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Research Report

Combining 10% propolis with carbonated hydroxyapatite to observe the RANKL expression in a rabbit's alveolar bone

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ABSTRACT

Background: *Periodontitis causes an increased receptor activator level in the nuclear factor-κβ ligand (RANKL), which is one of the inflammatory mediators that plays a role in osteoclastogenesis. The open flap debridement (OFD) technique is the preferred treatment when accompanied by regenerative periodontal treatment using guided tissue regeneration (GTR) and guided bone regeneration (GBR). Carbonated hydroxyapatite is a GBR material that serves as a scaffold and has strong osteoconductive properties for bone regeneration. Propolis is natural product that can decrease osteoclastogenesis in periodontitis by decreasing the RANKL expression.* ***Purpose:*** *To investigate the RANKL expression after open flap debridement by applying carbonated hydroxyapatite to 10% propolis in the alveolar bone of rabbits.* ***Methods:*** *Nine induced-periodontitis rabbits (Oryctolagus cuniculus) were divided into three treatment groups of Group A OFD, Group B OFD followed by the application of carbonated hydroxyapatite, and Group C OFD followed by application of 10% propolis-carbonated hydroxyapatite. Each group was selected one to euthanised on the seventh, 14th and 28th day, respectively, and prepared using histology slides. The data was analysed using a two-way ANOVA followed by a post-hoc LSD test ($p<0.05$).* ***Results:*** *The RANKL expression in each group showed significant differences ($p=0.00$; $p<0.05$) on the seventh, 14th and 28th day. The post-hoc LSD test showed that the RANKL expression in the treatment group with carbonated hydroxyapatite-10% propolis had significant differences ($p<0.05$) in the intergroup analysis at different time points.* ***Conclusion:*** *Combining 10% propolis with carbonated hydroxyapatite in OFD treatment can decrease the RANKL expression in a rabbit's alveolar bone.*

Keywords: *10% propolis; carbonated hydroxyapatite; periodontitis; RANKL expression*

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INTRODUCTION

Periodontal disease is caused by *Porphyromonas gingivalis (P. gingivalis)* and increases inflammatory cell infiltration, i.e. T lymphocytes, B lymphocytes and neutrophils in the connective tissues of the periodontium.[1] These inflammatory cells may lead to an increase in inflammatory mediators, such as prostaglandin E2 (PGE2), interleukin-1 (IL-1) and the receptor activator of nuclear factor-κβ ligand (RANKL). RANKL is an inflammatory mediator that plays a role in osteoclastogenesis. T lymphocytes can activate RANKL during inflammation in periodontal tissues, which causes the RANKL expression to increase during periodontitis.[2]

Periodontal treatment using the open flap debridement (OFD) technique is the preferred treatment when it is accompanied by regenerative periodontal treatment using guided tissue regeneration (GTR) and guided bone regeneration (GBR).[3] Carbonated hydroxyapatite is a GBR material that serves as a scaffold. It has strong osteoconductive properties for bone regeneration, can be well-resorbed by osteoclasts in the body and has good solubility in weak acidic conditions, i.e. when osteoclasts resorb bone by releasing H+ ions.[4] The addition of 10% propolis in carbonated hydroxyapatite can stimulate the growth of fibroblast cells, and it is a good candidate for alveolar bone regeneration.[5] A study by Kusumawati

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et al.[6], which loaded the propolis into the carbonated hydroxyapatite using the immersion method, found that the 10% propolis solution had the strongest chemical bond with carbonated hydroxyapatite. This experiment is in line with the study by Devitaningtyas et al.[7], which found that 10% propolis with carbonated hydroxyapatite had a strong antibacterial inhibition against *P. gingivalis bacteria.*

The largest active compounds in propolis are flavonoids and phenols. The phenolic content in propolis is usually called caffeic acid phenethyl ester (CAPE).[8] The CAPE compound can increase osteoprotegerin (OPG) in tissues and prevent osteoclastogenesis, as OPG works by preventing RANKL from binding to the receptor activator of nuclear factor-κβ (RANK).[9] Bone regeneration processes can be observed from bone formation biomarkers, one of which is RANK.[10] Carbonated hydroxyapatite can be combined with 10% propolis, which has anti-inflammation, anti-tumour and immunomodulator properties, to increase the bone graft material ability for regeneration by decreasing the RANKL expression pathway. This study aimed to determine the effect of the application of carbonated hydroxyapatite-10% propolis in an open flap debridement on RANKL expression.

MATERIALS AND METHODS

This study was an experimental study with a randomised control group design. The carbonated hydroxyapatite that was used in this study was Gama-CHA (PT. Swayasa Prakarsa, Yogyakarta, Indonesia). To acquired carbonated hydroxyapatite-10% propolis, the Gama-CHA block was divided into 10mg and immersed in 1ml of 10% propolis solution for 24 hours at room temperature.[6] The experimental animals that were used were nine male rabbits (*Oryctolagus cunicullus*) aged 5–8 months that weighed 1500–2000g. Periodontitis was induced in the experimental animals in the mandibular incisors using the ligation method with silk 3-0 and an injection of 0.05ml LPS *P. gingivalis* using a tuberculin needle into the interdental area three times a week for six weeks.[11] Clinical signs of the induced periodontitis that were observed in the rabbits were tooth mobility, gingival recession and redness of the gingiva. The sampling method was stratified random sampling. The experimental animals were divided into three groups after ligation. Group A had open flap debridement treatment, Group B had open flap debridement treatment with the application of carbonated hydroxyapatite and Group C had open flap debridement treatment with the application of carbonated hydroxyapatite-10% propolis.

Open flap debridement was done under anaesthesia using ketamine 40mg/kgBW and xylazine 5mg/kg BW. A sulcular incision was performed using scalpel no. 15 in the buccal sulcus of the mandibular central incisors, and the flap incision was then reflected using a small raspatorium. Debridement was performed on both soft tissues and hard tissues. Once OFD was done, irrigation with distilled water and flap repositioning was performed, followed by a suture using 4-0 nylon thread.[12] Groups B and C were given the same treatment; however, carbonated hydroxyapatite material was added to Group B and carbonated hydroxyapatite-10% propolis was added to Group C. After the treatment, the laboratory animals were administered soft food for 24 hours, tramadol at dose of 0.2–0.5mg/kgBW and one interflox antibiotic at a dose of 0.1 mg/kgBW after the treatment through intramuscular injection. One rabbit from each group was randomly selected to be decapitated on the seventh day after surgery, and the remaining rabbits were taken on the 14th day and 28th day after surgery. They were then euthanised using an intermuscular injection of an overdose of sodium pentobarbital, i.e. 120mg/kgBW. Mandibular decapitation was carried out and fixed with formalin before the mandible was cut to obtain mandibular incisor specimens and placed onto four microscopic slides. The immunohistochemistry examination was conducted using antibody polyclonal RANKL from Bioss USA to measure the RANKL expressions in the alveolar bone, which were viewed using a light microscope in 400x magnification on three different fields of view by two observers. Each field of view showed both positive and negative cells. The calculation used the following formula:[13]

$$\text{RANKL Expression} = \frac{\text{Number of Positive Cells}}{\text{Total Cells}} \text{ x } 100$$

The data was analysed using the software SPSS version 21 for Windows (IBM, Chicago, USA). The data analysis was performed using the normality test with the *Shapiro–Wilk* test. A homogeneous variation test was conducted to discover the data variation in the groups with Levene's test ($p>0.05$) with a two way-ANOVA and multiple comparison LSD test ($p<0.05$).

RESULTS

Figure 1 shows the alveolar bone of *Oryctolagus cuniculus* with a magnification of 400x at the seventh, 14th and 28th day in each group. Osteoblasts were found in the sides of the bone. Osteoblasts that positively expressed RANKL were marked by dark brown cytoplasm, whereas those that negatively expressed RANKL were marked by bright blue cytoplasm. The results from this experiment are shown in Table 1. The carbonated hydroxyapatite-10% propolis group had the lowest expression of RANKL.

The normality test showed that the RANKL expression in each treatment group and on each decapitation day had a significance greater than 0.05 ($p>0.05$). The homogeneity test also showed a significance greater than 0.05 ($p>0.05$). Based on the normality and homogeneity tests results, it could be concluded that the RANKL expression data was normally distributed and homogeneous. Therefore, a statistical test using the parametric test – two-way ANOVA – was carried out. The results of the two-way ANOVA

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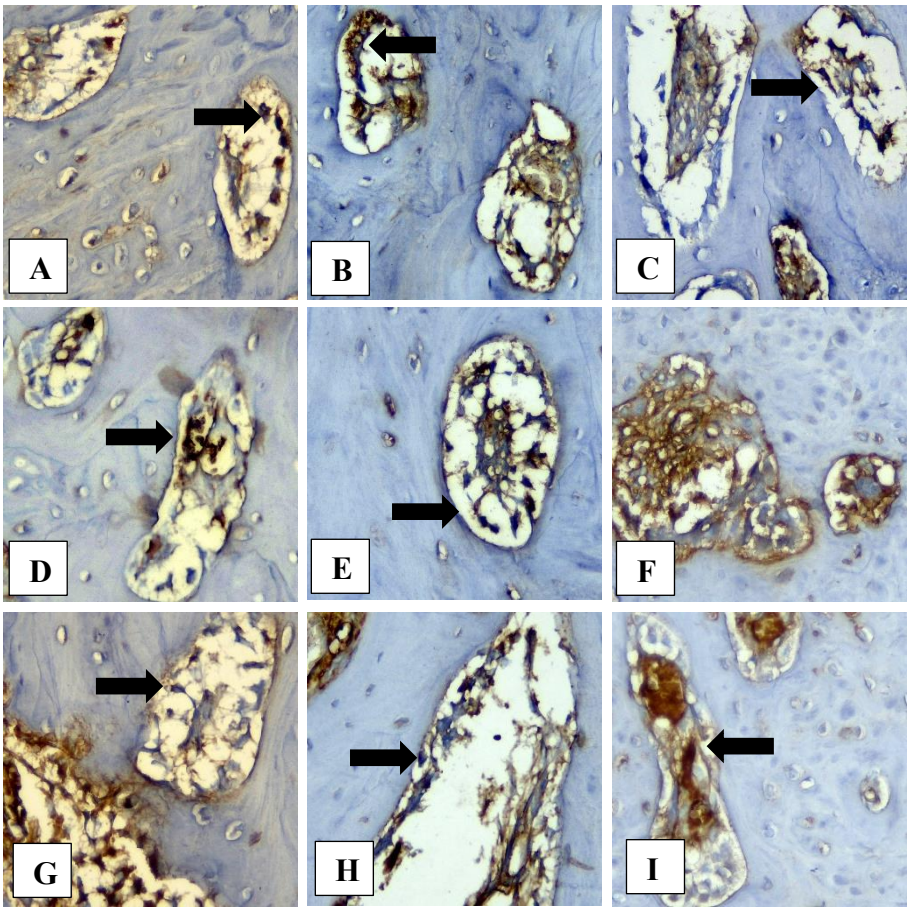

Figure 1. The expression of RANKL in the alveolar bone in each group on the 7th, 14th and 28th day of examination shows the following using a black arrow (osteoblasts which positively expressed RANKL were marked by dark brown cytoplasms): A. Group OFD 7th day; B. Group OFD 14th day; C. Group OFD 28th day; D. Group OFDCHA 7th day; E. Group OFDCHA 14th day; F. Group OFDCHA 28th day; G. Group OFDCHA-10% propolis 7th day; H. Group OFDCHA-10% propolis 14th day; I. Group OFDCHA-10% propolis 28th day.

Table 1. Mean and standard deviation of the RANKL expression

Groups	*X*±*SD*			P
	7th day	14th day	28th day	
OFD	43.21±8.72	30.79±2.76	46.77±6.35	
OFDCHA	42.90±4.52	26.08±4.66	37.49±6.81	0.00*
OFDCHA-10% propolis	22.60±6.77	15.670±6.42	14.42±2.97	

*significant (p<0.005)

Table 2. LSD test of the RANKL expression

Time Point	Group Treatment	OFD	OFDCHA	OFDCHA-10% Propolis
7th day	OFD		0.952	0.002*
	OFDCHA			0.002*
	OFDCHA-10% propolis			
14th day	OFD		0.203	0.002*
	OFDCHA			0.002*
	OFDCHA-10% propolis			
28th day	OFD		0.045*	0.000*
	OFDCHA			0.000*
	OFDCHA-10% propolis			

* significant (p<0.005)

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showed that there were significant differences ($p<0.05$) (Table 1). The result of the post-hoc LSD test showed that the OFD followed by application of 10% propolis-carbonated hydroxyapatite (OFDCHA-10% propolis) group had a significant value ($p<0.05$) compared to the OFD and OFD followed by application of carbonated hydroxyapatite (OFDCHA) groups at all time points (Table 2), as well as the OFDCHA to the OFD group at the 28th day ($p<0.05$). The OFDCHA group compared to the OFD group at the seventh and 14th day was not significant (Table 2).

DISCUSSION

RANKL is a cytokine that regulates bone remodelling and is expressed by osteoblast cells.[2] The RANKL expression is stimulated by cytokines that bind to gp130 signal transducers, such as IL-6, IL-1 and TNF-α. With periodontitis, increasing IL-6, IL-1 and TNF-α will stimulate *activators of transcription* (STAT) dan *mitogen-activated protein kinase* (MAPK) in osteoblast cells, so that the RANKL expression will increase and stimulate ostoclastogenesis.[14,15] Carbonated hydroxyapatite is a strong drug delivery system because it has a uniform pore size, high pore volume, mesoporous (2–5nm) and a large surface area. The –OH group in carbonated hydroxyapatite is an active compound that binds with the bioactive molecule on propolis and creates a hydrogen bond. These hydrogen bonds make the carbonated hydroxyapatite easier to load and release the propolis molecules.[16]

The results of the study showed that the RANKL expression in the group that was treated with OFD and had the application of carbonated hydroxyapatite-10% propolis was the lowest compared to the OFD and OFDCHA groups at all observation time points on day seven, 14 and 28. This indicates that propolis that is incorporated into carbonated hydroxyapatite decreases the RANKL expression in the alveolar bone of rabbits until day 28. The results of this study are in line with the study by Andrade et al.[17], which showed that propolis-incorporated alloplastic bone graft material has good porosity and is able to release active substances until the 30th day. The addition of propolis to a carbonated hydroxyapatite graft material aims to boost the performance of the graft material by reducing the inflammatory response and providing osteoinductivity. Propolis has antibacterial, antiviral, antifungal, anti-tumour and immunomodulatory properties.[18] Propolis is able to reduce the RANKL expression by activating Wnt signalling. Activation of canonical Wnt signalling leads to β-catenin over-expression in cytoplasms, which is translocated to the nucleus of osteoblasts. An increase in the β-catetnin expression suppresses the RANKL expression.[19] Furthermore, propolis is anti-inflammatory as it decreases pro-inflammatory cytokines, such as IL-1β, IL-1, IL-8 and TNF-α. Cytokine cause periodontal destruction by increasing the RANKL expression and inducing osteclastogenesis.[20]

The RANKL expression of the OFDCHA-10% propolis group decreased from day seven to day 28. This indicates that propolis addition can reduce the RANKL expression from the seventh day, whereas the reduction in the other groups began to take place on the 14th day. Day seven is the end of the inflammatory phase and the start of the proliferation phase. At the end of the inflammatory phase, the RANKL expression declines because the osteoblasts are preparing to secrete bone matrix. This is in line with research by Steen et al.[21], which showed that propolis could suppress the RANKL expression at the beginning of inflammatory phase between days three to six. This is in line with the study by Tang et al.[22], which showed that propolis has the ability to suppress and regulate RANKL in three phases of osteoblast development. Although there was an increase during the proliferation phase, this was not significantly different to the mineralisation and maturation phases. This condition allows osteoblasts to secrete more bone formation matrices, which optimises tissue regeneration. In addition, the caffeic acid phenethyl ester (CAPE) in propolis triggers the osteoclastogenesis process by inhibiting osteoclastogenesis at the early stage of differentiation by suppressing RANKL-induced NF-kB activation.[23] The limitations of this research are that there were no baseline conditions and the duration of the experiment was limited. The conclusion from the study was that combining 10% propolis with carbonated hydroxyapatite in OFD treatment can decrease the RANKL expression in a rabbit's alveolar bone. It is necessary to conduct further research on the effect of the application of carbonate hydroxyapatite-10% propolis in open flap debridement with a longer period of observation.

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