ORIGINAL ARTICLE

The Effect of Carbonated Hidroxyapatite-10% Propolis Application on Open Flap Debridement Towards Transforming Growth Factor B1 Expression

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ABSTRACT

Introduction: Carbonated Hydroxyapatite (CHA) is used as a bone graft material in the treatment of periodontitis in dentistry. Carbonated Hydroxyapatite has high biocompatibility, osteoconductive, and as a drug carrier. Propolic is a resinous material from bees that has antibacterial, anti-inflammatory, and accelerates wound healing. The incorporation of CHA with propolic is expected to accelerate alveolar bone regeneration better by increasing the expression of Transforming Growth Factor β1 in the treatment of periodontitis. This study's aim was to examine the effect of CHA-Propolic 10% application on open flap debridement (OFD) and TGF-β1 expression. **Methods:** This study use 24 sample from six male rabbits. The fourth tissue slices periodontitis from each treatment group was taken from the mandibular incisors of the rabbit Oryctolagus Cuniculus that had been induced by ligation and injected with LPS of Propyromonas gingivalis bacteria. Periodontitis conditions in rabbits were obtained after 2 weeks of induction of periodontitis then the rabbits were treated and divided into 3 groups, group I only OFD action, group II OFD action added CHA application, and group III OFD action added 10% Propolic-CHA application. Decapitation was done on the 7th day and 4th day. The data were analyzed using the Shapiro-Wilk test, Anava two paths test, and continued with the Least Significant Difference (LSD) test. **Results:** The results showed CHA incorporated with a 10% propolic group had a significant difference compared to other treatment groups (p<0,05). **Conclusion:** The conclusion obtained from this study was that the application CHA incorporated with a 10% propolic group shows the ability to increase the expression of TGF-β1 alveolar bone of rabbits.

Keywords: Carbonated Hydroxyapatite, Propolic, TGF-β1, Osteoblast

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INTRODUCTION

Periodontitis is an inflammatory disease that attacks the supporting tissues of the teeth (1). The pathogenesis of periodontitis is bacterial plaque which can initiate periodontal inflammation which is characterized by swelling, leukocyte penetration, and detach of inflammatory mediators. The inflammation results in periodontal pockets, tissue attachment loss, and alveolar bone resorption, leading to loose teeth. The treatment may include the removal of plaque and calculus by scaling and root planing, even some patients may require additional chemotherapy (2).

Periodontitis may cause alveolar bone loss. A treatment for bone loss is done with bone grafts which serve as bone regeneration. Bone graph materials are classified as autograft, allograft, xenograft, and alloplastic graft. Alloplastic graft or synthetic bone graft is worn to treat localized bone loss. Carbonate hydroxyapatite is included as a synthetic or alloplastic bone graft (3). Carbonate hydroxyapatite has osteoconductivity and biocompatibility, and low osteoinductivity (4), and it is a drug delivery system(5). Gelatin carbonate hydroxyapatite is a scaffold material, consisting of inorganic material (B-CHA) and gelatin which is a protein (6).

Propolic is a resin substance containing more than 300 different components in honey from various plant species. Propolic has anti-inflammation, immunomodulation (7), antioxidant, and antibacterial

properties (8). 10% propolic in a study by Suryono (9) was proven to reduce the number of poly-morphonuclear, increase the number of fibroblasts as well as the number of newnblood vessels. According to Damayanti et al., 2012 (cit 9) flavonoids contained in propolic serve to stimulate the production of TGF-β and vascular endothelial growth factor (VEGH) by stimulating the proliferation of fibroblasts and blood cells. A549 cells (alveolar lung cells) pretreated with propolic and then treated with TGF-β1 for 24 hours regained epithelial cell morphology, decreased the production of N-cadherin and ROS, and had reduced motility. Propolic prevents the effects of TGF-β1-induced Smad2 and AKT activation pathways and Snail expression (10).

Transforming Growth Factor-β1 (TGF-β1) is one of the essential cytokines which have pleiotropic effects as pro-inflammatory and anti-inflammatory agent in regulating inflammatory infiltration and it is also one of the multifunctional cytokines involved in the process of angiogenesis, immunosuppression, and extracellular matrix synthesis (11). Transforming Growth Factor-β1 plays various roles in bone regeneration, i.e. enhancing the proliferation of osteoblasts, osteoblast predecessor or matrix-producing osteoblasts by way of chemotactic attraction (12) and it is the most abundant in human tissues (13).

Open flap debridement (OFD) is a periodontal flap surgical treatment in deep periodontal pockets to stimulate tissue regeneration by debridement to the root surface of tooth to make less probing pocket depth and prevent tissue attachment loss (14). In fact, repair in bone tissue is different from repair in connective tissue healing. This is because repair in bone tissue involves osteoblasts and osteoclasts. The healing process in bone tissue is mainly found in the periosteum of connective tissue which covers the wound. Periosteum is a source of cell precursors which develop into condroblasts and osteoblasts which are crucial for bone healing (15). The expression of TGF-β increases during the intramembranous ossification and alveolar bone formation, and the increase starts on the 7th day after injury and increases to peak on the 14th day. The regulation of callus formation during bone repair is mediated by the expression of growth factors. One of which is TGF-β1 which is connected in the regulation of osteoblast distinction and extracellular matrix output (16). This study used rabbits as the laboratory animals. This is because the structure and composition of the oral mucous membrane of rabbits are histologically similar to those of humans (17). This study's aim was to examine the effect of CHA-Propolic 10% application on open flap debridement (OFD) and TGF-β1 expression.

MATERIALS AND METHODS

The procedure in this research has been accepted by the Ethical Committee of the Faculty of Dentistry, Universitas Gadjah Mada, No. 00207/KKEP/FKG-UGM/EC/2019.

1. Preparation of laboratory animals with periodontitis
The rabbits (Oryctolagus cuniculus) were allowed to environmentally adapt in a cage for 1 week. One week later, the mandibular incisors of the rabbits were ligated. The ligature placement was done under anesthesia by intramuscular injection of the rabbits' thigh with ketamine 40 mg / kg body weight and xylazin 3 mg / kg body weight. The ligation was performed using 3-0 silk thread placed on the cervical region of the anterior mandibular tooth, followed by inoculation ofn0.05 ml lipopolysaccharide of Porphyromonas gingivalis ATCC 33277 from Thermo Scientific, USA in the interdental area in triplicate, i.e. on the 1st, 3rd and 5th days for 7 days. Fourteen days after the ligation, various signs of periodontitis were clinically seen, i.e. reddish and rounded gingival margin, and gingival recession. Ligation aims to cause dental plaque accumulation which then induces periodontitis.

2. Making of bone graft specimens
Bone graft specimens (Gama-CHA®, PT. Swayasa Prakarsa, Yogyakarta) were prepared by cutting bone graft material with a weight of 10 mg. The specimens were then placed in a 1 ml microcentrifuge tube. Propolic (Propolic Brazilian®, Minas Gerais, Brazil) was diluted using sterile water with a ratio of 1:9 to obtain 10% propolic solution, then carbonate hydroxyapatite bone graft specimens were immersed in a 10% propolic solution for 24 hours. The specimens were prepared two days prior to the application to the laboratory animals.

3. Treatment on laboratory animal
Rabbits with periodontitis were anesthetized by intramuscular injection of ketamine 40 mg / kg BW and xylazin 5 mg / kg BW, followed by open flap debridement (OFD) in the anterior mandible, i.e. sulcular incision was performed on the labial region of the incisors using a blade no 15 with an envelope full-thickness flap. Next the gingival tissues were reflected using a small rasparatorium. Debridement was performed on both hard and soft tissues, then irrigated using distilled water. After open flap debridement was done, two rabbits were treated with Carbonate Hydroxyapatite and two other rabbits were given Carbonate Hydroxyapatite-10% propolis. Flap repositioning used suturing flap with 4.0 nylon thread. In fact, two rabbits in the control group

were treated only with OFD in the anterior mandible. After the surgery, these rabbits were administered a soft diet for the first 24 hours as well as Tramadol 0.2-0.5 mg / BW once intramuscularly, followed by feeding ad libitum during the study.

Three rabbits from each group were randomly selected for decapitation on the 7th day (H7). The decapitation used overdose of Sodium Pentobarbital, i.e. 120 mg / kg BW by intramuscular injection and the remaining three rabbits were decapitated on the 14th day (H14) after OFD and treatment.

4. Making of microscope slides

Alveolar bone resorption that occurs in the periodontitis model group is a horizontal type of bone resorption. The bone tissues of the rabbits were fixed with 10% buffered formalin and left for at least 24 hours. The bone tissues were then cleaned from the remaining soft tissues. The alveolar bones of the rabbits were then taken and fixed in a 10% formalin solution, then taken to the Laboratory of Anatomic Pathology, Faculty of Medicine UGM because the tissues were to be cut and microscope slides were to be made.

5. Immunohistochemical staining

The thick parts of five micrometers were chop and assembled on (Biocare, AS) positively- charged slides, then differentiated and rehydrated. For immunohistochemical staining by TGF-β polyclonal antibodies (US biologis); these parts were then dipped in 0.3% hydrogen peroxide (H2O2) to block endogenous peroxidase activity, washed in phosphate-buffered saline (PBS), then incubated in 10% normal serum to block non-specific antibody binding. The parts of the tissues were incubated with TGF-β mouse anti-human polyclonal antibody (thinned out 1:30) at night at 37°C. The antibodies bound were detected using the streptavidin-biotin complex method, after immunoreaction, these parts were stained using Hematoxylin. The IHC staining process was finished by covering the preparation using coverslip and the preparations were ready to be observed.

6. Calculation of TGFβ-expressing osteoblasts

The observations were performed under a light microscope. TGF- β1 IHC was observed in three visual fields. Each visual field allowed to see positive and negative cells. Positive osteoblasts which expressed TGF-β1 were seen to be dark brown while the negative ones were seen to be blue.

The data are presented in the form of a percentage of positive osteoblasts in TGF β1 IHC staining. The formula is (20).

$$\text{Percentage of osteoblasts} = \frac{\textit{number of positive cells}}{\textit{total cells}} \times 100\%$$

7. Data Analysis

This study use 24 sample. All the data obtained from the observations were quantitative interval data. The data were normal based on the Shapiro-Wilk test and were homogeneous based on the Lavene's test, followed by Two-Way ANOVA and post hoc test with a level of significance of 0.05.

RESULTS

This study obtained data on the percentage of TGF-β1 expression in the osteoblasts of the alveolar bone of Oryctolagus cuniculus as the laboratory animals on day 7 and day 14. The mean and standard deviation of each group on each observation day are shown in the following table.

The descriptive data of TGF-β1 consentration in all the groups are presented in Table I. The group treated with open flap debridement and carbonate hydroxyapatite-10% propolic on day 14 (49.07%) showed the highest TGF-β1 expression, while the lowest TGF-β1 was found in concentration the group treated with OFD on day 7 i.e.17.21%.

Based on the results presented in table II, it appears that there is a significant difference ($p<0.05$) between the treatment groups with TGF-β1 expression. The two line Anava test results also showed a significant difference between the days of measurement with TGF-β1 expression ($p<0.05$). The interaction between

Table I : Mean and standard deviation of TGF-β1 expression (%) in the treatment groups on day7 and day 14.

Groups	n	Mean± standard deviation of TGF-β1expression (%)	
		Day 7	Day 14
OFD-CHA-Propolis 10%	4	43.22±6.35	49.07±2.96
OFD-CHA	4	34.84±3.72	40.34±6.45
OFD	4	17.21±2.84	35.15±5.00

Where:
n is the number of samples

Table II : Anava test results of two mean pathways of TGF-β1 expression in all groups and treatment times of 7th and 14th days.

Variable	*P*	Description
Group	0.000*	*p < 0.05*
Day	0.000*	*p < 0.05*
Group*day	0.028*	*p < 0.05*

(*) : signifikan (p<0.05)

the treatment group and the measurement time had a significance value of 0.028 ($p<0.05$) which showed that the interaction between the treatment group and the measurement day affected the expression of TGF-β1. Data analysis continued with the post hoc LSD test.

The result in Table III shows that the group treated with open flap debridement and the application of Carbonate Hydroxyapatite-10% Propolic on day 14 obtained a significance of $p<0.05$ compared to other treatment groups and other observation days. However, this group did not have significant difference with the group treated with open flap debridement and the application of Carbonate Hydroxyapatite-10% Propolic on day 7 ($p>0.05$).

DISCUSSION

This study was conducted to analyze the effect of the application of Carbonate Hydroxyapatite-10% Propolic in Open Flap Debridement (OFD) on TGF-β1 expression. The results of the three groups showed that OFD with the application of carbonate hydroxyapatite-10% propolic was the best treatment, evident from the highest TGF-β1 expression i.e. 49.07 %. Transforming Growth Factor β stimulates the proliferation of mesenchymal stem cells, pre-osteoblasts, osteoblasts, and chondrocytes, as well as the production of extracellular proteins including collagen, proteoglycans, osteopontin, osteonectin, and alkaline phosphatase (18). Autocrine and paracrine stimulation by TGF-β is vital for the maintenance and

Table III : The result of Least Significant Difference (LSD) test on the mean of TGF-β1 expressionin each treatment group and day of observation.

Treatment & Day	OFD7 CHAProp	OFD7 CHA	OFD7	OFD14 CHAProp	OFD14 CHA	OFD14
OFD7	-	0.024*	0.000*	0.102	0.407	0.028*
CHAProp OFD7		-	0.000*	0.001*	0.122	0.928
CHA OFD7			-	0.000*	0.000*	0.000*
OFD14				-	0.019*	0.001*
CHAProp OFD14					-	0.143
CHA OFD14						-

Table III. (*) presents the results of the LSD test with a significance of $p<0.05$.

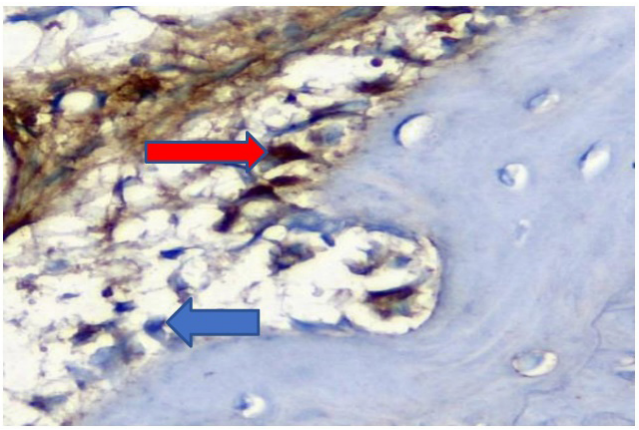

Figure 1 : Expressing TGF-β1 on day 7 of immuno-histochemical staining on osteoblast show positive if osteoblasts are dark brown (red arrows), but negative if osteoblasts are purplish blue (blue arrows).

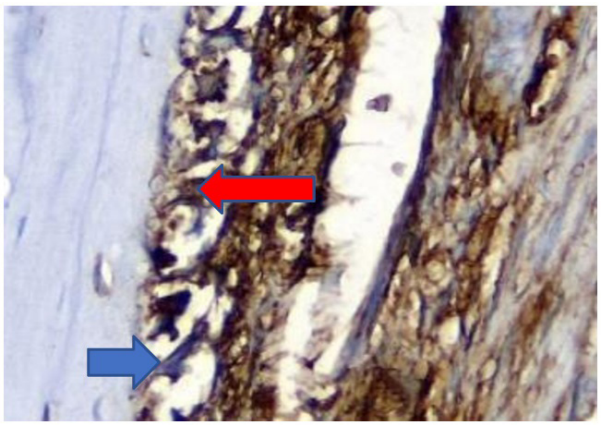

Figure 2 : Expressing TGF-β1 on day 14 of immuno-histochemical staining on osteoblast show positive if osteoblasts are dark brown (red arrows), but negative if osteoblasts are purplish blue (blue arrows)

expansion of mesenchymal stem cells. Bones and cartilages contain a large amount of TGF-β and target cells for TGF-β activity (12).

Signals from the activities of osteoclasts are received by TGF-β1 receptors on cell surface. There are two TGF-β1 receptors, namely receptor 1 and receptor 2. The receptors then forward the signal to the cells through the 2/3 smad pathway, producing an order to transcribe mRNA TGF-β1 (19). Next, Runt-related transcription factor 2 (RUNX-2) as a vital factor in a DNA which binds transcription to an osteogenesis process, will regulate TGF-β1 and BMP-2. The transcription process allows for the expression of large amount of TGF-β1 for both autocrine and paracrine signaling(20). An increase in TGF-β1 expression will stimulate the proliferation of mesenchymal stem cells to release osteoprogenitors. Bone morphogenic protein (BMP) plays a role in the maintenance of osteoprogenitors. Transforming Growth Factor-β1 plays a role in the differentiation of osteoprogenitors into osteoblasts (12).

Open Flap Debridement on day 7 in Table 6 shows a significant difference compared to the other treatment groups. This is likely because OFD is a periodontal flap surgical treatment in deep pockets to stimulate tissue regeneration by debridement to the root surface of the tooth so as to reduce probing pocket depth and prevent tissue attachment loss (14). The procedure for the elimination of periodontal pocket is by removing the necrotic tissues that are found in periodontitis and cause bleeding. According to Chaparro(18), local vascularization in the injury is one of the important parameters in healing processes, thus the decrease in granulation tissues after OFD will increase the regeneration and healing of periodontal tissues(7). On the other hand, Khausal(21) stated that OFD treatment is less optimal in the healing of periodontal tissues, so additional treatment is required so as to stimulate optimal healing.

The group treated with OFD and Carbonate Hydroxyapatite-10% Propolis on day 14 showed a significant difference compared to the other treatment groups. This shows that the addition of 10% propolic to hydroxyapatite carbonate can increase TGF-β1 expression in osteoblasts. According to Kasagi and Chen (12) high concentrations of TGF β1 can stimulate osteoblast proliferation and reduce regulation of receptor activator of NF-kappaB ligand (RANKL) expression in osteoblast while low concentrations of TGF β1 play a role in osteoclast maturation. This is in line with an in vitro study conducted by Elkhenany et.al.(22) showing that 10% propolic can increase the proliferation of mesenchymal stromal cells. The reason why propolic as an anti-inflammatory agent can minimize inflammation is due to the fact that it contains CAPE and quertecin which suppress T cell activity. CAPE inhibits Nuclear Transcription Factor Kappa B (NF-kB) and IL-2 stimulant which trigger the proliferation of T cells, while quertecin affects cyclooxygenase pathway. Propolic extract containing polyphenolic compounds, such as flavonoids and CAPE can increase number of osteoblasts and has anti-inflammatory properties activity (22). Propolic and its substance content can regulate lymphocyte growth through the Erk-2 signal pathway, namely by suppressing proinflammatory cytokines and suppressing cytokines originating from Th1 and Th 2 and stimulating the regulation of T lymphocytes through increasing TGF β1 numbers (23). Propolic contains flavanoids which act as antioxidants. Antioxidant therapy has been widely studied for its effect on bone metabolism by inhibiting osteoclast activity and increasing osteoblasts. In his research Guney et al. (24) examined the effects of propolic on the antioxidant system and fracture healing. Flavanoids play a role in the formation of new bone by stimulating osteoblast maturation.

The formula of carbonate hydroxyapatite is Ca10(PO4CO3)6(OH)2. Hydroxyapatite contains OH groups that can absorb bioactive molecules through hydrogen bonds by increasing the drug load and releasing the drug (25), thus propolic can be combined with carbonate hydroxyapatite. According to a study by Abdellatif et al.(26), propolic used as an orthotopic autograft coating has osteoinductivity of which the function is inseparable from osteoconductive materials. A study by Kusumawati et.al. (27) application of 10% propolic- carbonate hydroxyapatite in the OFD procedure can increase the expression of type 1 collagen in the rabbits's alveolar bone on the seventh and 14th days. The limitation of this study are the small number of samples and the shorter observation time. Further research is to investigate the variables over a longer time period with a larger sample size and needed on the effect of carbonate application 10% hydroxyapatite-propolic on clinical bone healing and immunologic analysis.

CONCLUSION

Addition of 10% propolic solution to carbonate hydroxyapatite bone graft material could increase the number of osteoblasts which express TGF-β1 in the alveolar bone of *Oryctolagus cuniculus* and the result is significant on day 14 compared to the other groups. Recommendation, It is necessary to conduct a study on the effect of the application of carbonate hydroxyapatite-10% propolic on clinical bone healing.

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