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The Effect of Propolis on Increasing the Number of Osteoblasts And Chondrocytes, And Decreasing the Number of Osteoclasts in Wistar Rats (Rattusnovergicus)with Femoral Bone Fracture

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Abstract
Background: *Bone healing is a complex biological process, involving many cell types, thousands of genes, and extracelullar matrix organization. Many studies had been done to enhance the healing of bone fracture, including biological intervention by using natural materials that can be found in the surrounding environment. Propolis is a substance produced by honeybees which have antioxidant, antimicrobial, and anti inflammation effects. This study was conducted to evaluate the effect of propolis inLawang on the bone healing process, reflected by the number of osteoblasts, osteoclasts, and chondrocytes.*
Methods: *This study used 24 male Wistar rats (Rattusnovergicus) that was randomly divided into 4 groups. Microscopic examination with HE staining was done on the 15th day to evaluate the number of osteoblasts, osteoclasts, and chondrocytes in the bone fracture site. The normality of the data was tested using Shapiro-Wilk test and the homogenity of the data was tested using Levene test. Data analysis was done using One-way ANOVA.*
Result: *Analysis of osteoblast count using Post Hoc test showed that the mean osteoblast count among groups were significantly different (p<0.05), except forIG1 and IG2 group (p=0.594). The difference in the mean osteoclast count among groups was statistically significant (p<0.05), except for IG2 and IG3 group (p= 0.936). The mean chondrocytes count was not significantly different (p>0.05), except for control and IG3 group (p = 0.00); and control and IG1 group (p = 0.007).*
Conclusion: *Propolis increase the number of osteoblast and chondrocyte, and decrease the number of osteoclast in RattusNo:*
Bone Fracture Healing, Propolis, Osteoblast, Osteoclast, Chondrocyte

I. Introduction

Bone fracture is a complete or incomplete discontinuity of bone. The most common cause for bone fracture is trauma or accidents. The overall incidence for bone fracture is 11,3 per 1000 individuals every year worldwide[2]. The incidence in men is 11.67 per 1.000 men each year, while the incidence in women is 10.65 per 1000 women each year[3].

Fracture healing is a complex biological process, involving many cell types, thousands of genes, and extracelullar matrix organization. All of these process have the purpose to restore bone mechanical strength and function. There are many therapeutic approaches focused on accelerating the formation of new bones. Despite advancements in orthopedic interventions and medical technologies, fracture healing is not always satisfactory with occasional prolongation of the treatment or nonunion[8].

A series of events occur during fracture healing including inflammation, repair and remodeling; and excessive amounts of freeoxygen radicals are released during these processes. Free radicals are highly reactive products and may result in cellular injury or death through their effects on almost all components of the cell such as lipids, proteins, DNA, carbohydrates and enzymes. They are known to have unfavorable effects in many conditions including fracture healing[33].

Recently, a study by Guneyet al evaluated the effect of propolis as antioxidant on the fracture healing process[2]. Propolisis collected by honeybees and is used for many purposes in the hive. It has antioxidant, anti inflammation, and other beneficial effects[2].It is a sticky, resinous substance with a specific odor and its color ranges from yellow to dark brown. In general, propolisconsists of 50% resin, 30% paraffin, 10% essential oil, 5% pollen, and 5% organicdebris[26].

Since ancient times, propolis has been used for the treatment of many diseases.Propolishas more than 300 chemical compounds, and flavonoids form the main content (25%). Flavonoids are polyphenolic compounds that exhibit their antioxidant action through inhibition of lipid peroxidation[20]. Caffeic acid phenethyl ester (CAPE) is also found as one of the useful components in propolis[14].

There are many studies that have investigated poplar propolis from Europe and baccharis from Brazil[2]. A study by Gunet et al (2014) has proved that poplar propolis can accelerate the fracture healing process.

However, the composition and effects of propolis differ in each geographical areas and plant sources[2]. Propolisfrom Indonesia contains moreflavonoids than propolis from Brazil. There is still no study investigating the effectivity of Indonesian propolis, especially propolis from Java, on the fracture healing. Therefore, the author aimedto investigate the effects of propolis of Java, particularly Lawang, on fracture healing using experimental rats with femur fracture[11].

II. Methods

This is an experimental study on 24 healthy Wistar rats (Rattus novergicus). Twenty-four rats with age 3-4 months and weighing 150-250 g body weight were included in this study. This study was conducted in Pharmacology Laboratory, Brawijaya University Faculty of medicine.

Rats were randomly grouped into 4 groups; Intervention group 1 (IG1) which would get propolis on day 3-7 post fracture; Intervention group 2 (IG2) which would get propolis on day 0-7 post fracture; Intervention group 3 (IG3) which would get propolis on day 0-14 post fracture; and control group (CG) which would not be given propolis therapy.

All rats had anesthesia with ketamine dose of 40mg/kg IM, underwent right closed femur fracture and closed reposition and immobilisation using long leg cast. Intervention groups were given 200 mg/kg body weight propolis by orally using a sonde. On the 15th day, fractured bone specimens were taken for histopathological examination. Specimens were stained by HE and evaluated under microscope for osteoblast, osteoclast, and chondrocyte cells/high power filed counts in fracture site.

The normality of the data was tested using Shapiro-Wilk test and the homogenity of the data was tested using Levene test.One way ANOVA was used to compare the osteoblast, osteoclast, and chondroyte counts among groups. Daa were analysed using SPSS version 15.0 for Windows. A value of $p<0.05$ was consideres as an indication of statistical significance.

III. Results

Osteoblast

Analysis of osteoblast count using Post Hoc test showed that the mean osteoblast count among groups were significantly different ($p<0.05$), except forIG1 and IG2 group ($p=0.594$)

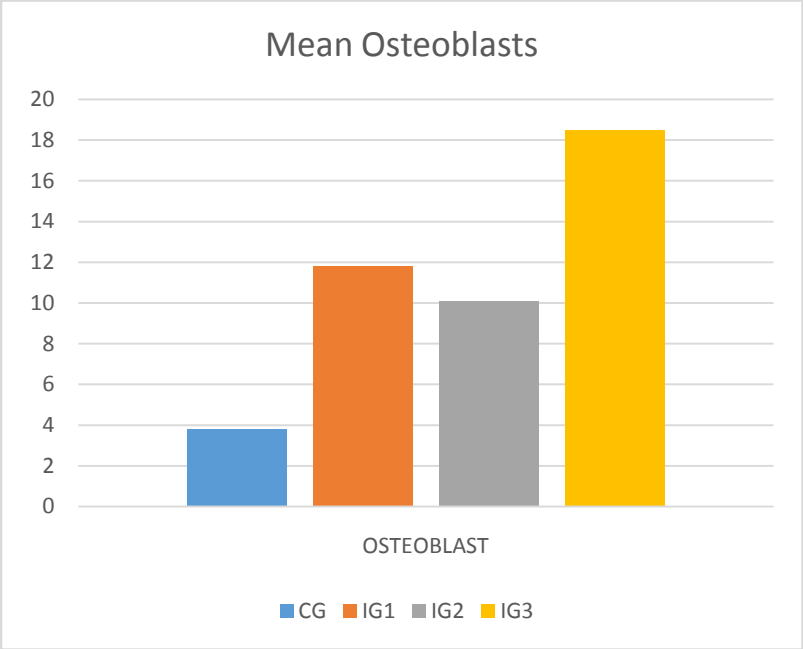

Figure 1. Mean osteoblast count in each group. IG 3 has the highest osteoblast count and CG has the lowest osteoblast count.

Osteoclast

The difference in the mean osteoclast count among groups was statistically significant ($p<0.05$), except for IG2 and IG3 group ($p= 0.936$).

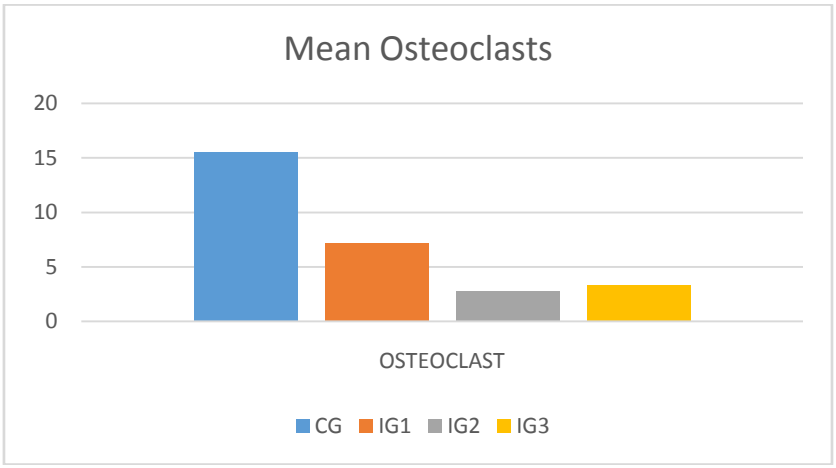

Figure 2. Mean osteoclast count in each group. CG has the highest osteoclast count and IG2 has the lowest osteoblast count.

Chondrocyte

The mean chondrocytes count was not significantly different ($p>0.05$), except for control and IG3 group ($p = 0.00$); and control and IG1 group ($p = 0.007$).

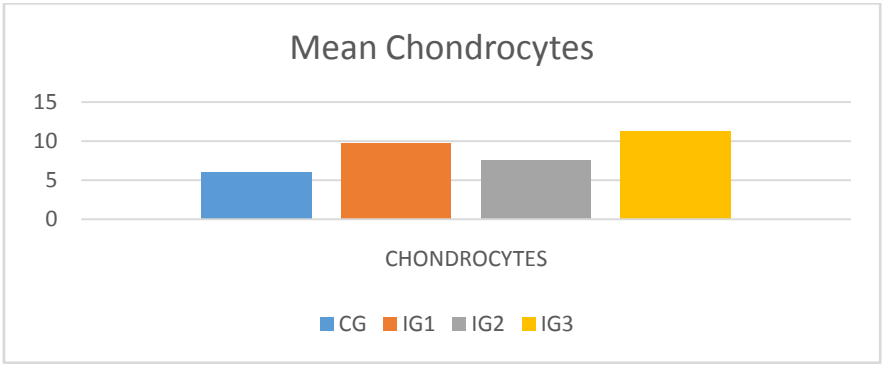

Figure 3. Mean chondrocyte count in each group. IG3 has the highest chondrocyte count and CG has the lowest chondrocyte count.

IV. Discussion

This experimental study demonstrated the favorable effects of propolis on fracture healing in an experimental setting, as evidenced histopathological findings. Some experts had found the association between oxidative stress and bone metabolism. Oxidative stress caused by excessive intracelullar ROS formation can give a negative biological effects on pre osteoblastic cell lines and stromal cell lines. ROS also supports the formation and activity of osteoclasts. ROS and tumor necrosis factor- α (TNF – α) supress osteoblastic differentiation[5]. Considering the harmful effects of oxidants, some antioxidants had been studied to evaluate its ability to overcome tissue destruction and its role in bone formation. The administration of antioxidants, such as vitamin D analog, thymoquinone, and boron had been proved useful in increasing new bone formation[3].

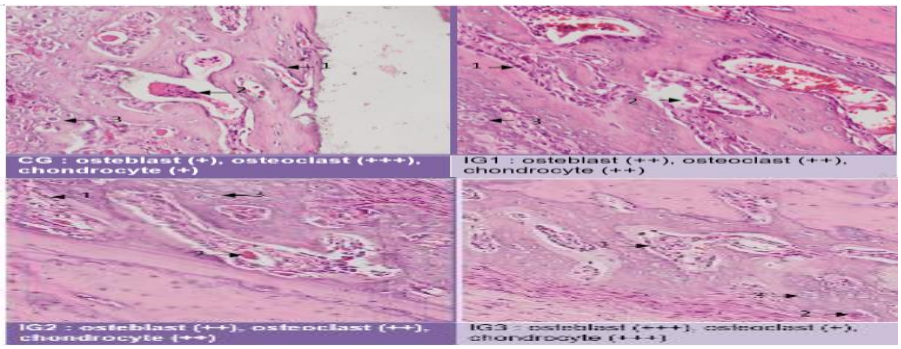

Figure 4.

mangnification microscope 8x. (1) Osteoblast; (2) Osteoclast; (3) Chondrocyte.

In the last decade, a few studies about the composition and biological properties of propolis had been published. Guneyet al.investigated the effect of propolis on the oxidant-antioxidant system and found that endogenous antioxidant level decreased both in blood and bone tissue when compared with the controls, reflecting the presence of exogenous antioxidants provided by propolis[25].

Although Kumazawa et al. related the powerful antioxidant properties of propolis to its kemferol and phenyl cafeate content, flavonoids are known to be the most effective and abundant antioxidant substances in propolis[20]. Moreno et al. showed a significant association between antioxidant activity and flavonoid content for Argentinean propolis. In addition, they found a positive relation between the flavonoid content and inhibitionof malondialdehyde (MDA), a substance taking part in oxidative process[23]. On the other hand, substances other than flavonoids have been reported as the contributors to the antioxidant properties of propolis[15]. Russo et al. compared the properties of propolis extracts with or without caffeic acid phenethyl ester (CAPE) and found that both types of propolis had a dose-dependent free radical scavenger effect and that both significantly inhibited xanthine oxidase activity and had anti lipoperoxidative capacity[32]. However, the extract containing CAPE was found to be more active when compared with the extract without CAPE, suggesting an important role of CAPE in the antioxidant effect of propolis. In addition, high concentrations of caffeic acid and phenyl cafeate have been associated with a powerful antioxidant effect[13].

Inflammation needs to be controlled so that it will not cause toxic consequences for organisms. Inflammation response produce free radicals through active macrophages and neutrophils.these molecules degrade fatty acids from plasm membrane, impair protein membrane, and cause DNA mutations. Nitric oxide is an inflammation mediator produced by endothelial cells and inflammation cells, which can harm body tissue if produced in a large amount.

Propolis has anti inflammation effect through the action of caffeic acid (CAPE),quercetin, andnaringenin. These substances supress the synthesis of prostaglandin and leukotriene by macrophage, and inhibit the activity of myeloperoxidase, NADPH-oxydase, ornithine decarboxylase and tyrosine protein kinase. Propolisalso inhibit the production of nitric oxide (NO). NO prolongs the inflammation reaction by causing vasodilatation and oedema. This results in increa-sed inflammation expression and increased prostaglandin synthesis.

Propolis affects some activities in inflammation process : inhibition of phospholipase A2, cyclooxyganase, and lipooxygenase activities, and countering free radicals. Flavonoid also proved to inhibit the expression of nitric oxide synthase. Another study suggested that CAPE can decreased NO production and GAG release into human cartilago tissues and can prevent the GAG synthesis inhibition in chondrocytes[15].

The cytokines family consists of 21 Interleukin (IL) types, called IL-1 to IL-21, which have distinct function and activities. IL-1 has 2 distinct forms, IL-1 α and IL-1 β. IL-1 α generally binds with membrane, while IL-1 β contributes in inflammation. IL-1 β is produced by fibroblasts and chondrocytes, and involved in inflammation process[24]. IL-1 β can induce the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) which will increase the arachidonic acid metabolites, ROS, and NO in fibroblasts and chondrocytes. NO stimulate the proenzymes production from chondrocytes, that will be converted into active enzymes (called matrix metalloproteinase [MMP]) , which can harm cartilagenous tissue and inhibit the synthesis of proteoglycans. A studyfound that propolis extract can prevent cartilago destruction. This natural compoounds can decrease NO production and release GAG in human cartilago culture, which was given IL-1 β[25].

Previous study stated that flavonoids can induce osteoblast differentiation. Osteoblast maturation is an imporant process in bone development. Flavonoids support bone formation by stimulating osteoblast maturation. Flavonoids induce osterix and Runx2 mRNA expression, which in turn will stimulate osteoblast differentiation[10].

Osteoblasts secrete and mineralize bone matrix. These extracelullar matrix consists of type I collagen fibers, osteocalcin osteopontin, sialo protein tulang, TGF – β, and hydroxylapatite minerals. The more osteoblast, the more bone can be formed and the stronger the bone will be[7].

In this study, intervention group had a significant more osteoblasts than control group ($p< 0.05$). this indicates that the administration of oral propolis increase osteoblast count. We also found a similar amount of osteoblast in IG1 and IG2. This may be caused by the negative feedback of osteoblast regulation, which limits the maximum osteoblast count in a given process. Protein gene CXXC finger protein 5 (CXXC5) has been identified as the negative regulator for osteoblast differentiation and bone formation. CXXC5 gives negative feedback on the Wnt/beta-catenin pathway, through its binding with Dishevelled (Dvl) during osteoblast differentiation[19].

IG3 has the most osteoblast count, which showed that the effect of propolis on osteoblast count is time-dependent; the longer the duration of propolis administration, the more osteoblast can be found on fracture site. This is consistent with the result of previous study by Guney et al[25].

Intervention groups have a significantly less osteoclast count than control group ($p< 0.05$). This result is consistent with the result from Pileggiet al (2009), which showed that propolis can inhibit osteoclasts and decrease bone resorption associated with trauma. IG2 and IG2 have the least osteoclast, where the difference between these groups is not significant. This inflicts that propolis administration affects the number of osteoclast in fracture site.

Previous studies had proven that propolis can inhibit the formation and maturation of osteoblast, through the inhibition of actin ring). Osteoclastogenesisneeds the activation of nuclear factor kappa B, which is the hallmark of inflammation[15].

Cardile et al examined the effects of the propolis extract on NO and glycosaminoglycans (GAG), the key molecules released during inflammation, in human cartilage tissue and chondrocyte culture. They found a significant effect of propolis and its active component caffeic acid phenethyl ester (CAPE) on IL-1β and confirmed the free radical scavenger effect of propolis protecting cartilage tissue owing to its active components with antioxidant properties[6].

In endochondral ossification, chondrocytes play an important role in callus formation and initiating the osteogenesis process[8]. Intervention groups have a significantly much more chondrocytes that control group ($p<0.05$); where IG3 has the most. This showed that propolis increase the chondrocyte amount in a time-dependent manner. This result was consistent with the finding from Arroyo in Spain[1], which found that the polyphenolic content in propolis supports the survivalof chondrocytes.

Propolis has been proven safe to use in small dose, but adverse effects can occur in dose >15 g/day. The most common adverse events are allergic reaction and irritation in skin and mucous (Kinerman, 2001). Our study used 200 mg/kg body weight/day and no adverse events occured.

From the analysis above, we can conclude that there is a consistency between theory and study results, that is propolis administration can increase the number of osteoblasts and chondrocytes, and decrease the number of osteoclasts significantly. Therefore, the hypothesis of this study can be accepted. The findings of this study can be the rasionalisation for propolis administration as supplemental therapy for patients with traumatic bone fracture.

V. Conclusion

Propolis increase the number of osteoblast and chondrocyte, and decrease the number of osteoclast in RattusNovergicusvarWistar, in vivo, compared to the control group.

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