The Effect of Cacao Bean Extract on the Number of Osteoblasts on Orthodontic Tooth Movement

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Abstract

The teeth that are given orthodontic forces, generally occur relapses. It is caused by resorption on the pressure side higher than the apposition on tension side. Cacao bean extract can increase bone apposition on bone remodeling through osteoblast proliferation.

This study aimed to determine the effect of extract cacao beans on the number of osteoblasts on orthodontic tooth movement.

This research uses an experimental laboratory posttest-only control group design. The extract comes from unfermented bulk cacao varieties. A strength of 10gf was measured using a tension gauge to move the upper molars in a mesial direction. 36 Wistar rats were decapitated after 7 days and 14 days of treatment. Immunohistochemical staining to show the expression of TGF-β, TNF-α, OPG, and RANKL by counting the number of expressed osteoblasts on tension side.

Compared with the positive control group, osteoblasts that are expressed by TGF-β, TNF-α, OPG, and RANKL were significantly ($p<0.05$) higher in the treatment group. The application of cacao bean extract of bulk variety on days 7 and 14 could significantly increase osteoblasts that are expressed by TGF-β, TNF-α, OPG, and RANKL in the treatment group than the positive control group on the alveolar bone.

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Introduction

The movement of orthodontic teeth is a complicated mechanical and biological process that involves many different molecules[1,2]. When the tooth gets orthodontically strengthened, it will experience a process of pressure by the osteoclasts, and the other side will experience the tension of the osteoblast, this is called the remodeling process. If the teeth are given optimal strength, the facts shown are on the side of the pressure that experiences excessive resorption, this will disrupt the balance of the bone remodeling process[3,4]. Therefore, herbal ingredients are needed that can balance bone remodeling. One of these ingredients is cacao beans (*Theobroma cacao L*) [5–7].

One of the ingredients of cacao bean extract and the most abundant is polyphenols, which can differentiate osteoblast cells. Teeth that are given orthodontic strength and given materials containing polyphenols, these polyphenols will stimulate the differentiation and proliferation of active osteoblast-progenitors, and bone apposition by activating macrophages which release the cytokine TGF 1 [8–10]. In addition, Receptor Activator of Nuclear Kappa Ligand (RANKL) will bind to the Receptor Activator of Nuclear Factor Kappa (RANK) to stimulate differentiation and activation of osteoclasts [11]. Osteoprotegerin (OPG) is a membrane that surrounds and secretes a protein attached to RANKL to inhibit its role on the RANKL receptor [12,13].

Bone resorption occurs due to the activity of osteoclasts, which are hematopoietic stem cells (HSCs) derived bone cells, also known as monocytes[14]. Due to orthodontic mechanical

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forces, inflammatory cells will be activated and produce proinflammatory cytokines including interleukin-1, tumor necrosis factor-alpha (TNF-α) and trigger osteoclast differentiation by receptor activator NF-kβ and ligands (RANK and RANKL) and osteoprotegerin (OPG), then stimulates alveolar bone resorption [15–18]. OPG is a RANKL decoy receptor that can inhibit RANKL-RANK binding so that osteoclast differentiation does not occur but osteogenesis occurs [19,20].

Orthodontic mechanical forces result in an imbalance between osteoclast and osteoblast activity, caused by an inflammatory process and an increase in oxidative stress [21]. This condition is portrayed by enhanced bone resorption and the occurrence of apoptosis of osteoblasts and osteocytes that support osteoclastogenesis [22,23]. As a result of excessive resorption, there can be an imbalance in the remodeling process. Therefore, antioxidants are needed to reduce bone resorption activity during orthodontic treatment [24,25].

One source of natural antioxidants that contain polyphenols is cacao beans (*Theobroma cacao L.*). Cacao beans that have not been fermented contain a variety of polyphenols [26,27]. Raw cacao beans contain flavanol monomers (epicatechin and catechin) and procyanidin oligomers, which are approximately 60% of the total polyphenols [28,29].

Based on the description above, the formulation of the problem involves determining whether or not (*Theobroma cacao L.*) can affect TGF-β, TNF-α, OPG, and RANKL expression on orthodontic tooth movement in the alveolar bone. This research aims to determine how orthodontic tooth movement in the alveolar bone was affected by the expression of TGF-β, TNF-α, OPG, and RANKL by the application of cacao bean extract (*Theobroma cacao L.*).

Materials and methods

Wistar Rat with Inflammation Induce by Orthodontic Tooth Movement

Thirty-six male Wistar rats aged 12-16 weeks, ranging in weight from 0.2 to 0.3 kilograms, were divided into the following category: experiment and control. The rats, which were in good health, were divided into six categories and given the names and characteristics outlined below:

Group with 7-days control (**K-7**): represented by rats R1, R2, R3, R4, and R5, which were sacrificed after seven days given orthodontic movement or not, and were not receiving cacao bean extract treatment.

Group with 14-days control (**K-14**): represented by rats R6, R7, R8, R9, and R10, which were sacrificed after fourteen days given orthodontic movement or not, and were not receiving cacao bean extract treatment.

Group with 7-days negative control (**K+7**): represented by rats R1, R2, R3, R4, and R5, which were sacrificed after seven days given orthodontic movement or not, and were not receiving cacao bean extract treatment.

Group with 14-days negative control (**K+14**): represented by rats R6, R7, R8, R9, and R10, which were sacrificed after fourteen days given orthodontic movement or not, and were not receiving cacao bean extract treatment.

Group with 7-days treatment (**P7**): represented by rats T1, T2, T3, T4, and T5, which were sacrificed after seven days given orthodontic movement, and were receiving cacao bean extract treatment.

Group with 14-days treatment (**P14**): represented by rats T6, T7, T8, T9, and T10, which were sacrificed after fourteen days given orthodontic movement, and were receiving cacao bean extract treatment.

A tension gauge was used to measure the distal direction of 10 grams of orthodontic force to the maxillary molar of male Wistar rats using using NiTi closed coil spring, Ormco® Glendora, USA. The Research Ethics Committee of the State University of Jember Dentistry Faculty approved the research protocol (No.1728/UN25.8/KEPK/DL/2022).

The process of making cacao bean extract comes from unfermented bulk (lindak) cacao varieties taken from the PTPN X Kertosari plantation, Jember, Indonesia.

Immunohistochemical Procedures

Formalin was used to fix the maxilaries, which were dissected, and EDTA was used to decalcify them. Paraffin-embedded and dehydrated samples were used. TGF, TNF, OPG, and RANKL were stained on tissue areas which under 180 μm from the distal root's furcation of the maxillary molar. After dewaxing, the sections were treated with antigen retrieval, blocked for endogenous hydrogen peroxidase,

and incubated with TGF, TNF, OPG, and RANKL secondary antibodies.

Statistical Analysis

The expressions (TGF-β, TNF-α, OPG, and RANKL) in the tension area were distinct from each group. The Kolmogorov-Smirnov test was used to demonstrate that the data were normalized ($p>0.05$). Then one-way Anova analysis was used to know if there was a significant effect on expression; if $p<0.05$, the results are significant.

Immunohistochemical Evaluation

This observation was examined Immunohistochemically, and a digital camera was used to take pictures of the area.

Results

Description of Research Data

Thirty-six male Wistar rats aged 12-16 weeks were used in this experimental study, which was classified as follows:

(**K-**) with an absence of treatment and movement of the maxillary molar tooth;
(**K+**) with maxillary molar tooth movement but not receiving treatment;
(**P**) with maxillary molar tooth movement and treatment by cacao bean extract. On days 7 and 14, the results of each group were examined.

Group Experiment	K-7 Mean ± SD	K-14 Mean ± SD	K+7 Mean ± SD	K+14 Mean ± SD	P7 Mean ± SD	P14 Mean ± SD	F	P
Expression of TGF-β	2.75±0.816	3±0.816	3±0.957	2.75±0.957	8.5±1.290	12.75±0.957	118.623	0.000
Expression of TNF-α	3±0.816	3.25±0.957	8±0.957	8±0.957	6.75±0.816	3±0.957		
Expression of OPG	4.75±0.816	5.05±0.957	4.05±0.957	5.1±0.957	5.15±0.816	7.8±0.957		
Expression of RANKL	4.75±0.816	5.05±0.957	4.8±0.957	7.77 ±0.957	8±0.816	9.15±0.957		

Table 1. Description of variable data of osteoblasts through the expressions of TGF-β, TNF-α, OPG, and RANKL.

The results of the one-way ANOVA analysis show a significant difference ($p<0.05$) between the negative control group (K-), positive control group (K+), as well as the treatment group (P). It showed that on the tension side, the immunohistochemistry had a positive reaction to TGF-β, TNF-α, OPG, and RANKL. The results expression's mean and standard deviation (**SD**) are shown in Table 1.

The results on day 7 indicate that applying cacao bean extract to the tension area can significantly increase the mean expression of TGF-β, OPG, and RANKL in osteoblast cells in comparison to (K-) and (K+), but decrease the mean expression of TNF-α in osteoblast cells in comparison to (K+) but higher than (K-).

The results on day 14 indicate that applying cacao bean extract to the tension area can significantly increase the mean expression of TGF-β, OPG, and RANKL in osteoblast cells in comparison to (K-) and (K+), but decrease the mean expression of TNF-α in osteoblast cells in comparison to (K-) and (K+).

This study's statistical analysis necessitates a normal distribution of the data. The homogeneity of the tested data was examined first using Levene Test.

The TGF-β, TNF-α, OPG, and RANKL's expressions of groups fulfilled the assumption of homogeneity, with the value of 0.080. Then the data for each variable was assessed by normality test. The normal distribution using QQ Plot for data analysis of TGF-β, TNF-α, OPG, and RANKL expressions from each variable shows in Figure 4 presents that the scatterplot forms both a straight and diagonal line, meaning that the data assumption of TGF-β, TNF-α, OPG, and RANKL expression by immunohistochemical staining is normal. Then subsequent statistical tests can proceed, namely the one-way ANOVA analysis.

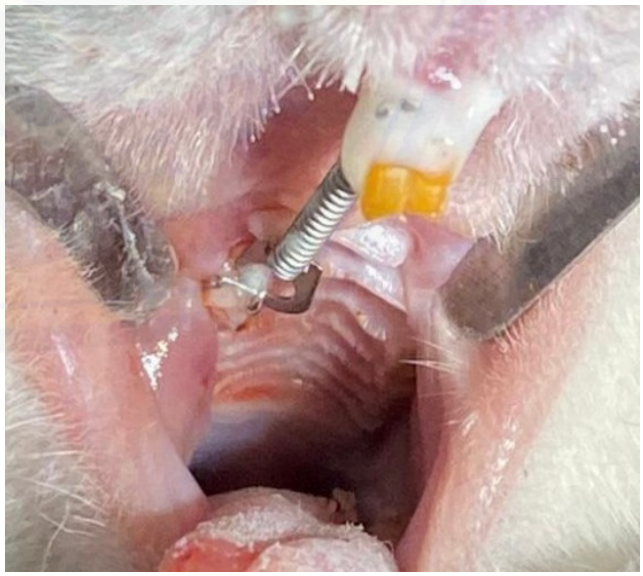

Figure 1. The model of orthodontic tooth movement using NiTi closed coil spring.

Various tests of variables between groups

The calculation of the immunohistochemistry results in osteoblast cells that exhibit positive results for TGF-β, TNF-α, OPG, and RANKL expression by immunohistochemistry staining is based on different test variables for each group in this study.

A one-way ANOVA analysis is used to conduct various variables tests between groups for TGF-β, TNF-α, OPG, and RANKL. Based on the results of the distribution normality test, Wilk's lambda is used to assess the data statistically; a value of 0.000, or ($p<0.05$), is obtained. This indicates that the groups have different effects.

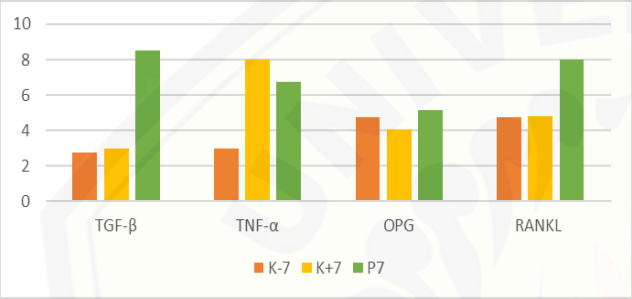

Figure 2. Average expressions of TGF-β, TNF-α, OPG, and RANKL on day 7.

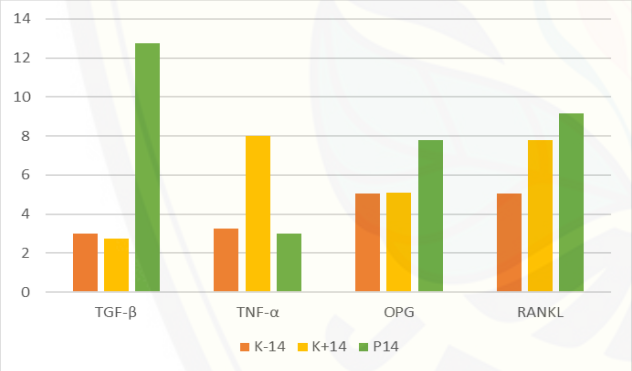

Figure 3. Average expressions of TGF-β, TNF-α, OPG, and RANKL on day 14.

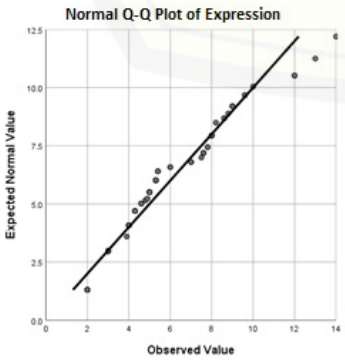

Figure 4. Data of normal distribution using test results of Plot Q-Q.

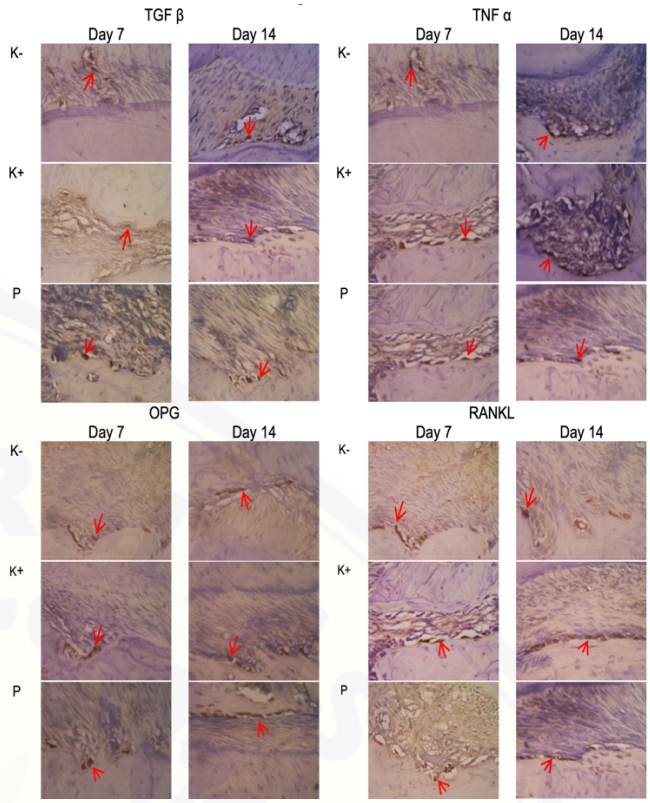

Figure 5. Osteoblast celss through the expression of TGF-β, TNF-α, OPG, and RANKL on day 7 and 14, with 400x magnification.

Discussion

Experimental studies on the expression of TGF-β, TNF-α, OPG, and RANKL in orthodontic tooth movement following the application of cacao bean extract (*Theobroma cacao L.*) conducted to the maxillary molar teeth in Wistar rats with an optimal orthodontic force of 10 g/cm^2.

The data is homogeneous, with a normal distribution for each group. This is demonstrated by the significance level of $p=0.080$ ($p> 0.05$), so the subsequent statistical test can proceed.

Optimal orthodontic force as the lightest force produces the most rapid tooth movement with the least tissue damage [30,31]. It gives varieties of reaction to the surrounding tissue of maxillary molar teeth. Neurotransmitter gene expression, signaling molecules, extracellular matrix components, and growth factors are involved in the balance between alveolar bone resorptions and deposition of new bone in orthodontic tooth movement. Tooth movement resulting in periodontal ligament pressure in some areas and tension in others. Optimal orthodontic force of movement reduces blood

flow, initiate osteoclast activation and further tissue resorption. Native paradental cells, leukocytes and platelets release proinflammatory factors such as TNF-α. TNF-α stimulate RANK through osteoclast to support resorption process [32]. The results on the tension area present that TNF-α expression on the treatment group (P) is lower compare to control positive (K+) but higher than control negative group (K-). It means that inflammatory process does exist by given an orthodontic movement to maxillary molar teeth and decrease after application of cacao bean extract. It implies that cacao bean extract able to inhibit the inflammatory process.

In tension area, alveolar bone deposition predominates, with an increase in osteoblast number. Tensile strain stimulates osteoblast progenitor proliferation in the periodontal ligament, induce cytokine IL-10, and boosting OPG. Osteoblast stimulate RANKL expression that will interact with RANK and induce resorption process. Osteoblast also produce OPG, a reseptor compete with RANK to bind to RANKL and inhibits its interaction with RANK so the process of osteoclastogenesis is inhibited (Alhasyimi & Rosyida, 2019). Osteoblast release osteocalcin and alkaline phospatase (ALP), ALP support mineralization to form collagen type 1, increasing bone formation. This theory is in line with the results that showed TGF-β, OPG, and RANKL had higher expression on the treatment group (P) compare to control positive (K+) and control negative group (K-). Osteoblasts in the treatment group experienced an increase due to the presence of antioxidant compounds in cacao beans. Cacao beans contain large amounts of polyphenolic compounds, whereas, in various studies, polyphenolic compounds can increase the number of osteoblasts. Polyphenols play a role in osterix and Runx2 expression. Runx2 will stimulate the differentiation of mesenchymal stem cells into osteoblast progenitors and then stimulate osterix to induce the differentiation of osteoblast progenitors into osteoblasts. During bone remodeling, antioxidants present in cacao are needed to increase osteoblast differentiation so that bone mineralization occurs quickly, and osteoclast effectiveness is reduced so that bone resorption is not too high [22,33].

Figure 1, 2 and 3 shows there is a significant difference in TGF-β expression between (P), (K+), and (K-). The application of cocoa bean extract was observed on days 7 and 14 and showed an increase of TGF-β expression. TGF-β is a part of the growth factor that can regulate cell growth, proliferation, and differentiation to maintain the homeostatic tissue as well as the process of wound healing. TGF-β may control the activity of collagen type 1, maintain osteoblast precursors, and enhance matrix protein in bone formation. This is due to the fact that cacao bean extract can enhance osteoblast, leading to an increased of TGF-β[1].

Conclusions

The application of cacao bean extract (*Theobroma cacao L.*) of lindak variety could significantly increase the expression of TGF-β, TNF-α, OPG, and RANKL in (P) compared to (K+) on the alveolar bone in the area of tooth tension in Wistar rats on orthodontic tooth movement.

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Ethical Approval

This experimental study has received ethical approval from the Ethics Committee of the Faculty of Dentistry Jember Universitas Jember No.1728/UN25.8/KEPK/DL/2022.

Declaration of Interest

The authors declare no conflict of interest.

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