the beginning and end of osteogenesis. So that alveolar bone remodeling can occur.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest.

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