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Original Article

Effect of cocoa administration during orthodontic tooth movement on RUNX2, calcium levels, and osteoclast bone-resorbing activity in rats

[Efecto de la administración de cacao durante el movimiento dental ortodóncico sobre RUNX2, los niveles de calcio y la actividad de reabsorción ósea de los osteoclastos en ratas]

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Abstract

Context: Cocoa contains caffeine-rich methylxanthine, which promotes accelerated orthodontic tooth movement. Caffeine affects calcium ion equilibrium, causing low bone density and shortening orthodontic treatment. It is unclear how cocoa affects periodontal tissue reconstruction when orthodontic stress is applied.

Aims: To evaluate the effect of cocoa administration during active orthodontic tooth movement (OTM) on RUNX2, calcium levels, and osteoclast bone-resorbing activity in rats.

Methods: A total of 48 Sprague Dawley rats were used. It was divided into four subgroups depending on the observation day: 0, 1, 7, and 14 (n = 3) for each group of 12 animals: group A (positive control, 2.3 mg of caffeine), group B (cocoa dose 1.37 g), group C (cocoa dose 2.05 g), and group D (cocoa dose 2.74 g).). The upper incisors of both groups were banded with a 3-spin loop spring that exerted 35 g of orthodontic force under anesthesia. Cocoa was given orally to the treatment group once a day based on the dose utilized. RUNX2 levels during OTM were determined by ELISA. Furthermore, lacuna resorption measured osteoclast bone-resorbing activity and the atomic absorption spectrophotometer assessed calcium levels. Data gathered were analyzed using two-way ANOVA and post hoc LSD ($p<0.05$).

Results: RUNX2 levels in the compression side were significantly different between the groups. Similarly, lacuna resorption depth was significantly different between the groups ($p<0.05$), but daily cocoa administration does not significantly downregulate calcium levels in rats during active OTM ($p>0.05$).

Conclusions: Cocoa supplementation during active OTM increases the RUNX2 levels and osteoclast bone-resorbing activity in rats.

Keywords: extraction; calcium; cocoa; orthodontic; osteoclast; RUNX2.

Resumen

Contexto: El cacao contiene metilxantina rica en cafeína, que promueve el movimiento ortodóncico acelerado de los dientes. La cafeína afecta el equilibrio de los iones de calcio, provocando una baja densidad ósea y acortando el tratamiento de ortodoncia. No está claro cómo el cacao afecta la reconstrucción del tejido periodontal cuando se aplica estrés de ortodoncia.

Objetivos: Evaluar el efecto de la administración de cacao durante el movimiento dental ortodóncico activo (OTM) sobre RUNX2, los niveles de calcio y la actividad de reabsorción ósea de los osteoclastos en ratas.

Métodos: Se utilizaron un total de 48 ratas Sprague Dawley. Se dividió en cuatro subgrupos según el día de observación: 0, 1, 7 y 14 (n = 3) para cada grupo de 12 animales: grupo A (control positivo, 2,3 mg de cafeína), grupo B (dosis de cacao 1,37 g), grupo C (dosis de cacao 2,05 g), y grupo D (dosis de cacao 2,74 g). Los incisivos superiores de ambos grupos se vendaron con un resorte de bucle de 3 giros que ejerció 35 g de fuerza ortodóncica bajo anestesia. Se administró cacao por vía oral al grupo de tratamiento una vez al día en función de la dosis utilizada. Los niveles de RUNX2 durante OTM se determinaron mediante ELISA. Además, la reabsorción de la laguna midió la actividad de reabsorción ósea de los osteoclastos y el espectrofotómetro de absorción atómica evaluó los niveles de calcio. Los datos recopilados se analizaron mediante ANOVA de dos vías y LSD post hoc ($p<0,05$).

Resultados: Los niveles de RUNX2 en el lado de compresión fueron significativamente diferentes entre los grupos. De manera similar, la profundidad de reabsorción de la laguna fue significativamente diferente entre los grupos ($p<0,05$), pero la administración diaria de cacao no regula significativamente los niveles de calcio en ratas durante la OTM activa ($p>0,05$).

Conclusiones: La suplementación con cacao durante la OTM activa aumenta los niveles de RUNX2 y la actividad de reabsorción ósea de los osteoclastos en ratas.

Palabras Clave: cacao; calcio; ortodoncia; osteoclasto; RUNX2.

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INTRODUCTION

The teeth can be straightened through a process known as orthodontic treatment, which aims to improve both the dentofacial appearance and the function of the bite (Brahmanta et al., 2021). Since tooth movement is associated with such complex changes, the orthodontic treatment typically lasts between one and three years. During this time, orthodontic appliances must be placed, making oral hygiene maintenance more difficult and time-consuming; consequently, patients are more susceptible to periodontal diseases, white spot lesion, caries, and root resorption (Arianda et al., 2020). Discovering novel approaches to improve orthodontic tooth movement and reduce treatment time has always been desired. In order to reduce the length of treatment, researchers have never stopped seeking ways to expedite the rate of OTM. Numerous interventions, including as the local administration of parathyroid hormone (Soma et al., 2000), hyperbaric oxygen 2.4 therapy (Brahmanta et al., 2021), low-intensity laser therapy biostimulation (Narmada et al., 2019), and extra or intraoral vibration devices (Katchooi et al., 2018), have been demonstrated to accelerate OTM. The therapeutic implementation of these methods, however, faces significant obstacles. In addition, the use of some medications will impose additional burdens on patients, such as potential side effects.

Recent years have seen a significant increase in the use, research, and production of natural materials for medical applications. Intriguingly, previous research suggests that cocoa administration may accelerate orthodontic tooth movement (OTM) (Alhasyimi and Rosyida, 2019). Cocoa includes methylxanthine, an active molecule with a high caffeine content (Franco et al., 2013). Caffeine's potential to promote tooth movement has long been known. Low bone density is caused by caffeine's effect on calcium ion balance, which causes bone remodeling to improve and shorten orthodontic treatment duration (Shirazi et al., 2017; Yi et al., 2012). Cocoa supplementation has been shown to modulate the rate of tooth movement, induce osteoclastogenesis by stimulating receptor activator of nuclear factor-κB ligand (RANKL) level and limiting osteoprotegerin (OPG) level, reduce alkaline phospatase levels, and decrease the number of osteoblasts in rats during active orthodontic movement (Alhasyimi and Rosyida, 2019; Arianda et al., 2020). However, it is still unclear what effect cocoa has on the reconstruction of periodontal tissue and other signaling pathways involved in osteoblastogenesis when orthodontic force is applied.

Bone remodeling occurs during orthodontic movement due to mechanical stress causing bone resorption on the pressure side and bone formation on the tension side. In this process, osteoclasts and osteoblasts play a crucial regulatory role (Alhasyimi et al., 2018). The Runt-related transcription factor 2 (RUNX2) signaling pathway is essential for osteoblast development and bone formation. Uncommitted mesenchymal cells have low levels of RUNX2 expression, which increases in preosteoblasts, reaches a peak in immature osteoblasts, and declines as the cells mature (Qin et al., 2018). RUNX2 overexpression in osteoblasts, on the other hand, enhances osteoblast-driven osteoclastogenesis. As a result, RUNX2 appears to be involved in osteoblastogenesis and osteoclastogenesis (Baniwal et al., 2012). *In vivo* studies discovered that orthodontic force can cause a transient elevation in RUNX2 expression levels within periodontal tissue, which plays a role in the osteogenesis and reconstruction of periodontal tissue during OTM (Han et al., 2015).

Root mineral density, influenced by calcium deposition, also affects OTM. Mechanical stress increases DMP1 and DSPP expression and levels, which may accelerate dentin mineralization and increase the mineral density of the peritubular extracellular matrix during OTM (Kong et al., 2010). According to previous research, calcium deficiency has been linked to an increased OTM rate (Goldie and King, 1984). Bone resorption on the compression side is thought to be the rate-limiting phase in orthodontic tooth movement. Histologic investigations suggest that osteoclasts are induced on the compression side during OTM (Mostafa et al., 2009). Accelerated tooth movement interventions considerably increase the number of osteoclasts (Alhasyimi and Rosyida, 2019). Pit (lacuna resorption) depth could be used to reliably quantify osteoclast activity irrespective of osteoclast differentiation. Therefore, this study aimed to explore the efficacy of cocoa consumption during orthodontic tooth movement on RUNX2 expression, calcium levels, and osteoclast bone-resorbing activity in rat models.

MATERIAL AND METHODS

Orthodontic tooth movement model in rats

All experimental animal methods were conducted under guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals to ameliorate any suffering of animals. The experiment met the current regulations and ethical approval of the Faculty of Dentistry's Research Ethics

Committee, UGM, Indonesia (No.085/KE/FKG-UGM/EC/2022). A total of 48 male Sprague Dawley rats aged 10 weeks weighing 250 and 300 g were enrolled. One week of acclimatization was conducted for housing and food pretreatment adaptation. During the studies, laboratory pellets were provided to the animals with tap water *ad libitum*. The maintenance room for the rats was kept at 25°C and 50% humidity. The room had approximately a 12 h light cycle.

The amount of caffeine used was based on what the Food and Drug Administration (FDA, 2018) says is a safe dose, which is between 100 and 200 mg daily. The dosage was then converted to rats dose, and found that 2.3 mg of caffeine was in 1.37 g of unsweetened cocoa, 3.45 mg was in 2.05 g of unsweetened cocoa, and 4.6 mg was in 2.74 g of unsweetened cocoa. There were four groups in the experiment, each of which contained a total of 12 animals: group A (positive control, 2.3 mg of caffeine), group B (cocoa dose 1.37 g), group C (cocoa dose 2.05 g), and group D (cocoa dose 2.74 g). Each group was then randomly split into four subgroups based on the day of observation: day 0, day 1, day 7, and day 14. There were three male rats in each sub-group. The Federer formula was used to figure out how many samples to take.

At doses of 5 mg/kg and 35 mg/kg body weight, xylazine and ketamine hydrochloride were used to sedate the rats before the orthodontic appliances were placed (Alhasyimi and Rosyida, 2019). A three-spin loop spring made of 0.012-inch stainless steel alloy wire and bands (American Orthodontics, USA) was banded to the rats' upper incisors with glass ionomer luting cement (Fuji I, GC, USA) in order to move the teeth distally (Fig. 1). This approach delivered nearly 30 g of constant orthodontic force, as determined by a tension gauge. The appliance was not reactivated throughout the testing. Using a gastric tube, caffeine and cocoa were supplied orally by dissolving in 3 mL of distilled water to the treatment group once a day, immediately following the placement of orthodontic appliances for up to 14 days (Alhasyimi and Rosyida, 2019).

Gingival crevicular fluid (GCF) collection and RUNX2 levels analysis

Air jets and cotton rolls were used to drain and separate the crevicular sulcus of each rat before GCF samples were collected. To obtain the GCF for each sample, the smallest diameter paper point (size #15, Sendoline, UK) was used four times: once (0, 1, 7, and 14 times) for each group. For 30 seconds, a paper point was gently inserted into the gingival sulcus on the teeth's distal (compression) side. Four paper points were then inserted in an Eppendorf tube of 1.5 mL containing 350 µL of saline solution. Finally, the tube was centrifuged for 5 min at 2000 rpm at 4°C with a microcentrifuge cooler to completely remove all GCF components from the paper points. In order to preserve the supernatants for further analysis, the paper points were removed, and the supernatants were kept at 80°C. To measure Runx-2 levels during active OTM, ELISA was employed. The analysis was performed using a quantitative anti-Runx-2 antibody-specific ELISA kit (ab76956, Abcam®). Comparing the total quantity of the transcription factor to its standard curve. Using a microplate reader, the optical density of the solutions was measured at 450 nm, and the total quantity of Runx-2 was expressed in pg/mL (Alhasyimi and Rosyida, 2019).

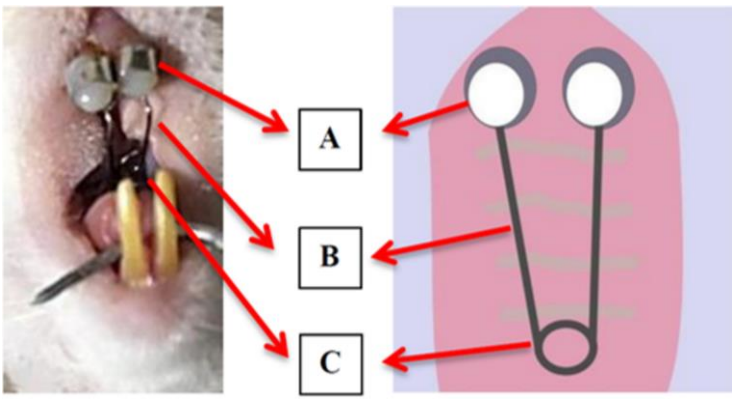

Figure 1. Orthodontic appliance design that has been attached to the rat maxillary incisors. **(A)** The incisor is surrounded by a matrix band; **(B)** The arm wire of 5 mm in length; **(C)** Coil with 2 mm diameter.

Table 1. Results of the one-way ANOVA comparing the Runx-2, calcium levels, and lacuna depth among four groups tested.

Parameter	G1	G2	G3	G4	*P*-value	Post hoc comparison
Runx-2 level						
Day 0	34.57 ± 4.32	36.11 ± 3.76	38.69 ± 4.23	41.21 ± 3.82	0.002*	G1,G2<G4
Day 1	49.11 ± 3.93	51.03 ± 5.13	55.19 ± 6.28	59.61 ± 5.07	0.000*	G1<G2<G3<G4
Day 7	38.19 ± 4.09	40.23 ± 4.49	40.09 ± 4.21	42.24 ± 4.17	0.056	NS
Day 14	27.04 ± 3.63	29.73 ± 3.08	33.21 ± 3.13	33.07 ± 2.99	0.004*	G1,G2<G3,G4
Calcium level						
Day 0	25.38 ± 1.29	24.53 ± 1.08	24.97 ± 1.42	24.66 ± 1.71	0.373	NS
Day 1	26.25 ± 1.76	26.16 ± 1.08	25.55 ± 1.13	24.48 ± 1.21	0.056	NS
Day 7	27.28 ± 1.16	27.39 ± 1.43	25.65 ± 1.02	25.02 ± 1.42	0.175	NS
Day 14	24.56 ± 1.53	24.42 ± 1.12	23.67 ± 1.21	23.21 ± 1.17	0.978	NS
Lacuna depth						
Day 0	13.73 ± 2.73	29.19 ± 2.13	23.77 ± 3.79	31.93 ± 1.67	0.000*	G1,G2<G4
Day 1	25.51 ± 2.31	25.48 ± 1.24	27.82 ± 2.53	36.38 ± 2.03	0.000*	G1,G2,G3<G4
Day 7	33.24 ± 4.08	40.99 ± 2.86	73.86 ± 5.04	76.78 ± 1.43	0.000*	G1<G2<G3<G4
Day 14	31.27 ± 3.73	36.77 ± 3.87	59.44 ± 6.39	66.39 ± 4.96	0.000*	G1,G2<G3,G4

The values are displayed using the mean along with the standard deviation. [a]Tested by two-way ANOVA and post hoc Tukey's LSD test; G1: the positive control group; G2: dose 1.37 g cocoa; G3: dose 2.05 g cocoa; G4: dose 2.74 g cocoa group. *p<0.05, significant difference between groups; NS: not significant.

Measurement of osteoclast bone-resorbing activity

The mandibular bones were dissected and preserved with 10% formaldehyde at 4°C for 24 h after all rats were sacrificed (0, 1, 7, and 14 days following orthodontic appliance installation) by an overdose of anesthetic and decapitation. Left-sided tissue pieces were then decalcified using formic acid for 2 days. Dehydrated specimens used graded alcohol at 4°C, xylol alcohol, pure xylol, and room temperature paraffin xylol. The paraffin block was made, coronal cut with thickness ± 6 µm parallel to the long axis of the tooth. After deparaffinization, hematoxylin-eosin staining was carried out. The osteoclast bone-resorbing activity was measured by counting the depth of lacuna resorption (the difference in height between the edge of the lacuna and the deeper point) in µm. Five areas of interest (ROIs) extending vertically from the cervical to the apical region of the distal surface's incisor alveolar bone were randomly selected from three histological sections obtained from each animal. This measurement was carried out using a 400-magnification light microscope outfitted with Optilab and Image Raster. Each measurement was performed three times by two blinded and independent observers. The mean depth of lacuna resorption across the five ROIs was computed and determined as the representative value.

Analysis of calcium levels

The distal of the right upper incisor and the tooth's root tip were removed from the right upper alveolar bone. They were calcined for 24 h to remove the organic matrix and dissolve minerals, then dissolved in concentrated HNO_3 at 80°C and diluted to concentrations of 1 to 5 parts per million. For the atomic absorption spectrophotometer assessment, the samples were collected and evaluated at 422.7 nm and 10 mA lamp current, determined in percentage units (%) (Figueiredo et al., 2011).

Statistical analysis

Statistics were used to assess differences and interactions between groups based on the results of this investigation, using two-way ANOVA. To establish whether there were significant differences between the groups, researchers used a post hoc LSD test. Statistical significance was defined as a p-value <0.05.

RESULTS

All experimental techniques were tolerated appropriately. In addition, there was no evidence of systemic toxicity following ingestion of caffeine and cocoa at the dose utilized. Table 1 compares the depth of lacuna resorption among groups, revealing a statistically significant difference between the control group and

the groups with varying doses of cocoa ($p<0.05$). On days 1, 7, and 14, histological examination reveals that subjects in the treatment group experienced a deeper lacuna resorption than those in the control group. The deepest lacuna resorption was experienced in the group that received a 2.74 g dose of cocoa within seven days of consumption. This can be shown in Fig. 2.

On days 0, 1, 7, and 14, during active orthodontic tooth movement, the RUNX2 level was found to be considerably greater than that of the positive control group ($p <0.05$), which corresponds with the observation made by histology ($p<0.05$) (Table 1, Fig. 3A). Comparison among groups revealed that the lowest RUNX2 level occurred in the control group with 14 days of cocoa consumption. In contrast, the highest occurred in the group that received a 2.74 g dose of cocoa with one day of consumption.

Meanwhile, daily cocoa administration did not affect the levels of calcium. Analyses with an atomic absorption spectrophotometer revealed that the calcium levels in the control group were somewhat higher than those in the other groups, but the observed differences were not statistically significant ($p>0.05$, Table 1, Fig. 3B).

DISCUSSION

The long duration and intricacy of orthodontic treatment may result in numerous complications. While surgical treatments for accelerating OTM have been proved to be beneficial, nonsurgical approaches have always been favored by clinicians and patients due to their noninvasiveness. As far as we know, no published research has looked at the link between daily cocoa consumption and RUNX2, calcium levels, and osteoclast bone-resorbing activity. Current evidence, however, relates cocoa intake to OTM acceleration and osteoclastogenesis *in vivo*. Cocoa has been shown to help with active orthodontic treatment by decreasing the rate of OTM rate by increasing osteoclastogenesis (suppressing the OPG level and stimulating the RANKL level) (Alhasyimi and Rosyida, 2019). Interestingly this is in accordance with this study's results, which showed cocoa significantly increases osteoclast bone-resorbing activity during active OTM. On the side of the compression, with light forces, numerous multinucleated osteoclasts in Howship's lacunae resorbed alveolar bone directly (Meikle, 2006). The methylxanthine caffeine found in cocoa may facilitate this process. This chemical can

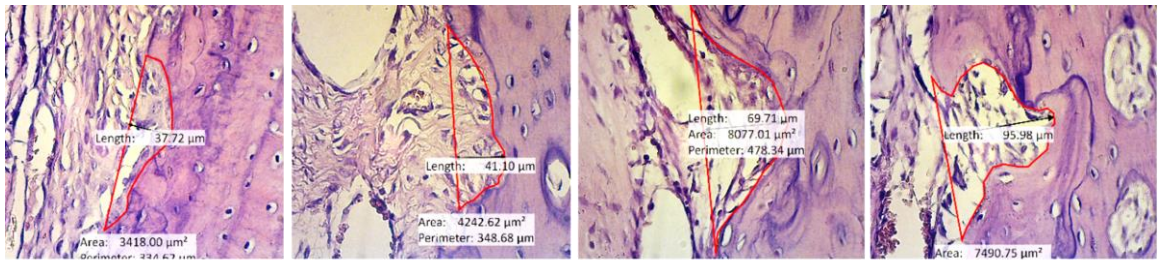

Figure 2. The depth of lacuna resorption was greater in nearly all groups after seven days of orthodontic tooth movement compared to other days of observation, with the greatest depth of lacuna resorption occurring in the group that consumed 2.74 g of cocoa daily for seven days. Arrows represent the depth or length of lacuna resorption.

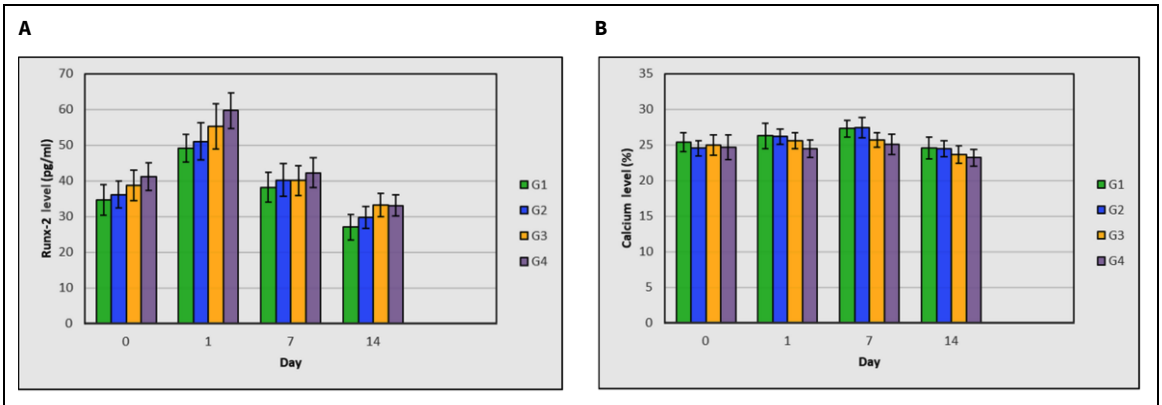

Figure 3. RUNX2 **(A)** and calcium **(B)** levels in the positive control (G1), dosage 1.37 g cocoa (G2), dose 2.05 g cocoa (G3), and dose 2.75 g cocoa (G4) groups from day 0 to day 14 of active OTM.

The data are presented in the format of mean ± standard deviation.

cause osteoblast apoptosis by increasing reactive oxygen species formation due to increased cyclic adenosine monophosphate production and increasing osteoclast numbers and resorption surface resulting in enhanced bone resorption (Pal et al., 2016). Additionally, caffeine may be capable of enhancing a COX-2/PGE_2-regulated RANKL-mediated osteoclastogenesis (Liu et al., 2011). A higher concentration of RANKL induces osteoclast differentiation, activation, and survival, resulting in enhanced bone resorption, which accelerates OTM (Alhasyimi and Rosyida, 2019).

This study also showed that daily cocoa consumption could increase the level of RUNX2 during active OTM. This condition is in line with the research conducted by Herniyati et al. (2018), which stated that the increase of RUNX2 occurred due to the caffeine content. Caffeine binds to adenosine receptors and modulates other receptors expressed in osteoblasts or progenitor cells. RUNX2 plays a vital function in periodontal tissue remodeling during orthodontic tooth movement. RUNX2 expression alterations resulted in periodontal tissue reconstruction and tooth position displacement (Han et al., 2015). According to the findings, RUNX2 stimulates osteoclastogenesis and bone resorption via the AKT/NFATc1/CTSK axis. AKT activation boosted NFATc1 nuclear translocation and increased the expression of downstream genes such as CTSK. AKT phosphorylation inhibition prevented osteoclast formation, but constitutively active AKT recovered osteoclast differentiation (Xin et al., 2020). In early studies, RUNX2 induction lowered the expression of OPG (Baniwal et al., 2012). As OPG functions as a decoy receptor for RANKL, a key activator of osteoclastogenesis, it is anticipated that a decrease in OPG level will allow RANKL activity and hence enhance osteoclastogenesis (Suparwitri et al., 2019). Enhancing osteoclastogenesis during orthodontic tooth movement facilitates rapid orthodontic tooth movement (Alhasyimi and Rosyida, 2019). The result showed that the top levels of RUNX2 in all doses occurred on day 1, which represents the initial phase of orthodontic tooth movement. RUNX2 is an important factor in DNA transcription in the early phase of stromal cells. RUNX2 can also trigger the expression of bone matrix genes, including osteocalcin and alkaline phosphatase, in the early stages of OTM (Ardani et al., 2022; Sitasari et al., 2020). The levels of RUNX2 show a decrease on day 7 and day 14. Han et al. (2015) stated that the decrease of RUNX2 may occur due to its biological functions. RUNX2 plays a cooperative role in the formation of periodontal tissue. After the transcription processes were completed, the signaling pathway induced by orthodontic force made phosphorylation of RUNX2. RUNX2 was activated and entered the nucleus to regulate the downstream effect elements to control the transcription process. RUNX2 and other proteins in the signaling pathway interact directly or indirectly with several molecules involved in bone remodeling.

This study showed that daily cocoa consumption did not affect the calcium level during active OTM. Physiological and controlled balancing tests reveal that caffeine has a clear depressive effect on intestinal calcium absorption; however, this effect is only very slight, and caffeine does not influence the overall amount of calcium excreted in the urine for 24 h. The detrimental effect of caffeine on calcium absorption can be completely neutralized by drinking as little as one or two tablespoons of milk. This is because caffeine's effect on calcium is rather minor. There is no indication that caffeine has any negative effect on either the state of the bones or the calcium levels in the body (Heaney et al., 2002).

Assessment of osteoclast activity represents a limitation of this investigation. No realistic estimate of resorption can be made in the absence of accurate 3-D visualizations of osteoclast lacunae. Therefore, a 3-D reconstruction of pits resorption is necessary to strengthen this result conclusion.

CONCLUSION

The results of this investigation show that cocoa administration can elevate RUNX2 levels and promote osteoclast bone-resorbing activity during active orthodontic tooth movement in rats. This was demonstrated by the fact that both effects occurred simultaneously. However, its clinical efficacy has yet to be verified, and further scientific proof from randomized trials is required before routine clinical acceptance.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Alhasyimi AA	Pudyani PS
Concepts or ideas	x	x
Design	x	
Definition of intellectual content	x	
Literature search	x	
Experimental studies	x	x
Data acquisition	x	x
Data analysis	x	
Statistical analysis	x	
Manuscript preparation	x	x
Manuscript editing	x	
Manuscript review	x	x

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