EFFECT OF CACAO SEED ETHANOL EXTRACT (THEOBROMA CACAO L.) ON OSTEOCYTE COUNT OF ALVEOLAR MANDIBLE BONE GIVEN TO POST-OVARIECTOMIZED RAT

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Abstract

Purpose: Estrogen is an essential hormone in human, especially on female that can keep the physiologic balance. Women with menopause are lack of estrogen that can lead post-menopausal osteoporosis and loss of bones. Bone loss occurs due to increase of osteoclast level and apoptosis of osteocyte that lead to bone resorption. Cacao seed contains polyphenol substances such as flavonoid, catechin and procyanidins. The aim of this study is to evaluate the effect of cacao seed ethanol extract on osteocyte of mandibular alveolar bone in postmenopausal osteoporosis using the ovariectomized rat model.

Methods: A Post-Control Group Design Experiment using 25 randomized female rats (Rattus norvegicus) divided into 5 different groups which is: normal group (negative control); 60 day old post-ovariectomy group (positive control); 60 day old post-ovariectomy group given cacao ethanol seed extract at the end of the day 30th with different dose respectively (p1 = 125 mg/kgBW, p2 = 250 mg/kgBW, p3 = 500 mg/kgBW) for another 30 day. A histologic view was used to count osteocytes of alveolar mandible bone.

Results: The result showed that there are significant difference between positive control, P2 with P3 and a strong correlation between extract of cacao given to rat and a increasing level of osteocytes (p < 0.05, correlation coefficient = 0.555) on mandibular bone after ovariectomy surgery.

Conclusion: This study concludes that cacao seed ethanol extract can increase mandibular osteocytes count of a post-ovariectomy rat.

Keywords: Alveolar mandible; Cacao seed; estrogen; osteocyte; ovariectomy

Introduction

During menopause, which usually occurs to women at the age of forty, there is a steady decrease in the size of ovary that can lead to some health problems (1). The problems that could arise from menopause include post-menopausal osteoporosis and the loss of periodontal tissues which function is to support the teeth (1,2).

Post-menopausal osteoporosis is a disease that causes a decrease in the bone mass and usually occurs on vertebrae and long bones because of estrogenic deficiency. At the process of post-menopausal osteoporosis, there is an imbalance in bones remodeling in which the creation of osteocytes by osteoblasts is lower than the tearing down of osteocytes by osteoclasts. If the imbalance goes on for long, it will lead to a bone loss (3).

During menopause, women also lose the protective effects of estrogen in jawbones (4). Research has shown that during menopause and post-menopausal osteoporosis, there is a decrease in the density of jawbones which is followed by an increase in progressive resorption of residual ridge and alveolar bone (5). This fact can be seen from ovariectomized rat in which a disappearing of alveolar bone is (6). However, we still cannot verify the mechanism of relationship between post-menopausal osteoporosis and the process of alveolar bone loss, which interests many researchers in the field of dental medical knowledge.

Cacao seeds are rich with phenolic compounds such as catechins (around 37%), proanthocyanidines (around 58%), phenolate acid, theanine, and other flavanoids (7). It is known that the rate of polyphenol per cacao serving size is higher compared with green tea which is due to the fact that people consume more serving size of cacao rather than green tea (8).

Polyphenol is known to not only help in preventing heart diseases and cancer, but also osteoporosis since its potency as antioxidant and abilities in regulating cytokines in inflammation responses (8).

Therefore, the present study aimed to evaluate the effect of cacao seed ethanol extract on osteocyte of mandibular alveolar bone in postmenopausal osteoporosis using the ovariectomized rat model.
Materials and Methods

This research was done at the Laboratory of Pharmacology, Laboratory of Anatomy-Histology, and Laboratory of Pathology-Anatomy in Medical Faculty, Universitas Brawijaya, Malang, Indonesia from February until May 2012.

Dose of polyphenol for repairing alveolar mandibular bone is not yet known, thus we are using serving size of human consumption in hope that per serving size of cacao for human can cause therapy effect. The administration of dosage is based on the amount of polyphenol in cacao serving size of 7.3 gram, which is around 1166 ppm (8). Serving of 7.3 gram cacao in the usual conversion of Indonesian diet is 4 gram (9). After that, we calculate t per serving with a conversion to rat by using NutriSurvey formula (10).

4000 mg cacao x 0.0128 = 51.2 mg cacao (rounded to 50 mg)

From the calculation above we take the lowest serving which is 25 mg/200 grBW as serving I; middle serving at 50 mg/200 grBW; and highest serving at 100 mg/200 grBW. The format is then converted to mg/kgBW, thus, serving I at 125 mg/kgBW, serving II at 250 mg/kgBW, and serving III at 500 mg/kgBW.

25 randomized female Wistar Rats (Rattus norvegicus), aged 3 months, weight 150-200 gram, were divided into 5 groups, each consisting of 5 rats. Group K(-) is the negative control group which has not received any treatment and is the normal control group in this research. Group K(+) is the positive control group where ovariectomy is done on the rats and waited for 30 days. During 30 days, the rats were not given ethanol extracts of cacao seeds. Group P1 is the group of rats with ovariectomy and Waited for 30 days. After that, rats are given ethanolic extracts of cacao seeds at 125 mg/kgBW for 30 days. Group P2 is the group of rats that have been ovariectomized for 30 days, and then given ethanol extracts of cacao seeds at 250 mg/kgBW for 30 days. Group P3 is the group of rats that have been previously ovariectomized for 30 days and given ethanol extract of cacao seed 500mg/kgBW for 30 days.

Cacao seeds were obtained from PT. PTPN XII Blitar, East Java, Indonesia, in the form of coarse dry cocoa powder and re-milled for a finer powder. Ethanol extraction was done using maceration method, filtered using ethanol and concentrated using rotary evaporator. Results of ethanolic extract are in the form of pasta which should be mixed with aqua dest to make 3 different dose. Delivery of ethanol extract of cacao using a modified syringe which can be mounted sonde at the end of it, and can be inserted through the mouth to the stomach of rats.

Ovariectomy of rats was done under anesthesia, the rats were injected with ketamine (Ketamine Hydrochloride Injection, Bioniche Pharma, Ireland) (20 mg/BW), the furs that covering surgical site were shaved approximately 1 cm above the ovary imaginary line. Surgical site were sterilized using povidone-iode (Betadine, Mahakam Beta Farma, Indonesia). Incision was done using sharp scalpel and both right and left ovaries were taken. Incision was sutured using catgut thread (Catgut Chrom, B. Braun, Germany), smeared with another povidone-iode and neomycin-bacitracin powder (Nebacetin, Pharos, Indonesia) and dressed with sterile gauge. Then, rats were injected intramuscularly with Metamizole Na (Novalgin, Sanofi Aventis, Indonesia) (20mg/kgBW) directly after surgical procedure, and were injected with gentamicin sulfate IM (Gentamicin, B. Braun, Germany) (60mg/kgBW) for the following 3 days after surgery.

After completing 60 days of experiment, all rats were sacrificed using ether and mandibular bone of each rats were resected, fixed in 10% neutral buffer formalin, decalcified with HCl 6% for 1 week, embedded in paraaffin, sectioned at 4 μm thickness. The sections were stained with Hematoxylin-Eosin and photos of each section were taken under modified light microscopy (Olympus Photo Slide BX51, Olympus, Japan). Quantitative count of osteocyte was performed using Cell Count for Windows (freeware).

Data analysis for the amount of osteocytes in alveolar mandibular bone of rats post-ovariectomy was done using SPSS19 for Windows (SPSS Inc.). Tests which were used consists of One Way ANOVA, Post-Hoc LSD, and Pearson correlation test.

Results

1. Osteocytes Calculation Result

The calculation of osteocytes is done under microscope with 400x enlargement from four views, supported with Cell Count for Windows application, to count the total and average of osteocytes per view. The calculation result of osteocytes in rat mandible (Rattus norvegicus) control and measurement can be seen in Table 1. The post hoc statistics test showed a significant difference between K(-) and K(+) groups (p = 0.006). There was no significant difference between P1 and K(+) groups (p = 0.059). Post-hoc test showed a significant difference between P2 and K(+) groups (p = 0.027). A significant difference was found between P3 and K(+) groups (p = 0.007).

The correlation analysis result shows that there is a strong (r=0.555) and significant (p = 0.006) correlation regarding the increased dosage of ethanol extract of cacao seed with the number of osteocytes in rats. The correlation is positive, which means the bigger the serving or dosage of ethanol extract of cacao seed given, the more osteocytes that the rats will have.

2. Histologic Observation Result

Histologic observation result in group K(-) shows osteocytes seen inside lacuna and osteocytes are spread evenly in microscope slides. This can be seen in Figure 1.

In group K(+), (Figure 2) the number of osteocytes can be seen sparse and less than that in K(-). The intercellular tissue of group K(+) is darker than group K(-). This is an effect of the ovariectomy on group K(+).

Group P1, in figure 3, shows the histologic view of the jawbone 60 days after it was ovariectomized and given ethanol extract of cacao
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Group P1, in figure 3, shows the histologic view of the jawbone 60 days after it was ovariectomized and given ethanol extract of cacao seed. Compared with group K(+), the number of osteocytes is higher and are spread evenly. The intercellular tissue of group P1 looks better than that of group K(+).

In Figure 4, group P2 is proved to have a higher number of osteocytes and an even spread. The intercellular tissue of group P2 looks better than that of K(+). This happens because group P2 has been given higher dose (250 mg/kgBW) of ethanol extract of cacao seed than what was given to group P1.

In figure 5, group P3 has more osteocytes and are spread more evenly compared to group K(-). The intercellular tissue is also better than group K(-). This is because group P3 has been given cacao seed therapy of 500mg/kgBW.

Discussion

This research is conducted with an aim to acknowledge the effects of ethanol extracts of cacao seed towards the number of osteocytes in alveolar mandibular bones of ovariectomized rats.

According to the research, the average count of osteocytes of group K(-) stands at 99.6 and has become a normal standard of the numbers of osteocytes in alveolar bones in this research. The histologic observation of group K(-) shows that osteocytes are seen inside the lacuna, and they are spread evenly on the microscope slides as seen on Figure 1. This suits with Harjana, which argues that matured osteocytes are located in the lacuna (11).

The alteration of osteocytes are seen clearly in group K(+), which is the group of rats that were ovariectomized and 60 days later their alveolar bones were observed under microscopes. The result of an average of 57.85 osteocytes shows a decline compared to the average of group K(-). Histologic observation shows a less number of osteocytes compared to group K(-) and a sparse spread. Intercellular tissues of group K(+) looks darker than group K(-) (Figure 2). The post hoc statistics test between K(-) and K(+) showed a significant difference between those groups. This situation matches with the research of Ejiri and Yang, where ovariectomized rats go through a decrease in jawbones osteocytes, like post-menopause (5,6). In addition, both Tanaka and Hekimsoy also mentioned that the loss of estrogen after ovariectomy causes the bone resorption surpasses the bone creation (12,13). The loss of estrogen inducts osteoclastogenesis and inducted an apoptosis of osteocytes. The decrease of estrogen causes increase in oxidative stress so that osteoblasts fall, which in turn fast-forwards bone destruction (14). Women who have gone through menopause also show an increase in proinflammatory cytokines concentration such as TNF- α, IL-1, and IL-6 (15).
This increase stimulates differentiation and maturation of osteoclasts, increasing bone resorption (16).

Group P1 is the group of rats that had been ovariectomized for 30 days and were given ethanol extracts of cacao seeds at 125 mg/kgBW for 30 days. The average osteocytes count at 85.2 is higher compared to that of the positive control group. Result of histologic observation shows the osteocytes rate to be higher than the negative control. Interacellular tissues of P1 look better than K(+) (Figure 3). Post-hoc statistics test between P1 and K(+) showed no significant difference between the two groups even though the average osteocytes of P1 is higher than that of K(-). This shows that the administration of ethanol extracts from cacao seeds on this group is not yet significant.

In group P2, which is the group of rats that have been ovariectomized for 30 days and then given ethanol extracts of cacao seeds at 250 mg/kgBW for 30 days. The average osteocytes of P2 at 90.5 shows significant increase compared to that of K(-). Result of histologic observation shows higher osteocytes than P1. Interacellular tissues of P2 looks better than K(+) (Figure 4). Post-hoc test between P2 and K(+) showed significant difference between those groups and goes towards the normal state. This matches the theory that says ethanol extract of cacao seeds contain polyphenol substances that are known to prevent bone destruction caused by post-ovariectomy osteoporosis because the potential antioxidants and anti-inflammatory activities (8). Flavonoid (procyanidins) in cacao is also known able to regulate proinflammatory cytokines in inflammation responses (8). Catechin types of polyphenol also prevents the production of TNF-α, IL-1 and IL-6 (proinflammatory cytokine) through the process of intracellular persecution from level ERK1/2 and NF-xB activation as well as reduction of oxidative stress with a persecuting activation mechanism of Nuclear Factor-xB (NF-xB), which is a transcription factor that is used to induce iNOS (inducible Nitric Oxide synthase) (17).

An optimal result was observed in the group P3, which is the group of rats that have been previously ovariectomized for 30 days and given ethanol extract of cacao seed 500mg/kgBW for 30 days. The calculation result of P3 osteocytes of 98.9 shows that there was a significant increase compared to group K(-) and almost levels up with the average osteocyte number on normal state. Histologic observation shows that the number of osteocytes is higher than that of group P2. Interacellular tissues of group P3 looks better than that of group K(+) (Figure 5). Post hoc statistic test between P3 and K(+) showed that there is a significant difference between those two groups.

The correlation analysis showed that the bigger the serving or dosage of ethanol extract of cacao seed given, the more osteocytes that the rats will have. This result strengthens the hypothesis of the research which says that the ethanol extract of cacao seed servings can increase the count of osteocytes. In the serving of ethanol extract of cacao seed at 250mg/kgBW, the cacao extract has shown an increase in the number of osteocytes. In the serving of ethanol extract of cacao seed at 500mg/kgBW, the cacao extract has been able to increase the number of osteocytes up until it almost reaches the normal state. Other factor that should be taken into account in the present study is that the microscope slides results that are rather unsatisfying.

This is due to the decalcification method that used HCI 6% which acts as an aggressive decalcification.

Further studies are required to focus on cacao processing method that can be used in human diet to eliminate ther contents that are not needed or the ones that influence the effect of cacao therapy.

Based on the explanation above, it seems that the serving of ethanol extract of cacao seed can increase the number of osteocytes in alveolar mandible bones in post-ovariectomized rats which can be due to the polyphenol’s mechanism that works as an anti-inflammatory as well as oxidative stress reducing agent. This mechanism might increase the number of osteocytes in the reaction of the destruction of alveolar mandible bones. In other words, it can be said that cacao polyphenol can cause an increase in the number of osteocytes.

**Conclusion**

According to the present results it can be concluded that:

1. There is a decreased number of osteocytes in alveolar mandible bones in post-ovariectomy wistar rats (Rattus novergicus)
2. There is an increase of the number of osteocytes in alveolar mandible bones in post-ovariectomy rats (Rattus novergicus) after given ethanol extract of cacao seed.
3. The dose of ethanol extract of cacao seed that can increase the number of osteocytes in post-ovariectomy wistar rats (Rattus novergicus) to the almost normal state is at 500mg/kgBW.
4. The correlation between the dose of ethanol extract of cacao seed (Theobroma cacao L) with the number of osteocytes in wistar rats is positive, quite strong and significant which means the more dose of ethanol cacao seed extract is given, the more number of osteocytes is alveolar mandible bones the rats will present.

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![Figure 5. microscopic view on alveolar bones of group P3 with 400x enlargement, Hematoxylin - Eosin staining](image)
Table 1. Calculation results of the number of osteocytes in alveolar mandible bones in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Rats</th>
<th>Average</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>K(-)</td>
<td>75,5</td>
<td>99,5</td>
<td>77,5</td>
</tr>
<tr>
<td>K(+)</td>
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<td>44,5</td>
<td>44,5</td>
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<tr>
<td>P 1</td>
<td>76</td>
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<td>78,75</td>
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<td>81,75</td>
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<td>P 3</td>
<td>121,75</td>
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References