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Indonesian Propolis Reduces Malondialdehyde Level and Increase Osteoblast Cell Number in Alveolar Bone of Wistar Rats with Orthodontic Tooth Movement

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Abstract

AIM: The aim of this study was to determine the antioxidant activity of Indonesian propolis gel 5% in Wistar rats alveolar bone, toward malondialdehyde serum levels and osteoblast cells number caused by orthodontic tooth movement (OTM).

METHODS: This was an experimental study using the post-test only group design. The samples were 28 male Wistar rats, divided into four groups: G1 (control group) – group without OTM and without propolis, G2 – group without OTM and with propolis, G3 – group with OTM and without propolis, and G4 – group with OTM and with propolis. Propolis available in the form of 5% gel and 30 gf helical spring force of OTM applied. Spring was applied in rat maxilla incisors. OTM treatment was given 17 days, and on day 18, blood samples were taken for the measurement of malondialdehyde levels, then tested using the ELISA test. Variable of osteoblast was calculated histologically using hematoxylin-eosin staining. The data of malondialdehyde level and the osteoblast number obtained were tested using one-way ANOVA.

RESULTS: The result indicated that osteoblast number was higher with propolis application compared to those without propolis in the control group and orthodontic tooth treatment group (G2>G1, 23.97 ± 2.95 vs. 18.63 ± 3.04 and G4>G3, 34.17 ± 5.57 vs. 28.26 ± 2.62) with significant difference (p < 0.05). Propolis application also reduces malondialdehyde serum level when compared to both groups without propolis (control and OTM group) (G2<G1, 1.02 ± 0.18 nmol/ml vs. 1.55 ± 0.24 nmol/ml and G4<G3 1.29 ± 0.22 nmol/ml vs. 1.83 ± 0.21 nmol/ml) and significantly different (p < 0.05). OTM increased the malondialdehyde level compared to the control group, with a significant difference (p < 0.05).

CONCLUSION: Propolis gel 5% application can reduce malondialdehyde serum level and could increase the number of osteoblast.

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Introduction

There is a continuous attempt to accelerate dental orthodontic treatment. The main principle on the orthodontic tooth movement (OTM) is the tooth that can move when alveolar bone resorption occurs in tension side and apposition in tensile side [1], [2]. Mechanical stress applied to a tooth results in an inflammatory response that stimulates the formation of receptor activator of nuclear factor-κB (RANK), receptor activator of nuclear factor-κB ligand (RANKL), and osteoprotegerin (OPG). When the RANK binds to RANKL, stimulation process of osteoclast differentiation occurs. OPG is a feedback receptor of RANKL, inhibiting the bonding to RANK, preventing the osteoclast differentiation process as a resorption cause. In the tensile side, OPG binds to RANK, stimulating the occurrence of apposition. OPG functions also inhibit resorption [1], [3].

On the tension side of OTM, the tissues undergoing ischemia and acute inflammation provoke the emergence of reactive oxygen species (ROS), though in a small amount [4]. Physiologically, this condition can stimulate transduction signals for cytokines, growth factors, neurotransmitters, and colony-stimulating factors (CSFs). The positive effects are vascular reactivity, reduced edema, growth factors and cytokine effect modification, vasculogenesis stimulation, increased cell proliferation, and bacterial killing [1], [2]. However, when the OTM persists for a long time, increased number of ROS could raise the inflammation level. Chronic inflammation level results in increased bacterial colony potential, which negatively affects the increased number of ROS. Increased number of ROS caused imbalance of oxidant and antioxidant inside the body, so it produces oxidative stress. In this situation, required antioxidants [5].

Propolis is a bee product consisting of resin substances collected from flowers, buds, and

exudates of various plant sources. Bees mix these substances with enzymes excreted from their mandible gland [5], [6]. Propolis has many beneficial activities such as antioxidant, anti-inflammation, antibacterial, immunomodulatory, antitumor, and analgesic and anesthetic properties [4], [5], [6]. According to its composition, propolis consists of a various components mixture such as amino acids, phenolic acids, ester phenolic acid, cinnamic acid, terpene, flavonoid, and caffeic acid [5], [6], [7], [8]. Propolis in this study was derived from Yogyakarta (Indonesia) and has been proved to contain flavonoid (quercetin) [9]. The previous studies have reported powerful antioxidant capacity [9], [10] and quercetin that reported in the literature has potent anti-inflammatory properties [10]. In this study, the application of propolis gel extract (from Indonesia) as an antioxidant and anti-inflammatory was studied. Therefore, the aim of this study was to evaluate the effect of propolis gel 5% application on oxidative stress level and its effect in bone remodeling osteogenesis on OTM in Wistar rats.

Materials and Methods

This study was approved by the Gadjah Mada University Ethics Committee for animal care and use, number 425/KEC-LPPT/II/2016.

Propolis extraction

Raw propolis material was taken from beekeeping in the Moyudan area, Yogyakarta, Indonesia (Figure 1a). The farmed bee type was *Apis mellifera* sp. The raw propolis was thinly cut, blended using 96% ethanol. Next, it is hushed up for 24 h and then filtering was conducted. The filtrate was evaporated with a vacuum rotary evaporator at the temperature of 60°C water bath heater. The viscous extract result was poured into a porcelain cup. Then, it was heated with a water bath at the temperature of 70°C with continuous stirring. The propolis extract result was weighed and packed (Figure 1b).

Preparation of propolis extract gel 5%

First solution, each carboxymethyl cellulose and nipagin, was dissolved into water. Propolis extract weighed 2 g and mixed with glyceryl and triethanolamine, and this solution was then added into the first solution while stirring and adding water to form a gel mass of 40 g (Figure 1c).

Helical spring

The OTM devices which applied to experimental animals were a helical spring made of 0.12 U stainless

steel wire (ClassOne Orthodontics, USA). The diameter of the coil was 2 mm with 10 mm wire sleeve length. The teeth animal made from a 2 mm matrix band (Meba, Germany) mounted on the wire end [11]. The mechanical force measured at 30 gf using a tension gauge device (Dentaurum, Germany) (Figure 1d).

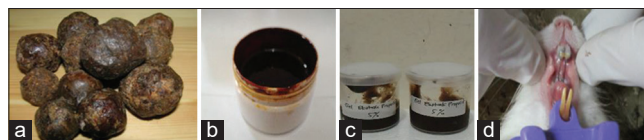


Figure 1: (a) Raw propolis; (b) propolis extract; (c) content of propolis gel 5%; (d) helical springs mounted in Wistar rat maxilla incisors teeth

Experimental and samples

The experimental animals used in this study were white rats (*Rattus norvegicus*) of Wistar strain obtained from the 4th Integrated Research and Testing Laboratory (LPPT-4), Gajah Mada University, Yogyakarta. The sample inclusion criteria for the experimental animals in this study were (a) healthy, (b) male, (c) 3 months old, and (d) body weight 200–250 mg. Then, the sample exclusion criteria were uncooperative rats, namely, after the installation of the OTM device, the mouse clawed the device so that it was released. A rat was dropped out if it died during the study.

The sample in this study was 28 rats (divided into four groups, totaling seven rats for each group). The study design consisted of G1 group (control/without propolis and without OTM), G2 group (provided with propolis and without OTM), G3 group (without propolis and with OTM), and G4 group (supplied with propolis and with OTM).

Before the study began, the rats were adapted for 1 week in individual cages. Furthermore, the rat was mounted with a helical spring, preceded by anesthesia with ketamine HCl 90 mg/kg BW and xylazine 3 mg/kg BW intramuscularly on the left rear leg (quadriceps muscle).

Installation of springs in the maxillary incisors was carried out perpendicularly and fixed with glass ionomer cement (Fuji IX GC, Japan). OTM was carried out for 17 days, and on the 18th day, 1 mL of blood samples were collected intra orbital for the examination of malondialdehyde (MDA) levels. Furthermore, capititation was performed for the maxillary bone collection and followed by histological examination of osteoblasts in the alveolar bone.

Determination of MDA

The immunohistochemical test was performed with an ELISA kit (Bioassay, USA). The study procedures followed the performance standard of bioassay. The results were read using ELISA reader.

Histological procedure

The samples were fixed using 10% formalin for 24 h, decalcified using 14% EDTA for 3 days, dehydrated, cleared, and embedded in paraffin. Then, helical springs were removed; and the sample was sliced perpendicular to a thickness of 4 microns for hematoxylin-eosin staining procedures. The staining procedure was used to determine the histological properties of osteoblast cells. Osteoblast cells count was calculated using a microscope (Olympus BX-51, Japan) at $\times 100$, in the five visual fields selected and averaged.

Statistical analysis

Calculation data included the average number of osteoblast cells and MDA levels in the four groups were collected and tabulated. Statistical analysis using one-way ANOVA test in SPSS version 22. Statistical test results were considered statistically significant at $p < 0.05$. Analysis then continued using *post hoc* test with LSD test to test the difference in mean levels of MDA and the number of osteoblasts between groups.

Results

The effect of propolis gel application on osteoblast level

The average number of alveolar bone osteoblasts was observed on the periodontal ligament (PDL) tensile side in four groups subject (Figure 2)

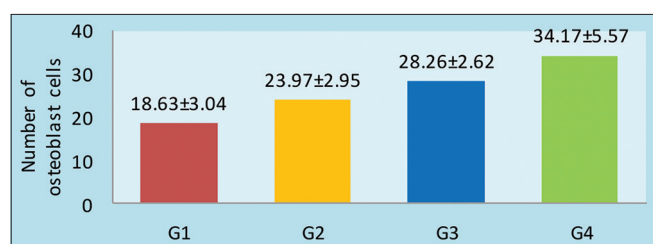


Figure 2: Diagram of the average and standard deviation osteoblast cells number in the four groups. G1 (control/without OTM and without propolis); G2 (without OTM and with propolis); G3 (with OTM and without propolis); and G4 (with OTM and with propolis)

and its standard deviation observed. The diagram shows that the average number of osteoblasts in rats with propolis treatment appears to be numerous when compared with the group without propolis application ($G2 > G1$ and $G4 > G3$). The results of the statistical test one-way ANOVA showed that there were significant differences between groups without treatment G2 versus G1 and the treatment group with OTM, G4 versus G3 ($p < 0.01$). The *post hoc* test results showed that the differences between groups were significant.

The activity of osteoblasts number in groups with OTM (G3 and G4) was higher than those groups without OTM ($G3 > G1$, $G4 > G2$, and $G3$ and $G4 > G1$ and $G2$). This indicates that in OTM treatment, on the tensile side, bone apposition osteogenesis occurs. With propolis application, the number of osteoblast cells increased in the OTM group. Thus, propolis showed a positive effect on the number of osteoblasts.

Histological features of osteoblast cells are shown in Figure 3. Overview slide on the tensile side:



Figure 3: Osteoblast cell (sign) on tensile side with $\times 100$. G1 group (control/without OTM and without propolis); G2 (without OTM and with propolis); G3 (with OTM and without propolis); and G4 (with OTM and with propolis gel). Note description: (a) Root, (b) vascularization area of PDL, (c) new woven bone area, (d) alveolar bone

The root of the tooth (a), defined on PDL (b + c), and PDL with the alveolar bone (d). G2 (group without OTM and with propolis treatment) showed PDL thickness with real vascularization (b) and new bone webbing (c) compared to G1 (control). G1 osteoblast cells are elliptical. The OTM-treated groups (G3 and G4) have osteoblast cells that are flatter in shape due to OTM tensile side response; in the alveolar bone surface, new trabeculae appear for osteogenesis.

In G2 and G4 (with propolis), newer trabecular is produced arising from the alveolar bone surface (c), with thicker vascularization area (b) and more define than the G1 and G3. This shows that propolis treatment increases vascularization and new bone weaving.

The effect of propolis application on MDA level

The average value of MDA level and standard deviations is shown in Figure 4. The results of the

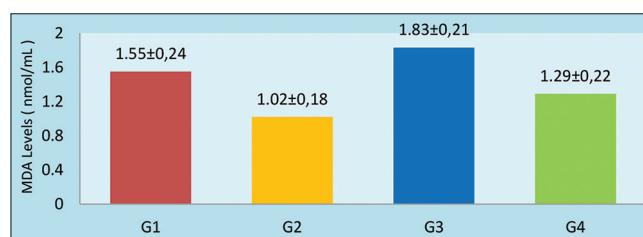


Figure 4: The average and standard deviation of MDA levels on propolis application. G1 group (control/without OTM and without propolis); G2 group (without OTM + 5% propolis gel); G3 group (OTM + without propolis); and G4 group (OTM + 5% propolis gel)

one-way ANOVA test indicate the difference between groups ($p < 0.01$). The analysis was continued with the

post hoc test, which showed a significant difference in all groups. This indicates that the application of propolis (G2 and G4) can reduce MDA levels (G4 vs. G3 in OTM; G2 vs. G1 in control).

Discussion

Based on the theory, OTM interventions cause molecular cell changes on the tension side and tensile side. On the tension side, there is a resorption process due to osteoclast activity. On the opposite side, the tensile side will experience new bone formation (apposition) because the activation of osteoblasts, differentiation, and matured osteoblasts will form osteoid and cause mineralization [1], [3].

OTM induced inflammatory response and remodeling which influenced PDL, alveolar bone, cell differentiation, and cell apoptosis. RANKL and macrophage CSF expressed by osteoclast apoptotic osteoblasts are an essential part of inflammatory cytokines responsible for the process of activation, differentiation, and survival of osteoclasts. On the tension side, osteoclasts produce RANK through communication of osteoblast-osteoclast. On the tensile side, osteoblasts will produce OPG, which inhibit the interaction between RANK and RANKL to prevent osteoclast genesis and accelerate the maturation of osteoclasts apoptosis [1].

RANKL and RANK bind on the tension side causes bone resorption, and OPG on the tensile side will inhibit resorption and trigger new bone apposition [1], [2], [3]. Propolis was applied locally to Wistar rats from the 1st day until the 17th day. From the previous studies, it has been proven that it can increase the number of osteoblasts on the tensile side both in the OTM treatment and in control.

OTM usually needs 2–3 years (depends on the malocclusions). OTM with superimposed pressure for an extended period causes an increase in ROS and free radicals. That triggers oxidative stress and resulting in the occurrence of lipid peroxidation [4], [6]. This lipid peroxidation is the ending result of a process that involves the breakdown of fatty acid strain into various aldehydes such as malondialdehyde (MDA) [12], [13]. The increase level of MDA is a marker of oxidative stress. The rapidly increasing amount of ROS is obtained through various pathways such as the NADPH oxidase pathway, the xanthine oxidase pathway, the Fenton reaction pathway, the Haber–Weiss reaction pathway, and the nitric oxidase pathway. Based on the previous studies, OTM can increase MDA levels [14]. Propolis has been shown to reduce the levels of MDA [4], [12], [14], [15].

Local propolis from Indonesia contains flavonoids, quercetin (dominant content), rutin

(small number), and gallic acid (small amount) [9]. Flavonoids play an important role in eliminating oxidative stress [4], [14], [15] in various ways, including the direct scavenging of ROS that can prevent ROS, to protect the lipophilic antioxidants, and increase enzymatic antioxidants. Application of antioxidants like quercetin could inhibit lipid peroxidation [4], [14], [15], also rutin (strength antioxidant) and gallic acid, thus preventing damage to gingival tissue, PDL, and bone tissue [5], [6], [7]. Oxidative stress can also damage bone metabolism because the inhibition of the Wnt/ β -catenin pathway in differentiation, proliferation, and maturation will inhibit the occurrence of osteoblast cells so that the process of bone remodeling is disrupted.

Quercetin also plays a role in angiogenesis to increase VEGF [16]. In the event of ischemia and hypoxia inside of tension with oxidative stress, the blood supply will decrease. Hypoxia-inducible factor-1 will inhibit Wnt signaling, thus inhibit differentiation osteoblast. Propolis plays an important role in the regulation of bone remodeling, possibly through angiogenesis [4], [6]. Increased VEGF can increase RANK so that flavonoids propolis also stimulates the OPG binding formation to the side. In this study, propolis can improve bone remodeling so the duration of OTM becomes probably shorter.

Conclusion

OTM intervention can increase oxidative stress, and 5% propolis gel can reduce blood MDA levels and increase the number of osteoblasts. In the future, Indonesian local propolis can be developed as an antioxidant to enhance OTM osteogenesis or another field in science.

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