Effect of Propolis on Bone Quality and Cortical Bone Thickness of Ovariectomized Female Wistar White Rats as a Model for Osteoporosis

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ABSTRACT
Estrogen deficiency increases the rate of osteoporosis, especially in menopausal women, by altering the bone tissue microarchitecture. Propolis has compounds that could be used as an alternative therapy to treat estrogen deficiency and to protect against bone damage. This study aims to determine the effect of propolis on bone quality and cortical bone thickness of femoral metaphysis in ovariectomized female Wistar white rats as a model for menopausal osteoporosis. The rats were divided into five groups: negative control group (not subjected to ovariectomy), sham group (subjected to ovariectomy), and treatment groups that were subjected to ovariectomy and given propolis orally at a dose of 180 mg/kg BW, 360 mg/kg BW, and 720 mg/kg BW for 30 days. Bone quality and cortical bone thickness testing were undertaken on the 31st day. The osteoblast and osteoclast cell examination was evaluated using an Olympus BX 51 light microscope at 400x magnification for bone quality and the Betaview program, Beta 3.1MP Sony Exmor CMOS Sensor camera at 40x magnification for cortical bone thickness. Data were analyzed using the one-way ANOVA and continued with Duncan’s multiple range tests. It was found that propolis had a significant effect on the ratio of osteoblast and femur bone osteoclast (p<0.05). The administration of propolis at a dose of 180 mg/kg BW, 360 mg/kg BW, and 720 mg/kg BW had an effect in decreasing the ratio of osteoblasts and metaphysical osteoclast cells of femoral metaphysis. However, propolis administration did not affect the thickness of the femoral metaphysical cortical bone.

Keywords: propolis; bone quality; osteoblast cells; osteoclast cells; cortical bone thickness

INTRODUCTION
Osteoporosis is a disease characterized by low bone mass and microarchitecture damage to bone tissue, causing bone fragility and increased fracture risk (NIH, 2018; Liu et al., 2017). According to WHO data, around 200 million people worldwide have osteoporosis, and it is classified as a “silent disease” that causes secondary health problems to death (Sozen et al., 2017; Kemenkes RI, 2015). An imbalance in bone formation and bone resorption during the remodelling process causes osteoporosis (Geng et al., 2019), which is influenced by two essential factors; aging factors and a decrease in gonadal function (Sihombing et al., 2012). Osteoporosis is more commonly found in menopausal women. In Indonesia, two out of every five women are at a higher risk of osteoporosis (Kanis et al., 2000; Ginta et al., 2015). Women are more likely to lose bone mass than males due to a reduction in estrogen hormone production, particularly in women who have gone through menopause (Ermati, 2018; Gambacciani et al., 2018; Thulkar et al., 2015). Estrogens are antiresorptive agents and stimulators of bone formation. It works by reducing apoptosis and osteoclast differentiation, inhibiting the reduction of osteoblasts and osteocytes, and reducing oxidative stress. The deficiency of postmenopausal hormone estrogen can increase the rate of bone resorption and cause an imbalance in the bone remodelling process (Sihombing et al., 2012; Mescher, 2012; Modder et al., 2011; Okman, 2015). Loss of bone tissue is characterized by an increase in the number of osteoclasts (increased resorption) or a decrease in the number of osteoblasts (deficiency of estrogen) (Grabowski, 2015). In animal experiments, ovariectomy was performed as a postmenopause modelling (Yudaniayanti et al., 2019). Nevertheless, osteoporosis therapies such as estrogen supplements and MHT (menopause hormone therapy) have side effects on the cardiovascular system and increase the risk of breast and uterine cancer on long-term use (Geng et al., 2019; Fait, 2019). Therefore, we need other alternative therapies, especially natural compounds with minimal side effects, one of which is propolis that is widely found in nature. Propolis is a natural substance collected by bees from the buds and plant exudates, then mixed with the secretion of saliva and the enzyme β-glycosidase (Segueni et al., 2011). The chemical content of propolis are flavonoids, flavonols, phenolic, flavones, benzoic acid, and its derivatives, such as benzaldehyde derivatives, cynamil alcohol, cinnamic

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acid and its derivatives, tannins, saponins, nicotinic acid, amino acids, esters, vitamins, and minerals (Ishtiaq et al., 2018).

Flavonoids are the main compounds in propolis which are the most studied (Luka et al., 2015). Flavonoids found in propolis are quercetin, routine, galangin, naringenin, isorhamnetin, pinocembrin, pinobanksin, kaempferol, luteolin, apigenin, catechins, daidzein and delphinidin (Ishtiaq et al., 2018; Luka et al., 2015; Kocot et al., 2018). These compounds serve as antioxidants to protect bone tissue and prevent increased bone resorption by reducing bone fragility through reactive oxygen species (ROS) clearance. Moreover, it inhibits pro-inflammatory factors such as nuclear factor-kappa-B (NF-κB) and cyclooxygenase-2 (COX-2), which play a role in osteoclast activation. It also impedes the receptor activator of nuclear factor-kappa-B ligand (RANKL) production and the process of osteoclastogenesis.

Furthermore, flavonoids stimulate osteoblastogenesis by increasing the production of nitric oxide (NO) and osteoprotegerin (OPG). OPG will bind to RANKL and reduce its function for bone resorption (Luka et al., 2015). Meanwhile, NO can prevent osteoporosis by increasing the proliferation and differentiation of osteoblasts and increasing bone mass through the reduction of osteoclasts and thus decreasing bone resorption (Joshua et al., 2014). With these mechanisms, propolis can prevent osteoporosis and could be a candidate for a natural compound to treat estrogen deficiency. Therefore, this research was conducted to see how the protective effect of propolis on osteoporosis treatment caused by menopause by observing bone quality (the ratio of osteoblast and osteoclast cells) and cortical bone thickness of ovariectomized female rats.

**METHODS**

**Materials**

Propolis solution from *Apis mellifera* (150 mg/mL) was purchased from local bee farmers in Solok, West Sumatera, Indonesia. Ketamine (Kimia Farma, Indonesia), alcohol 96% (Sigma Aldrich, Singapore), aqua injection (Otsuka, Indonesia), sterile sodium chloride 0.9% (Otsuka, Indonesia), formaldehyde (Sigma Aldrich, Singapore), sodium carboxymethylcellulose 0.5% (PT. Brataco Indonesia), hematoxylin and eosin (Merck, Germany).

**Ethical Approval**

This research was approved by the Animal Ethics Committee of the Faculty of Medicine, University of Andalas (No. 185 / KEP / FK / 2020).

**Experimental Animals**

This study used 20 female Wistar rats weighing 150-200 grams at three months of age. The rats were acclimatized for seven days. During the acclimatization process, experimental rats were given standard feed and drinking water sufficiently. The ovariectomy procedure was performed on 16 rats. Observations from the ovariectomy procedure were carried out for 24 hours. The experimental rats were randomly divided into five groups: group I, negative control that was only given food and drink; group II, a sham group that was subjected to ovariectomy and was not given propolis; group III, subjected to ovariectomy and was given propolis orally at a dose of 180 mg/kg BW; group IV, subjected to ovariectomy and given propolis orally at a dose of 360 mg/kg BW; and group V, subjected to ovariectomy and given propolis orally at a dose of 720 mg/kg BW. All groups were treated for 30 days.

**Ovariectomy Procedure**

Ovariectomy was performed to make female rats become menopausal (Yudaniayanti et al., 2019) by removing the ovaries of female rats. The experimental rats fasted for six hours. Ketamine 50 mg/kg BW and xylazine 10 mg/kg BW were given intramuscularly (IM) as anaesthesia. The mouse was placed in its supine position, and the hair on the left flank area was shaved with a length of ± 15 cm; then, the shaved area was cleaned with a clean cotton swab and alcohol 70%. Povidone-iodine was rubbed into the surgical incisions. During the surgery, the subcutaneous skin and linea alba from cranial to caudal direction were incised with a length of ± 4 cm, followed by ligation of the ovaries and removal of the left and right ovaries. The cut end of the cervix was inserted back into the abdomen, and the alba line and subcutaneous skin were sewed again. Gentamicin was given to avoid infection and leave one day after the surgery to heal the incision wound.

**Femoral Bone Preparations**

On the 31st day, the rats were sacrificed and dissected to remove the left femur for cortical thickness analysis and bone quality. The steps in making bone preparations were fixation of rats femur bone tissue with 10% formalin, then decalcification in 8% HCl. Furthermore, it was processed into paraffin blocks, cut with a microtome with a thickness of 4 µm, and placed on the slide. The slides were deparaffinized with xylene for 2x5 minutes, then were rehydrated with graded alcohol starting with ethanol 100%, 96%, 70%, and distilled water for 5 minutes per solution. The slides were then stained with hematoxylin for 8 minutes and rinsed with distilled water for 10 minutes. Subsequently, the slides were dehydrated with 70% alcohol for 5 minutes and 96% alcohol for 5 minutes and immersed in eosin solution for 2 minutes.
Then, the slides were rinsed in ethanol 96% and 100% for 5 minutes each. Lastly, the slides were cleared in xylene for 2 x 5 minutes and mounted in the deck glass with entellant for examination under a microscope (Liu et al., 2017).

**Histological Analysis of Bone and Cortical Bone Thickness**
Measurements were made by capturing hematoxylin-eosin slides. The osteoblast and osteoclast cell examination was evaluated using an Olympus BX 51 light microscope at a magnification of 400x. The assessment of cortical bone thickness was evaluated using the Betaview program, Beta 3.1MP Sony Exmor CMOS Sensor camera at 40x magnification. Photomicrographs were taken in a representative area.

**Data Analysis**
The qualitative data was represented as microscopic images of bones tissue. The cortical bone thickness data and the ratio of osteoblast cells and osteoclasts were analyzed using a one-way analysis of variance (ANOVA) to see the relationship with the dose. Duncan’s multiple range test was carried out to see the significant differences between all groups.

**RESULTS**

**The Ratio of Osteoblasts and Osteoclasts Cells**
Based on the results of the histology analysis, as seen in Figure 1, there was a difference in the histological features of the femur. In the negative control group, osteoblasts were seen regularly arranged on the bone surface with few osteoclasts. Both osteoblasts and osteoclasts are small with a small amount of cytoplasm, suggesting a stable bone homeostasis process. The sham treatment showed a bone area with an increased osteoclast population with larger cells and a slight decrease in the osteoblast population. Interestingly, the administration of propolis could affect the population and morphology of osteoblasts and osteoclasts more like negative control rats.

One-way ANOVA statistical test results showed that the treatment group had a significant effect on the ratio of osteoblasts to the osteoclasts in the femoral metaphysis (p<0.05). The average value of the ratio of osteoblasts and osteoclasts in the negative control group had the highest value, about 12.89 J/mm². In contrast, the sham group had an average value of 6.71 J/mm². The results in the treatment groups showed an increase in the average ratio

![Figure 1](image-url)
of the number of osteoblasts to osteoclasts compared to the sham group, with the dose of 360 mg/kg BW gave the greatest increase in the average ratio of osteoblasts to osteoclasts 10.83 J/mm², which statistically have the same subset with the negative group in Duncan’s multiple range test (p>0.05). Meanwhile, the doses of 180 mg/kg BW and 720 mg/kg BW were 7.43 J/mm² and 8.69 J/mm², respectively, as shown in Figure 2.

Cortical Bone Thickness
The cortical bone thickness results were presented in Table 1 and Figure 3. Our data showed that the cortical bone thickness in the negative control group was 201.82 µm and in the sham group was 175.92 µm. Surprisingly, the bone thickness in the treatment groups was not increased linearly as expected following the dose increment, with a dose of 360 mg/kg BW giving a slight decrease with 168.95 µm compared to a dose of 180 mg/kg BW and 720 mg/kg BW that showed 187.42 µm and 207.35 µm, respectively. One-way ANOVA statistical test results showed no effect of propolis administration on the cortical bone thickness of the femoral metaphysis (p>0.05).

DISCUSSION
Our study aimed to evaluate the effect of propolis on the ratio of osteoblasts and osteoclasts in ovariectomized female Wistar white rats. Ovariectomy is a menopausal golden standard in experimental animals as a model for osteoporosis. The ovaries, which are the main part of the producer of estrogen, are removed from the rats to mimic the pathophysiology of humans (Sophocleous and Idris, 2014; Yousefzadeh et al., 2020).

Estrogen is essential for maintaining bone formation. The mechanism in bone metabolism is to reduce apoptosis of mature osteoblast cells (osteocytes), thereby reducing the process of bone turnover (anti-remodelling). This hormone also promotes osteoclast apoptosis escalation and RANKL differentiation reduction, thus resulting in decreasing bone resorption. Moreover, it is necessary to reduce oxidative stress, where oxidative stress reactions play a role in postmenopausal bone loss. In contrast, the loss of estrogen increases the production of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-7 (IL-7), and tumor necrosis factor-alpha (TNFα). TNFα can increase osteoclast formation, and IL-7 can increase osteoclastogenesis, which both of them lead to bone loss (Mödder et al., 2011; Okman, 2015). An elevated number of osteoclasts and a lower number of osteoblasts are signs of bone loss, which subsequently drives a significant change in the architecture of the cortical bone and trabecular bone due to an imbalance in the bone remodeling process (Grabowski, 2015). The ratio of osteoblasts to osteoclasts illustrates the higher number of osteoblasts in the bone formation process as opposed to the number of osteoclasts that are accountable for breakdown cells in the bone resorption process.

Our study showed that the sham group significantly decreased the ratio of osteoblast cells metaphysis femoral cells (p>0.05) compared to the negative control group, suggesting a change in the bone remodeling process dominant bone resorption process. The decrease in estrogen hormone causes a reduction in osteoclast apoptosis and promotes the differentiation of RANKL induction. RANKL will bind to the receptor activator of nuclear factor-kappa-B (RANK) and stimulate osteoclast
Table 1. Effect of propolis on cortical bone thickness of femoral metaphysis

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Cortical Bone Thickness (µm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>201.82 ± 21.31</td>
</tr>
<tr>
<td>Sham</td>
<td>175.92 ± 13.24</td>
</tr>
<tr>
<td>Dose 180 mg/kg BW</td>
<td>187.42 ± 4.90</td>
</tr>
<tr>
<td>Dose 360 mg/kg BW</td>
<td>168.95 ± 5.02</td>
</tr>
<tr>
<td>Dose 720 mg/kg BW</td>
<td>207.35 ± 36.40</td>
</tr>
</tbody>
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Figure 3. Femur bone thickness in the metaphysical area of experimental rats. (a) negative control group, (b) sham group (ovariectomy control), (c) treatment group with propolis at dose 180 mg/kg BW, (d) treatment group with propolis at dose 360 mg/kg BW, (e) treatment group with propolis at dose 720 mg/kg BW. Bone cortex (t), Medullary cavity (m), connective tissue and muscles outside the bone (o).

As expected, the results in the treatment groups showed an increase in the average ratio of osteoblasts to osteoclasts compared to the sham group. It indicates an increase in forming new bone compared to the resorption process due to an increase in osteoblasts and a decrease in osteoclasts. Surprisingly, the dose of 360 mg/kg BW gave the most significant increase, which amounted to 10.83 J/mm². The ratio of osteoblasts and osteoclasts at this dose is close to the ratio in the negative control group, thus making the most optimal dose to increase the ratio of osteoblasts and osteoclasts. On the other hand, the doses of 180 mg/kg BW and 720 mg/kg BW were much lower, which accounted for 7.43 J/mm² and 8.69 J/mm², respectively. One of the possible mechanisms of the propolis effect could be the ability to improve fracture healing by clearing up free oxygen radicals or ROS. The antioxidant properties of propolis as exogenous antioxidants may aid the endogenous
connections. Endocortical and trabecular resorption also results in fewer endocortical and trabecular bone resorption. Trabecular bone resorption caused by ovariectomy sometimes occurs due to high-level free oxygen radicals, conditions such as infection, inflammation, and trauma (Guney et al., 2011). Antioxidants can overcome tissue damage and play a role in bone formation by preventing ROS activity. ROS increases osteoclast formation and activity and, together with TNF-α, suppresses osteoblast differentiation. Therefore, the inhibition of ROS is necessary to reduce bone resorption. Consequently, the number and activity of osteoclasts decrease while the osteoblast differentiation process continues (Luka et al., 2015; Darmadi and Mustamsir, 2016).

Apart from the antioxidant mechanism, quercetin (one of the flavonoid compounds) could suppress bone resorption by inhibiting osteoclast differentiation and activation. Quercetin suppresses the activation of the transcription factors NFkB and activator protein-1 (AP-1). These transcription factors play a role in modulating osteoclast differentiation, survival, and activation to decrease osteoclast formation. Quercetin also inhibits osteoclast formation by acting on osteoclast progenitors (inhibiting preosteoclast tartrate-resistant acid phosphatase (TRAP) activity). In addition, propolis can stimulate osteoblastogenesis and thus increase the formation of OPG and NO. OPG is an anti-osteoclastogenesis similar to RANKL, and NO can increase the proliferation and differentiation of osteoblasts and reduce osteoclasts (Luka et al., 2015; Joshua et al., 2014).

Interestingly, we found that the cortical bone thickness of the femoral metaphysis gave no significant difference between treatment groups (p> 0.05). We assumed it was due to a short period of experimental design, which was only 30 days of treatment after ovariectomy. The earliest change in the width of the cortical femoral bone could be seen between 90 and 120 days after ovariectomy and takes 180 days or more to reach a steady state. Even though Pei-Yu Hsu et al. research in 2016 reported a decrease in cortical thickness at the femoral neck three months after ovariectomy, this result was not statistically significant (Hsu et al., 2016). Cortical bone has an outer surface called the periosteal and an inner surface called the endosteal. The periosteum is a sheath of fibrous connective tissue that surrounds the outer surface of the cortical bone. It contains blood vessels, nerve fibers, osteoblasts, and osteoclasts. The periosteum also protects, nourishes, and aids the process of bone formation. Therefore, the periosteum plays a vital role in the growth and repair of fractures.

In addition, the decrease in cortical bone thickness caused by ovariectomy sometimes occurs due to high-endocortical resorption. Trabecular bone resorption also results in fewer endocortical and trabecular connections. Endocortical and trabecular resorption would eventually lead to loss of endocortical and trabecular connections, where this connectivity plays an essential role in bone strength. This process is due to the deficiency of estrogen, which increases the resorption process beyond the formation process. However, these changes usually occur 2-3 months after ovariectomy (Hsu et al., 2016). Therefore, further study needs to be considered by extending the observation period to see the actual decrease of bone thickness by estrogen deficiency, which mimics osteoporosis progression in menopausal women. Since we only investigated the histological analysis and the cortical thickness of the femoral bone, the following study, including scanning electron microscopy (SEM) examination, in vitro assay, more specimens, power tests, and in vivo scans, need to be taken to assess osteoclastogenesis, bone microarchitecture, and morphology. If these limitations are resolved in the future, the mechanism of propolis in preventing osteoporosis in menopausal women could be fully elucidated, and we could further extend this potent effect by confirming it on the human body.

CONCLUSION

The administration of propolis orally at a dose of 180 mg/kg BW, 360 mg/kg BW, and 720 mg/kg BW had an effect in decreasing the ratio of osteoblasts and metaphysical osteoclast cells of femoral metaphysics in ovariectomized female Wistar white rats as menopause modelling. Propolis at dose 360 mg/kg BW gave the highest ratio of osteoblasts and osteoclasts, which is statistically the same with the negative control group. However, propolis administration did not affect the thickness of the femoral metaphysical cortical bone.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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