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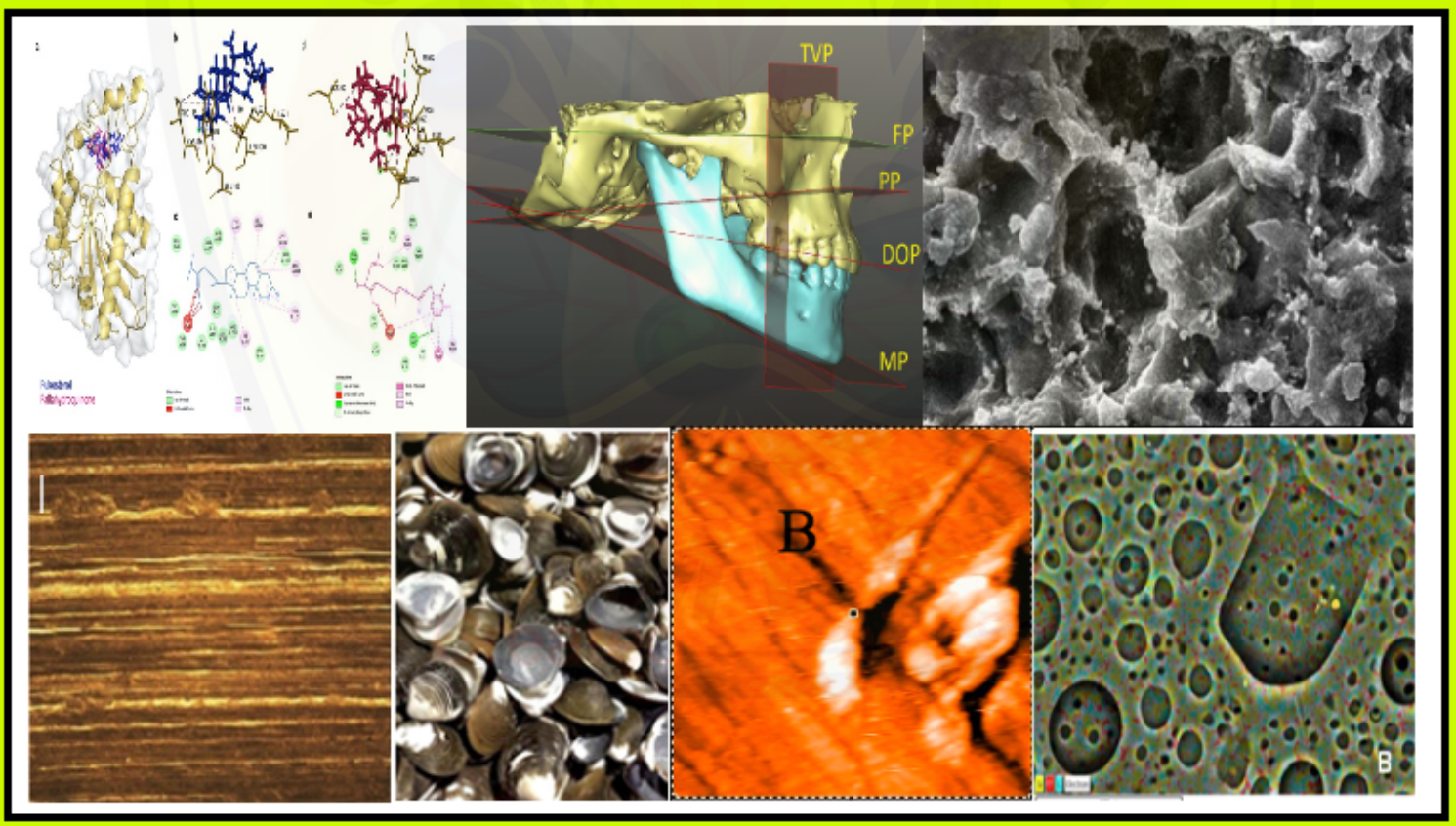

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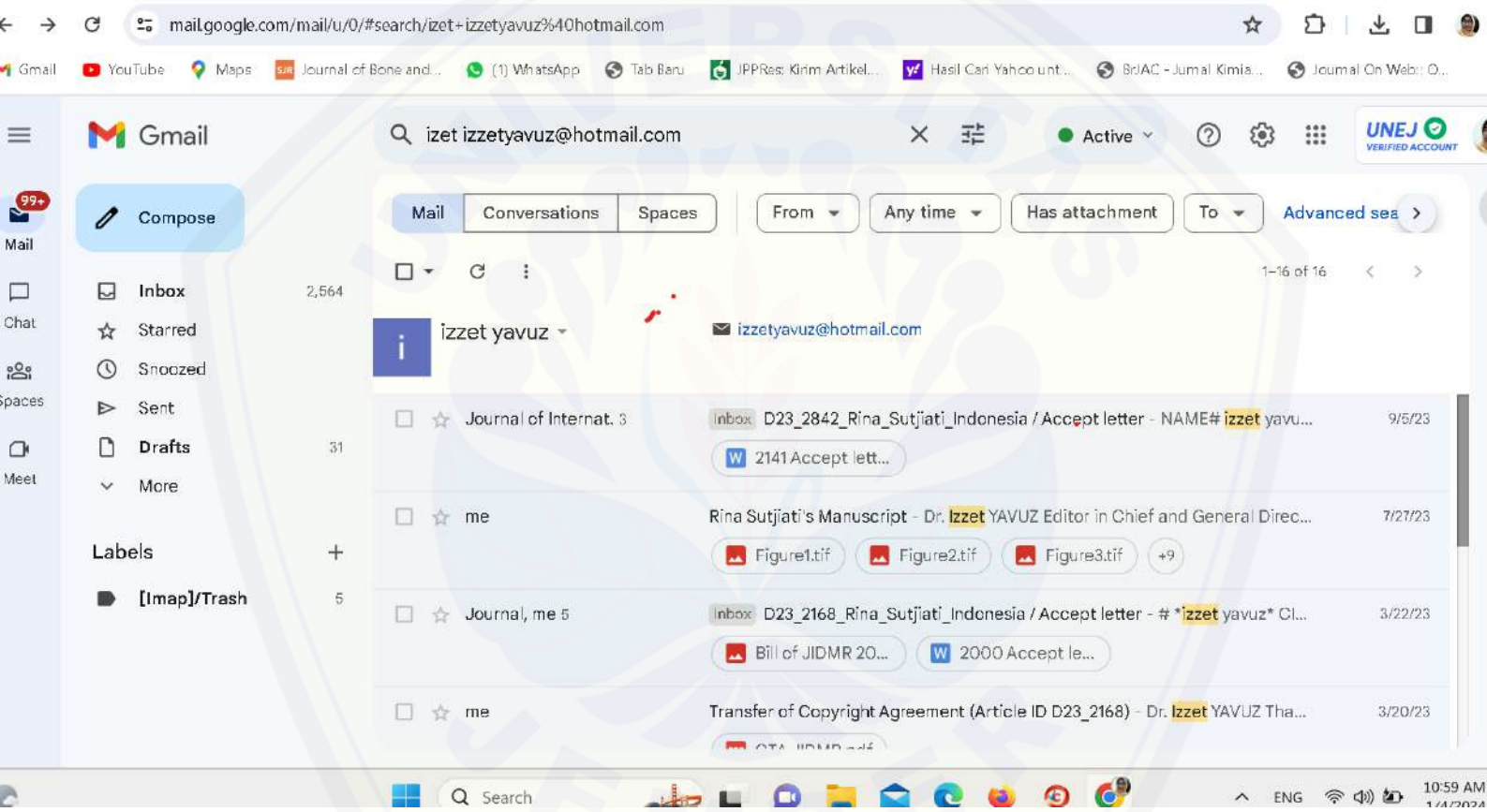

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OPG and RANKL Expression on Orthodontic Tooth Movement after Cacao Bean Extract Administration

Rina Sutjiati[1]*, Leliana S. Devi[1], Herniyati[1], Rudy Joelijanto[1], Vanda Ramadhani[1], Dwi Prijatmoko[1], Shierin V. Fiolita[2], Syafika N. Fadiyah[2], Millenieo Martin[2]

1. Department of Orthodontics, Faculty of Dentistry, University of Jember, Jember, Indonesia.
2. Graduated Student of Dentistry, Faculty of Dentistry, University of Jember, Jember, Indonesia.

Abstract

Teeth that are given orthodontic forces can relapse because of excessive resorption on the pressure side. Cocoa bean extract increases bone apposition during bone remodeling by enhancing osteoblast proliferation.

This study aimed to determine the role of cacao bean extract (Theobroma cacao L.) on the expression of OPG and RANKL on orthodontic tooth movement in the alveolar bone.

This research is a laboratory experiment applying the posttest-only control group design. The NiTi closed coil spring was applied between the right maxillary 1st molar and the maxillary incisor. Strength of 10gF was measured using a tension gauge to move the molars in a mesial direction. Wistar rats were decapitated after 7 days and 14 days of treatment. Immunohistochemistry staining was done to determine the expression of OPG and RANKL by counting the number of expressed osteoclasts. The data analysis used the one-way ANOVA test followed by the LSD test.

The research showed that the expression of OPG and RANKL enhanced significantly in the treatment group compared to the positive control group. Conclusion: The administration of cacao (Theobroma cacao L.) bean extract of the lindak variety to the alveolar bone could significantly enhance the expression of OPG and RANKL in the 7 and 14 days of the treatment group than the positive control group.

Experimental article (J Int Dent Med Res 2023; 16(4): 1425-1430)

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Introduction

Orthodontic treatment is one type of treatment carried out in dentistry that aims to obtain an aesthetically dentomaxillofacial appearance. The process of bone remodeling, namely alveolar bone resorption in the pressure area and bone formation in the tension area, is triggered when orthodontic mechanical force is applied to the teeth. Both occur in the periodontal ligament [1].

It is known that orthodontic treatment takes a long time. In fixed orthodontic treatment, the duration of the treatment can last as long as 24.9 months . Even in the case of extraction, it can reach up to 35 months [2]. The length of orthodontic treatment is related to the bone remodeling process, and good bone remodeling results can prevent excessive bone resorption on the pressure side [3].

Bone resorption occurs due to the activity of osteoclasts, which are bone cells generated from hematopoietic stem cells (HSCs), also known as monocytes [4,5]. Due to orthodontic mechanical forces, inflammatory cells will be activated, produce proinflammatory cytokines such as 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 [6,7]. OPG is a RANKL decoy receptor that can inhibit RANKL-RANK binding so that osteoclast differentiation does not occur but osteogenesis does [8,9].

The occurrence of this inflammatory process is also capable of causing an expansion in the synthesis of free radicals with the

Corresponding author:
Rina Sutjiati,
Department of Orthodontics, Faculty of Dentistry, University of Jember, Kalimantan Tegalboto Street No.37, Krajan Timur, Sumbersari 68121, Jember, Indonesia.
E-mail: rinasutjiati@unej.ac.id

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consequence of oxidative stress [10,11]. During the bone remodeling process, the RANKL/OPG ratio is sensitive to an increase in oxidative status that induces upregulation RANKL and decreased OPG [12,13]. This situation can activate the differentiation of preosteoclast thereby increasing bone resorption [12,14].

An imbalance in activity between osteoclasts and osteoblasts is caused by the inflammatory process and an increase in oxidative stress brought on by orthodontic mechanical forces [2,15]. Increased bone resorption and apoptosis of osteoblasts and osteocytes that support osteoclastogenesis are hallmarks of this condition [2,16]. As a result of excessive resorption, there can be an imbalance in the remodeling process [17]. Therefore, antioxidants are needed in reducing bone resorption activity during orthodontic treatment [18,19].

One source of natural antioxidants that contains polyphenols/flavonoids is cacao beans (*Theobroma cacao* L.) [20–22]. Polyphenols are a group of different substances found in unfermented cacao beans. Raw cacao beans contain flavanol monomers and procyanidin oligomers, which account for approximately 60% of the total polyphenols [18].

The purpose of this research is to prove whether cacao bean extract (*Theobroma cacao* L.) can affect the expression of OPG and RANKL on orthodontic tooth movement on the pressure side of the alveolar bone.

Materials and methods

This research used an experimental laboratory method with a posttest-only control group design. The total samples of male Wistar rats were 36 which were divided into 6 groups. Group A : group of negative control rats was the group that was not given any treatment (K-7, K-14); group B : the positive control rat group was given orthodontic mechanical force (K+7, K+14); and group C : the treatment group was given orthodontic mechanical force and cacao bean extract (P7, P14). This research has received permission in the form of Ethical Clearance No. 1727/UN25.8/KEPK/DL/2022.

The cacao beans were unfermented lindak cacao varieties obtained from the PTPN X Kertosari plantation, Jember, Indonesia, which identified in the form of Plant Identification No. 153/PL17.8/PG/2021. Cacao beans were extracted using the maceration method. The whole cacao beans used were 1 kg extracted with a ratio of 1:4 to 96% ethanol solvent, carried out for 3 days, and covered with aluminum foil with occasional stirring. The results of the macerate were concentrated using a rotary evaporator for 2 hours at a temperature of 40-50. The final result was 10.3 grams of cacao bean extract. The administration of cacao bean extract at a dose of 250 mg/kg BW to the rats could reduce the level of oxidative stress, which is in line with the optimization of bone remodeling through the increased osteoblast number [23]. The sample of this study was 36 rats weighing 200 g, so the appropriate dose of cacao bean extract was 50 mg. Meanwhile, according to their stomach capacity, the recommended maximum volume of fluid given to rats is 10 ml/kg BW, so 200 g rats will need 2 ml of aqua [24]. As a result, the cacao bean extract given to each rat was 50 mg diluted in 2 ml of aqua once a day orally.

As a mechanical force, NiTi closed coil spring was placed between the rat's maxillary incisor and the right maxillary of the first molar to move the molar mesially. The strength given to this NiTi closed coil spring was 10gF as measured using a tension gauge. Wistar rats were decapitated after 7 days and 14 days of treatment. Furthermore, the sample was taken on the right region of the maxilla and then fixed using 10% BNF for 24 hours before making histological preparations. Tissue blocks made of paraffin were cut using a rotary microtome, carried out from the coronal to the apical direction to show the shape of the teeth and alveolar bone intact in the tension and pressure area until it became 5 µm of thickness. OPG and RANKL expression from the sample on the right region of the maxilla was examined by immunohistochemical staining. After the tissue slice was deparaffinized, it was incubated using primary antibody anti-OPG & anti-RANKL with Universal Horseradish Peroxidase (HRP) as its secondary antibody. These tissue slices in the pressure and tension area of the alveolar bone were then divided into three visual fields: cervical, middle, and apical third to be observed by three observers, respectively, under a light microscope at 400× magnification. The positive number of each OPG and RANKL expression in osteoclasts can be seen from the brown-stained osteoclasts. The data obtained were then analyzed using a one-way ANOVA test followed by an LSD test.

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Results

Description of Research Data

Thirty male Wistar rats (*Rattus norvegicus*) aged 3 to 4 months were divided into three groups: (K-) without treatment and movement of maxillary incisor teeth; (K+) with the movement of maxillary incisor teeth without treatment; (P) with treatment and movement of maxillary incisor teeth by cacao bean extract. Variables in each group were observed on day 7 and 14. OPG and RANKL were found to have a positive immunohistochemistry response, according to the result. The mean and standard deviation (SD) of the immunohistochemistry staining results for the osteoclast in the expressions of OPG and RANKL are shown in Table 1.

Group Experiment	K-7 Mean ± SD	K-14 Mean ± SD	K+7 Mean ± SD	K+14 Mean ± SD	P7 Mean ± SD	P14 Mean ± SD
Expression of OPG	3±0.816	3±0.816	2.75±0.957	2.75±0.957	8.5±1.290	12.75±0.957
Expression of RANKL	3±0.816	1.75±0.957	3.25±0.957	2.75±0.957	8±0.816	11.25±0.957

Table 1. Description of variable data on the number of osteoclasts in the expressions of OPG and RANKL (K-), (K+), and (P) by administration of cacao bean extract.

The normal distribution of the data is required for certain statistical analyses carried out in this study. Before additional information examination on the outflow of OPG and RANKL, information on homogeneity was assessed first. The Box's Test analysis was used to determine whether the data were homogeneous. The significant value of 1.000, which is greater than 0.05. This indicates that the expressions of OPG and RANKL have completed the homogeneity assumption. Then, all variables were tested for normal distribution. The test results, presented in Fig.1, show that the results of osteoclasts in the expressions of OPG and RANKL data using a QQ Plot for data analysis have a normal distribution. A straight and diagonal line seems likely to be formed by the scatter plot in Fig.2. The test measurement Z shows a higher value than 0.05, and that implies that the information OPG and RANKL articulation by immunohistochemistry staining has satisfied the normal assumption, so the following examination stage, in particular one-way ANOVA examination, can be proceeded.

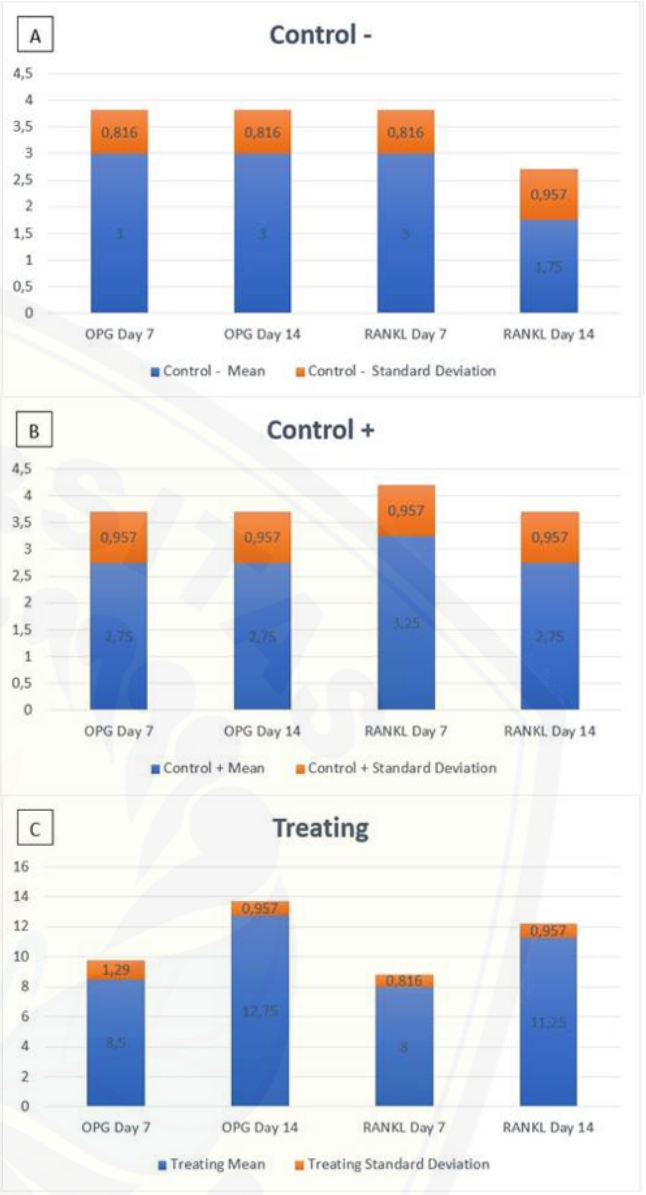

Figure 1. Average data of osteoclasts on the expressions of OPG and RANKL on days 7 and 14, on (A) negative control groups; (B) positive control group; and (C) treatment groups.

The Results of Immunohistochemistry Examination of the OPG Expression

The observation of osteoclasts cells generated positive expression data of OPG by immunohistochemistry methods in the negative control group (K-), positive control (K+), and the treatment group (P) because of the administration of cacao bean extract. Image A to F showing the expression of OPG on osteoclast's alveolar bone on day 7 and 14 is described below.

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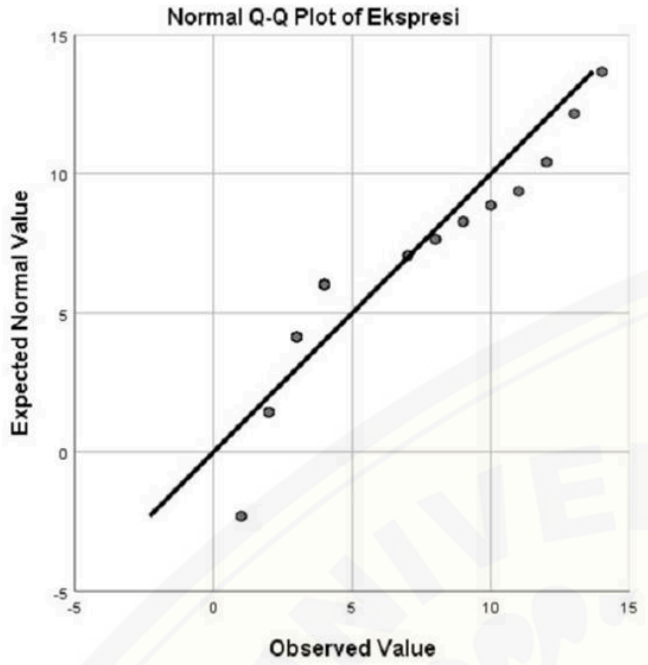

Figure 2. The results using the normality distribution with Plot QQ test.

The results of one-way ANOVA analysis show a significant difference ($p<0.05$) between the (K-) and (K+) as well as the (P). The results show that the administration of cacao bean extract on the pressure area can cause an increase in the mean expression of OPG in osteoclasts cells significantly compared to the positive control on day 14 as shown in Fig.1.

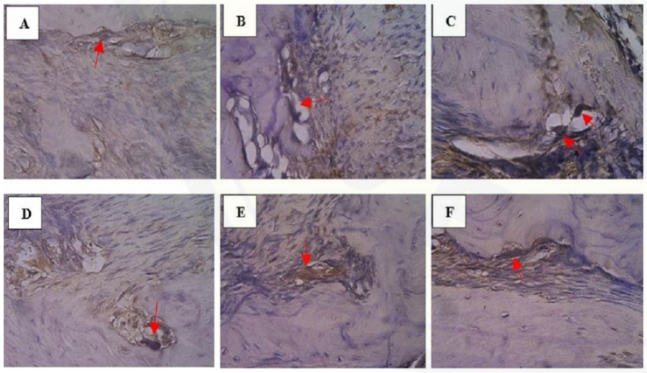

Figure 3. The red arrow marks the expression of OPG on osteoclast alveolar bone cells. A is the (K-), B is the (K +), C is the (P) by administration of cacao bean extract on day 7, D is the (K-), E is the (K +), F is the (P) by administration of cacao bean extract at day 14, with 400X magnification.

The Results of Immunohistochemistry Examination of the Expression of RANKL

RANKL positive expression data are derived from observations of osteoclast cells by immunohistochemistry methods in the (K-), (K+), and (P) by administration of cacao bean extract on the pressure region. Fig. 4 shows the RANKL expression in osteoclasts' alveolar bone on day 7 and day 14.

There is a significant difference ($p<0.05$) between the (K-), (K+), and (P) groups in the one-way ANOVA analysis, shown on table 1. The results show that the mean expression of RANKL in osteoclasts cells can be enhanced significantly by the administration of cacao bean extract on the pressure area compared to the positive control on day 14 as shown in fig.1.

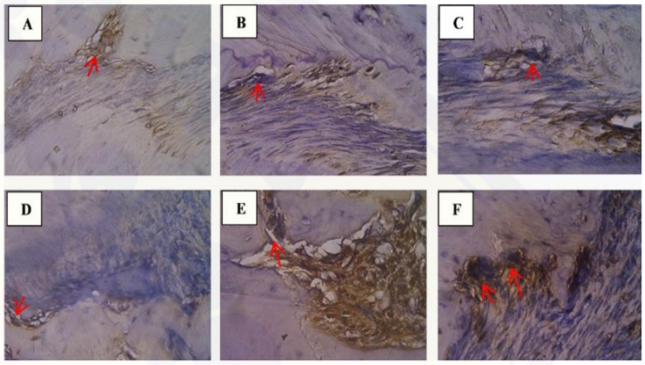

Figure 4. The red arrow indicates the expression of RANKL on osteoclast alveolar bone cells. A is the (K-) group, B is the (K+) group, and C is the (P) group by the administration of cacao bean extract on day 7, D is the (K-) group, E is the (K +) group, F is the (P) group with administration cacao bean extract on day 14, with 400X magnification.

Discussion

Orthodontic treatment is one of dental treatment procedures that takes a long time, ranging from 25-35 months. Many efforts have been made to accelerate orthodontic treatment because it not only shortens the duration of treatment but also correlates to root resorption and excessive resorption on the pressure side [1,25]. This study aims to examine the role of cacao bean extract (*Theobroma cacao* L.) on the number of osteoclasts expressed OPG and RANKL on the alveolar bone on the tooth pressure side of male Wistar rats on orthodontic tooth movement for 7 days and 14 days. The method is administering diluted cacao bean extract using a gastric probe as much as 2 ml.

The results of the ANOVA test (Table 1) show that group 1 had a significant difference

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with a significance value of 0.020 ($p<0.05$). The increase in the osteoclast number of the positive control group was due to the mechanical force applied to the rat teeth. This mechanical force can trigger an inflammatory process associated with an increase in free radical synthesis with consequences of oxidative stress [26]. Cytokines produced during the inflammatory process can cause osteoblasts to express the protein RANKL which will then bind to RANK. RANKL-RANK binding will stimulate osteoclast formation [6,9]. The increase in osteoclasts is also caused by oxidative stress, which can activate the differentiation of preosteoclasts into osteoclasts by increasing the RANKL expression, in line with the increasing number of osteoclasts and the occurrence of bone resorption [11].

The number of the OPG and RANKL expressed osteoclast count in the treatment group was higher than that in the positive control group. This is supported by the results of the ANOVA test (Table 1) presenting a significant difference between the K+7 group and the P7 group ($p=0.021$) and between the K+14 group and the P14 group ($p=0.028$). This significant difference shows that cacao bean extract can increase the expression of OPG and RANKL during orthodontic treatment.

The increase in OPG and RANKL expression that occurred in the treatment group was due to the potential chemical compounds possessed by cacao beans. Cacao beans are rich in flavonoids with antioxidant activity reaching 80% at a certain concentration [27]. Flavonoids are proven to be able to reduce alveolar bone resorption with a mechanism that can reduce the interaction between RANK and RANKL. Furthermore, it will increase the expression of OPG thereby reducing the process of osteoclast formation. The increase in OPG gives a great impact to the activation of osteoclast. As a decoy receptor for RANKL, OPG block the binding between RANKL and RANK in the cell membrane of osteoclast precursor, therefore inhibiting osteoclastogenesis. The inhibition of osteoclastogenesis can decrease the number of osteoclast, so that bone resorption can be reduced, and reported that cacao bean extract could significantly inhibit the activation of NF-κB [6,28]. NF-κB is a transcription factor which is translocated to the nucleus through RANK-RANKL binding. It plays a crucial role in the initial stages of osteoclast development. There will be no osteoclast differentiation in mice with a small amount of NF-B.

Overall, the treatment group has a significantly increased expression of OPG and RANKL compared to the positive control group. Cacao bean extract of the lindak variety can potentially increase the expression of OPG and RANKL on the pressure side of Wistar rat's alveolar bone in orthodontic tooth movement [3,6]. This automatically reduces the process of osteoclastogenesis, thus reducing the excessive resorption process on the pressure side when the teeth are moved orthodontically and a balance can be achieved between the resorption and apposition of alveolar bone to speed up the remodeling process of alveolar bone during orthodontic tooth movement.

Conclusions

The expression of OPG and RANKL on the alveolar bone in the area of tooth pressure in Wistar rats on orthodontic tooth movement was significantly higher in the treatment group than that in the positive control group when cacao bean extract (*Theobroma cacao* L.) was given to day 7 and 14 of the treatment group.

Acknowledgements

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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. 1727/UN25.8/KEPK/DL/2022.

Declaration of Interest

All the authors hereby declare that there is no conflict of interest.

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