Immunohistochemistry assay of osteocalcin on bone healing with CHA-10% propolis

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Abstract

Objective: To evaluate the effect of the application of carbonated hydroxyapatite-propolis 10% on open flap debridement towards osteocalcin expression of periodontitis induced Oryctolagus cuniculus. Material and Methods: Six rabbits (Oryctolagus cuniculus) were divided into three groups, which are carbonated hydroxyapatite-propolis 10% group, carbonated hydroxyapatite group, and open flap debridement group. Periodontitis on animal models was induced with a combined technique using ligation and LPS injection of P. gingivalis. On each group, the animals were decapitated on day 7 and day 14 after the designated treatment. The immunohistochemistry assay was used to measure the osteocalcin expression. The data were analyzed statistically with Two Way Anova and continued with post hoc LSD.

Results: The results showed that the carbonated hydroxyapatite-propolis 10% group had the highest osteocalcin expression on day 7, followed by carbonated hydroxyapatite group and the open flap debridement group (p<0,05). There was no significant difference in the expression of osteocalcin on day 7 and day 14 (p>0,05) on the carbonated hydroxyapatite-10% propolis group.

Conclusion: The application of carbonated hydroxyapatite-propolis 10% could increase the osteocalcin expression on periodontitis induced Oryctolagus cuniculus.

Keywords: ESR, Periodontitis (MeSH), PISA, Platelet count (MeSH), Type 2 diabetes mellitus (MeSH)

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Introduction

Periodontitis is a chronic inflammation disease of the periodontal tissue that results in the progressive destruction of gingiva, periodontal ligament, and alveolar bone. This condition was initially caused by bacterial colonization in the gingival sulcus. However, the immune response towards bacterial invasion is responsible to the destruction of bone and connective tissue.

The ultimate goal of periodontal therapy is to regenerate periodontal tissue and maintain the anatomical shape and function of the tissue itself. Open flap debridement (OFD) is one of the periodontal surgery techniques used to achieve an ideal condition for periodontal tissue regeneration to take place. This treatment has been proven to show an outstanding result and has been used as a control procedure on clinical trials regarding regenerative therapies. Bone graft is one of the regenerative therapies that often used to treat alveolar bone defect.

Bone graft could be made from natural or synthetic materials (alloplastic). Carbonated hydroxyapatite is one of the alloplastic bone grafts. Alloplastic bone grafts have an excellent osteoconductive property by functioning as a scaffold for bone regeneration and also an osteoinductive ability.

Propolis is a natural product produced by honeybees (Apis mellifera) that has advantageous effect for bone regeneration. Previous study found that propolis was able to increase osteoblasts cell count and decrease osteoclasts cell count on bone fracture. Propolis was also proven to increase the expression of bone forming biomarkers and decrease bone resorption ones. One of the components of propolis, caffeic acid phenetyl ester has been proven to increase the healing of induced-bone defect on animal models. Propolis has an excellent osteoinductive ability and capable of increasing osseointegration on bone healing process. Another study proved that 10% propolis was able to stimulate the healing of gingivitis by reducing polymorphonuclear leukocyte cell count, increasing fibroblasts number and increasing neovascularization.

Osteocalcin is one of the calcium-binding proteins and also the most abundant non-collagen protein found in mineralized tissue. Osteocalcin is secreted specifically by osteoblasts and has important role in bone turnover. The increase of osteocalcin serum was found during the high rate of bone turnover. Another study found that propolis was able to increase the expression of osteocalcin and type I collagen. Bone mineralization process takes place from day 7 to day 14. The expression of bone matrix protein such as type I collagen, osteocalcin and osteopontin was found increasing

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from day 7 to day 14 in vitro. Itagaki et al. proved that expression of osteocalcin reached its peak on the second week and decreased gradually during the next observed weeks.

Rabbits are the representative animal model for studies about inflammation and periodontal regeneration. This is due to the similar physiological features of periodontal tissue with human. Induction of periodontitis on rabbits could be achieved by combining ligation technique with injection of bacterial lipopolysaccharide. 19

This study aimed to evaluate the effect of the application of carbonated hydroxyapatite-propolis 10% on open flap debridement towards osteocalcin expression of periodontitis induced Oryctolagus cuniculus.

Material and Methods
Rabbits were anesthetized using intramuscular injection of ketamine HCL 40 mg/kg and xylazine 3 mg/kg. Periodontitis was induced by ligating silk suture 3.0 on lower anterior teeth. The injection of 0.05 mL Porphyromonas gingivalis lipopolysaccharide was done three times a week in the interdental area.

Carbonated hydroxyapatite was cut into 10 mg specimen. Propolis was diluted using sterile aquabidest to acquire 10% concentration. Carbonated hydroxyapatite then soaked into 1 mL of propolis solution for 24 hours on room temperature.

Six rabbits were anesthetized using ketamine 40 mg/kg and xylazine 5 mg/kg intramuscularly. Open flap debridement was done using the envelope design on the lower anterior teeth of all rabbits. Carbonated hydroxyapatite-10% propolis was applied on the first two rabbits (group A), carbonated hydroxyapatite was applied on the next two rabbits (group B), and on the last two rabbits were not applied anything (group C). Flap was repositioned and sutured using nylon 4.0. Analgesic was administered intramuscularly right after the OFD procedure. One rabbit from each group was chosen randomly to be decapitated 7 days after OFD, and other rabbits from each group was decapitated 14 days after OFD. Euthanasia was done by overdose administration of sodium pentobarbital intramuscularly. The alveolar bone on the defect area was harvested and then fixated in 10% formalin for at least 24 hours, and then continued with microscope slides preparation and immunohistochemistry staining.

Counting of osteoblast-expressed osteocalcin was done by observing 3 field of view on each microscope slides. Osteoblasts that were positively expressing osteocalcin would be seen in dark chocolate, whereas osteoblasts that were not expressing osteocalcin would be seen in purplish blue. Osteoblasts counting was done by two calibrated experts. The data was presented in percentage of osteoblasts positively expressing osteocalcin, with the formula:

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\text{Percentage of osteoblasts positively expressing osteocalcin} = \frac{\text{positive osteoblasts cell counts}}{\text{total osteoblasts cell counts}} \times 100\%
\]

The data was analyzed statistically using Two Way ANOVA and then continued with post hoc LSD.

Results
Table 1 shows the descriptive data of osteocalcin expression on all groups on each observation time. The highest osteocalcin expression was seen on the carbonated hydroxyapatite-10% propolis
group on day 14 (71.85%) whereas the lowest osteocalcin expression was seen in the OFD group on day 7 (41.75%). Figure 1 shows the immunohistochemistry staining for osteocalcin. Osteoblasts that express osteocalcin are seen in dark brown shade.

Based on the data shown in table 2, there was significant difference (p<0.05) in the expression of osteocalcin between all treatment groups. The two-way ANOVA test showed there was also significant difference in the expression of osteocalcin between all observation time (p<0.05). Interaction between treatment groups and observation time had significance value of 0.047 (p<0.05) which proved that the interaction between treatment groups and observation time had an effect towards the expression of osteocalcin. These results suggested that there was an effect between treatment groups and observation time towards osteocalcin expression. Statistical analysis was continued with post hoc LSD test on each treatment group and each observation time.

Based on table 3, there was significant difference on osteocalcin expression on day 7 in all treatment groups (p<0.05). Thus, the carbonated hydroxyapatite-propolis group expressed the highest level of osteocalcin on day 7. The osteocalcin expression on day 14 showed that there was significant difference between carbonated hydroxyapatite-propolis group and open flap debridement group (p<0.05), yet there was no significant difference between carbonated hydroxyapatite-propolis group and carbonated hydroxyapatite group (p>0.05). This suggested that both carbonated hydroxyapatite-propolis group and carbonated hydroxyapatite group have the same effectivity in expressing osteocalcin.

**Discussion**

Statistical analysis showed that there was significant difference in the osteocalcin expression on day 7 between all treatment groups. This means that application of carbonated hydroxyapatite-10% propolis on open flap debridement is proven to have the highest effectivity in increasing osteocalcin expression on day 7, compared to application of carbonated hydroxyapatite group and open flap debridement group. This result is in agreement with Abdellatif et al.\(^2\) that proved that the use of propolis as allograft coating contributed to a better osteoconductivity and osteoinductivity compared to uncoated propolis. Another study by Al-Saeed and Mohamed\(^3\) found out that the application of propolis on animal models could increase the expression of osteocalcin. Biological activity of propolis mostly due to its flavonoid content.\(^4\) Flavonoid functions by activating mitogen-activated protein kinases (MAPKs) signaling pathway.\(^5\) Activation of this signaling pathway results in activation the next mechanism, which is the Wnt/β-catenin signaling pathway.\(^6\) Once activated, this signaling pathway would result in the expression of runt-related transcription factor 2 (Runx2). Expression of Runx2 would in turn increase the expression of type I collagen, alkaline phosphatase, bone sialoprotein, and osteocalcin.\(^7\) The high osteocalcin expression on the carbonated hydroxyapatite-10% propolis group proved that flavonoid in propolis could increase the bone formation process on animal models. The chemical formula for carbonated hydroxyapatite is Ca\(_{10}\)(PO\(_4\)CO\(_3\))\(_6\)(OH)\(_2\), in which the –OH group functions as an active site for absorbing molecules of propolis, such as flavonoid, through the hydrogen bond. Carbonated hydroxyapatite has interconnected porous structure which comes with high surface area and high porous volume which allow the incorporation of active molecules into the porous and release it to the surrounding tissue in a controlled manner.\(^8\)

Osteocalcin expression on day 14 showed that there was significant difference between carbonated hydroxyapatite-10% propolis group and OFD group (p<0.05), yet there was no significant difference between carbonated hydroxyapatite-10% propolis group with carbonated hydroxyapatite group (p>0.05). This result showed that carbonated hydroxyapatite-10% propolis group
and the carbonated hydroxyapatite group has the same effectivity in increasing the expression of osteocalcin on day 14. Open flap debridement is one of the periodontal surgery therapies that has been proven to be effective in reducing intrabony defect. In this study, the OFD group showed the increase in osteocalcin expression from day 7 to day 14. However, the increase of osteocalcin level in this group was not statistically significant. A study by Needleman et al. stated that open flap debridement as control group showed a good clinical result. Clinical improvements that are achieved from OFD are increase in clinical attachment level (CAL), probing depth (PD) reduction, improvement in gingival recession, and alveolar bone gain in 12 months. However, these results may vary between patients, thus giving a chance for increasing its ability in regenerating periodontal tissue by adding regenerative material in the OFD procedure.

On this study, the osteocalcin expression on day 7 of carbonated hydroxyapatite group was found higher than the OFD group. This result is in agreement with Jebahi et al. that proved that the use of carbonated hydroxyapatite on rabbits could increase the bone formation and showed a good tissue response. In the same study, it was found out that the calcium and phosphate level on animal models treated with carbonated hydroxyapatite were higher than the control group. This result is in correspondence with Ana et al. that stated that carbonated hydroxyapatite could increase the calcium and phosphate level in bone, which are crucial in the bone formation process. Besides its excellent osteoconductive ability, carbonated hydroxyapatite also possesses osteoinductive ability. Therefore, the use of carbonated hydroxyapatite could be combined with other material to improve its osteoinductive ability.

There was no significant difference on osteocalcin expression on day 14 between the carbonated hydroxyapatite-10% propolis group and the carbonated hydroxyapatite group. This result is similar with a study by Al-Molla et al. that found out that application of propolis could improve the osseointegration ability by increasing osteocalcin expression on day 7, but did not show significant result on the second week to the sixth week. This outcome could be because propolis has reached its saturated point on day 7 so its effectivity in increasing bone formation has decreased. Carbonated hydroxyapatite has been known to possess excellent osteoconductive ability, and also osteoinductive ability, therefore the carbonated hydroxyapatite group has the same effectivity with the carbonated hydroxyapatite-10%propolis group in expressing osteocalcin on day 14.

**Conclusion**

The application of carbonated hydroxyapatite-10% propolis could increase the osteocalcin expression on open flap debridement. However, there was no significant difference of osteocalcin expression on day 7 and day 14. Therefore, it is recommended to conduct another study with longer observation time to evaluate the effect of carbonated hydroxyapatite-10% propolis on bone regeneration.

**Acknowledgment**

None.

**Conflict of Interest**

The authors report no conflict of interest.

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