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Original Article

Anti-inflammatory effect of caffeic acid phenethyl ester supplementation on TNF-α and NF-κB expressions throughout experimental tooth movement *in vivo*

[Efecto antiinflamatorio de la suplementación con éster fenetílico de ácido cafeico en las expresiones de TNF-α y NF-κB a través del movimiento dental experimental *in vivo*]

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Abstract

Context: Orthodontic tooth movement (OTM) changes the periodontal tissue and increases the incidence of root resorption (OIRR). Caffeic acid phenethyl ester (CAPE), an antioxidant and anti-inflammatory chemical generated from honey propolis, might be useful in controlling inflammation during OTM and so reducing the risk of OIRR.

Aims: To evaluate if CAPE supplementation has an anti-inflammatory impact on tumor necrosis factor-α (TNF-α) and nuclear transcription factor κB (NF-κB) during experimental OTM in male Wistar rats (*Rattus novergicus*).

Methods: Forty-eight healthy male Wistar rats were divided into positive control group (OTM 10 g/mm^2 force application) and experimental group (OTM application and CAPE administration). Each groups were observed for 3, 7, 14 days. A nickel-titanium closed coil spring that was 8.0 mm long, thick was inserted between the upper left first molar and upper central incisor in order to move the molar mesially. A 20 mg/kg body weight dose of CAPE was taken orally. Using immunohistochemistry, the expression of TNF-α and NF-κB was examined on the compression side of the OTM. Both the Tukey's honest significant difference test and the one-way analysis of variance test were applied ($p<0.05$).

Results: TNF-α and NF-κB expression in the compression side differed considerably across groups ($p<0.05$). Daily administration of CAPE significantly downregulates TNF-α and NF-κB expression on the compression side.

Conclusions: Administration of CAPE throughout OTM can successfully reduce the number of TNF-α and NF-κB expressions in the compression side *in vivo*.

Keywords: caffeic acid phenethyl ester; experimental tooth movement; medicine; nuclear transcription factor-κB; tumor necrosis factor-α.

Resumen

Contexto: El movimiento dental ortodóncico (OTM) cambia el tejido periodontal y aumenta la incidencia de reabsorción radicular (OIRR). El éster fenetílico del ácido cafeico (CAPE), un químico antioxidante y antiinflamatorio generado a partir del propóleo de la miel, podría ser útil para controlar la inflamación durante la OTM y así reducir el riesgo de OIRR.

Objetivos: Evaluar si la suplementación con CAPE tiene un impacto antiinflamatorio sobre el factor de necrosis tumoral-α (TNF-α) y el factor de transcripción nuclear κB (NF-κB) durante OTM experimental en ratas Wistar macho (*Rattus novergicus*).

Métodos: Cuarenta y ocho ratas Wistar macho sanas se dividieron en un grupo de control positivo (aplicación de fuerza de 10 g/mm^2 de OTM) y un grupo experimental (aplicación de OTM y administración de CAPE). Cada grupo se observó durante 3, 7, 14 días. Se insertó un resorte helicoidal cerrado de níquel-titanio de 8,0 mm de largo y espesor entre el primer molar superior izquierdo y el incisivo central superior para mover el molar mesialmente. Se tomó por vía oral una dosis de 20 mg/kg de peso corporal de CAPE. Usando inmunohistoquímica, se examinó la expresión de TNF-α y NF-κB en el lado de compresión del OTM. Se aplicaron tanto la prueba de diferencia significativa honesta de Tukey como la prueba de análisis de varianza de una vía ($p<0,05$).

Resultados: La expresión de TNF-α y NF-κB en el lado de compresión difirió considerablemente entre los grupos ($p<0,05$). La administración diaria de CAPE reguló significativamente a la baja la expresión de TNF-α y NF-κB en el lado de la compresión.

Conclusiones: La administración de CAPE a través de OTM puede reducir con éxito las expresiones de TNF-α y NF-κB en el lado de compresión *in vivo*.

Palabras Clave: éster fenetílico del ácido cafeico; factor de necrosis tumoral-α; factor de transcripción nuclear-κB; medicamento; movimiento dental experimental.

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Abbreviations: OTM: orthodontic tooth movement; OIRR: orthodontically induced root resorption; CAPE: caffeic acid phenethyl ester; TNF-α: tumor necrosis factor-α; NF-κB: nuclear factor κB.

INTRODUCTION

Mechanical loading with varying durations, frequencies, and amplitudes results in orthodontic tooth movement (OTM), which is induced by the application of orthodontic force for the correction of malocclusion (Krishnan and Davidovitch, 2021; Triwardhani et al., 2021a). Orthodontic force alters the local environment and blood flow, which has an impact on the homeostasis of the periodontal ligament region. The oxygen tension (O_2:CO_2 level) and the chemical environment are accelerated by the production of physiologically active substances such cytokines, growth factors, neurotransmitters, colony stimulating factors, and arachidonic acid metabolites (Arqub et al., 2021; Rahmawati et al., 2020). The biochemical and cellular processes that maintain the stable condition of the alveolar bone are set in action by this transformation. This will subsequently provide the conditions for molecular and cellular signaling in periodontal tissue (Yamaguchi and Fukasawa, 2021).

In the compression and tension parts of the periodontal ligament (PDL), chemical mediators activate cellular activity differentially, leading to bone resorption on the compression side and bone formation on the tension side. For example, a heavy force can stop the blood flow and cause cell death. Compression force is linked to a variety of other biological impacts as well (hyalinization) (Hisham et al., 2019; Narmada et al., 2019).

Orthodontically induced inflammatory root resorption, or OTM, is a kind of root resorption that occurs more frequently and changes the periodontal tissue (OIRR). It is unknown what causes OIRR, an iatrogenic condition that develops after orthodontic therapy, but it is known that it is brought on by intricate inflammatory processes involving a number of factors including mechanical pressures, tooth root shape, alveolar bone, PDL, cementum, and known biological messengers (Kojima et al., 2013; Krishnan and Davidovitch, 2021).

Tumor necrosis factor-α (TNF-α) is one among the chemical mediators and is referred to be the "master regulator" of cytokines. It participates in innate immunity and inflammatory responses. TNF-α levels were found to be higher during OTM in gingival crevicular fluid (GCF) (Kojima et al., 2013). Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα). IκBα is phosphorylated as a result of the IKK complex being stimulated by TNF-α. Nuclear factor- *kappa* B (NF-κB) is a protein that binds to it in the cytosol, leaving it inactive and leading it to remain in the cytoplasm. The proteasome breaks down phosphorylated IκBα, which causes cytosolic NF-κB to go to the nucleus and activate osteoclastogenesis while blocking osteoblast activity (Liu et al., 2017).

In almost all cell types, exposure of cells to TNF-α induces NF-κB activation. A number of genes associated to inflammation are then expressed while NF-κB moves from the cytoplasm to the nucleus (Armutcu et al., 2015). Additionally, this will stimulate the development of osteoclasts, which might lead to bone resorption during orthodontic tooth movement (OTM) (Nareswari et al., 2019). Therefore, in order to get the best treatment results, shorten OTM, and avoid OTM's negative consequences, efforts should be made to enhance alveolar bone remodeling during OTM (Inayati et al., 2020; Pramusita et al., 2020).

Propolis is a natural remedy that has been used for hundreds of years and is now available as dietary supplements (Vagis, 2014). One of the most bioactive substances detected in bee propolis is caffeic acid phenethyl ester (CAPE). CAPE has anti-inflammatory and antioxidant, that make it a viable option to root canal irrigation solutions (Tosun and Karataslioglu, 2010). CAPE treatment during the animal model's tooth extraction resulted with faster repair of alveolar bone abnormalities (Günay et al., 2014). In endotoxin-induced periodontitis, CAPE also improves bone repair. Previous research has also discovered that CAPE suppresses osteoclast activity in orthodontic tooth movement (Kızıldağ et al., 2019). In orthodontics, CAPE may also increase the proportion of osteoblasts on the tension side while reducing the proportion of osteoclasts on the compression side (Narmada et al., 2021). Additionally, this study aims to examine if the administration of CAPE affects TNF-α and NF-κB during experimental OTM in male Wistar rats (*R. novergicus*).

MATERIAL AND METHODS

The experimental study design and ethical clearance approval

This study used an analytical experimental design with a randomized post-test only control group. Healthy male Wistar rats (n = 48), weighing 200–250g and aged 16–20 weeks, were divided into two groups of 24 rats each, group K (positive control, OTM application) and group KP (OTM application and CAPE

dose of 0,5 mg). Each group was then divided into three subgroups based on the day of observation: days 3, 7, and 14. The age of Wistar rats was determined by the size of the rats' jaws, which must be large enough to hold medications (Turner et al., 2011). Lameshow's formula was used to determine the minimum sample size, and eight samples were given to each group to allow for the possibility of animal dropout (Narmada et al., 2021). The Airlangga University (UNAIR) Faculty of Dental Medicine's health ethical clearance committee approved the use of experimental animals in this study with approval number 529/HRECC.FODM/IX/2021.

Preparation and provision of caffeic acid phenethyl ester

Phenethyl caffeate ($C_{17}H_{16}O_4$) (CAPE) was dissolved in 10 mL distilled water at a dose of 20 mg/kg (cat. no. 104594-70-9, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The solution was given once daily by oral gavage for 1, 7, or 14 days, depending on the experimental group (Park et al., 2004). The dose of CAPE used was chosen because it has previously been shown to cause the maximum effect against inflammation in rodents (Al-Hariri et al., 2021; Park et al., 2004).

Experimental tooth movement in the animal model

The Faculty of Veterinary Medicine UNAIR provided all of the experimental animals used in this study, and the Faculty of Dental Medicine UNAIR's Research Center will carry out the immunohistochemistry analysis. To lessen the stress brought on by the changing setting, the male Wistar rats were habituated to the new habitat for seven days before treatment. Each rat was kept in a controlled environment with a 12-hour light/dark cycle, a constant temperature of 25°C, and a humidity of 50% using polycarbonate cages (0.90 × 0.60 × 0.60 m). All test animals were fed a standard pellet diet and had unrestricted access to drinking water. Every day, the animal cages were examined for signs of food and drink consumption as well as fecal characteristics, and cage hygiene was upheld. Using a digital scale, the weight of each animal was recorded before and after therapy.

The experimental animals were given ketamine (100 mg/kg bw) and xylazine (5 mg/kg bw) to make them more comfortable during the installation of the OTM apparatus. The strength of the NiTi closed coil spring was assessed prior to installation using a tension gauge to deliver a force of 10 g/mm^2. An 8.0 mm long nickel titanium closed coil spring from American Orthodontics (AO), the United States, was placed between the upper left first molar and upper central incisor to move the molar mesially, producing the experimental tooth movement in animal models. A 0.07 stainless steel ligature wire was used to hold the maxillary central incisive and first molars in place (Hermawan et al., 2020; Triwardhani et al., 2021b). Oral gavage was used to administer the 20 mg/kg bw CAPE supplementation.

The cervical dislocation method was then used to assess all samples on the specified days (days 1, 7, and 14) for each group. Making the animal as comfortable as possible throughout the termination operation is the aim of this action. The damaged premaxilla tissue was removed and immersed in 10% formalin for 4 days as part of the fixation procedure (OneMed; Sidoarjo, Indonesia). The premaxilla was immersed in 10% EDTA (OneMed) for two months after the fixation process. After the sample had been prepared for tissue, it was dried in a graduated sequence of ethanol before being embedded in paraffin. On a 5 m rotary microtome, the sections were cut (RM2235; Leica, United States). Before being dried at 60°C for 16 days, paraffin ribbons were flattened in a 40°C water bath and put on Polysine microscope slides by Thermo Scientific in the United States (Sakura Heater; Tokyo, Japan) (Savi et al., 2017).

TNF-α and NF-κB expression immunohistochemistry staining

TNF-α (1:200 dilution) antibody was employed in this study to detect TNF-α expression, whereas NF-κB (1:200 dilution) antibody was used to detect NF-κB expression. In addition, 3.3′-diaminobenzidine stain kit (Sigma Aldrich, United States) was used for immunohistochemical staining, and hematoxylin-eosin (HE) was used for counterstaining (Sigma Aldrich). The number of TNF-α and NF-κB expressions in the alveolar bone were detected and estimated on the compression side (Narmada et al., 2021). Using a Nikon H600L light microscope (Japan) at 400× magnification, two observers manually observed and computed in five perspective fields of vision.

Statistical analysis

Results are expressed as mean ± SD (n = 7). Analysis of the research data was preceded by a normality test using the Kolmogorov–Smirnov's test ($p>0.05$), after which the homogeneity test using the Levene's statistic test ($p>0.05$) was conducted. A one-way analysis of variance test was conducted to determine whether there was a difference between the two treatment groups ($p<0.05$). Furthermore, Tukey's honest significant difference test was performed to find out the differences in each treatment group ($p<0.05$). The Statistical Package for Social Science (SPSS) 20.0 version (IBM Corporation; Illinois, Chica-

go, United States) software was used in this study to analyze the data.

RESULTS

CAPE did not cause overall toxicity, edema, mortality, or changes in the animal model bw at the given doses. It is worth mentioning that during OTM on the compression side, all experimental groups revealed positive TNF-α expression in the Wistar rat's alveolar bone (Fig. 1). NF-κB was likewise shown to be expressed favorably in the osteoclasts of the Wistar rat's alveolar bone during OTM on the compression side in all experimental groups (Fig. 2). The data in this investigation were all homogenous and evenly distributed ($p>0.05$). The K1 group had the greatest TNF-α expression, whereas the KP3 group had the lowest. As a result, the K3 group had the highest NF-κB expression, whereas the KP3 group had the lowest. There was a substantial difference between the groups on the TNF-α and NF-κB expression compression side ($p<0.05$). (Table 1). Table 2 compares TNF-α and NF-κB expression on the compression side of the groups.

Table 1. TNF-α and NF-κB expression in the compression side during OTM.

Group	TNF-α	p-value	Group	NF-κB	p-value
K1	11.71 ± 2.81		K1	9.28 ± 2.49	
K2	13.14 ± 2.34		K2	11.57 ± 2.07	
K3	14.00 ± 3.00	0.01*	K3	12.71 ± 2.69	0.001*
KP1	5.86 ± 1.57		KP1	5.71 ± 1.25	
KP2	5.43 ± 1.90		KP2	4.00 ± 1.41	
KP3	5.43 ± 1.99		KP3	3.43 ± 162	

Data are expressed as mean ± SD (n = 7) KP1: 3 days of OTM and CAPE; KP2: 7 days of OTM and CAPE; and KP3: 14 days of OTM and CAPE. K1: 3 days of OTM; K2: 7 days of OTM; K3: 14 days of OTM. OTM: Orthodontic tooth movement; CAPE: Caffeic acid phenethyl ester.

Table 2. Tukey's HSD comparison between TNF-α and NF-κB expression on the compression side during OTM of the groups.

Group	Compared Group	TNF-α	NF-κB
K1	K2	0.258	0.039*
	K3	0.074	0.030*
	KP1	0.001*	0.003*
	KP2	0.001*	0.001*
	KP3	0.001*	0.001*
K2	K3	0.495	0.292
	KP1	0.001*	0.001*
	KP2	0.001*	0.001*
	KP3	0.001*	0.001*
K3	KP1	0.001*	0.001*
	KP2	0.001*	0.001*
	KP3	0.001*	0.001*
KP1	KP2	0.732	0.117
	KP3	0.732	0.039*
KP2	KP3	1.000	0.596

The comparison between groups is significantly different by ANOVA followed by a Tukey's test ($p<0.05$). KP1: 3 days of OTM and CAPE; KP2: 7 days of OTM and CAPE; and KP3: 14 days of OTM and CAPE. K1: 3 days of OTM; K2: 7 days of OTM; K3: 14 days of OTM. OTM: Orthodontic tooth movement; CAPE: Caffeic acid phenethyl ester.

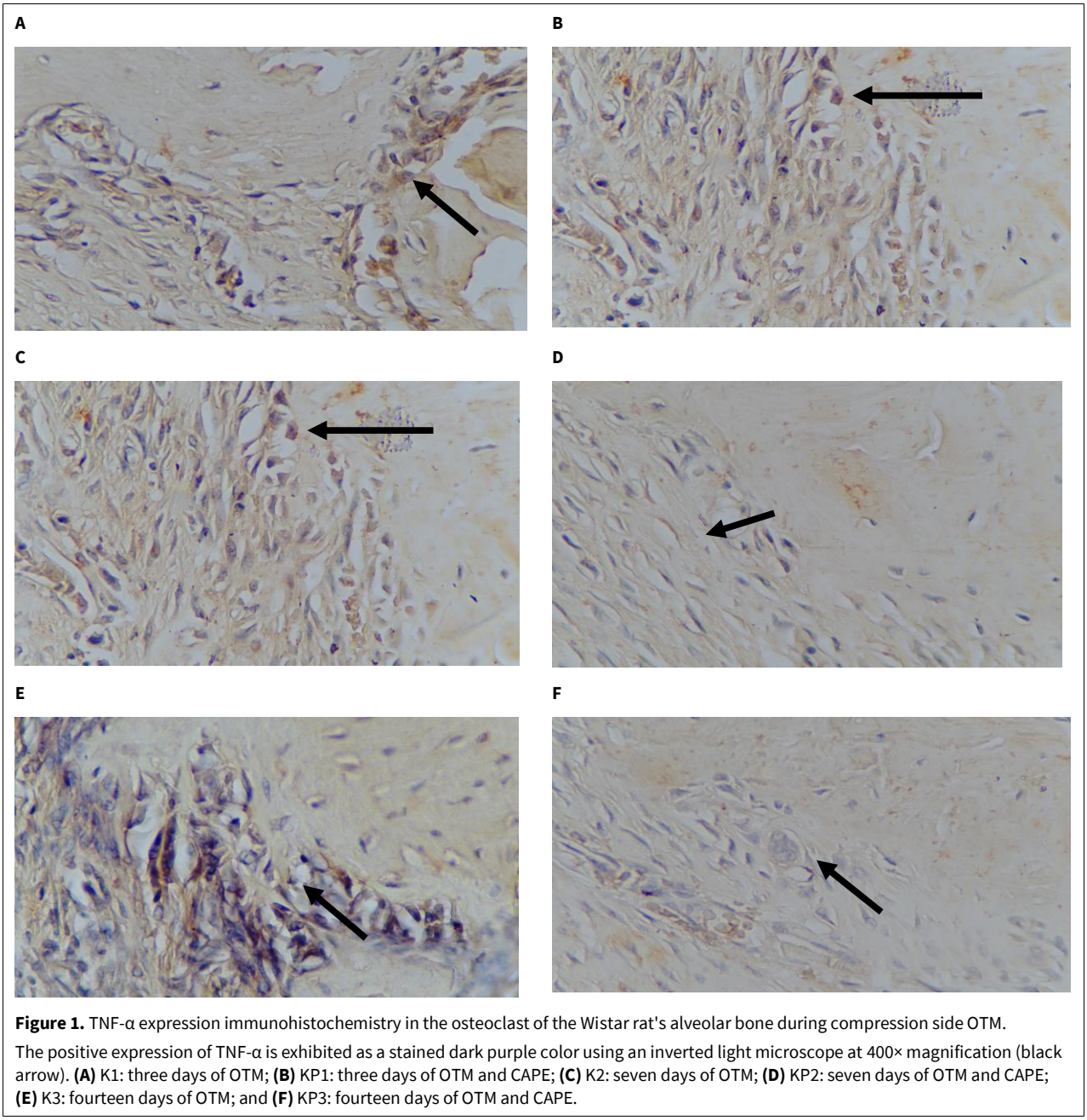

Figure 1. TNF-α expression immunohistochemistry in the osteoclast of the Wistar rat's alveolar bone during compression side OTM.

The positive expression of TNF-α is exhibited as a stained dark purple color using an inverted light microscope at 400× magnification (black arrow). **(A)** K1: three days of OTM; **(B)** KP1: three days of OTM and CAPE; **(C)** K2: seven days of OTM; **(D)** KP2: seven days of OTM and CAPE; **(E)** K3: fourteen days of OTM; and **(F)** KP3: fourteen days of OTM and CAPE.

DISCUSSION

During the OTM procedure, tissue resorption and development in the surrounding bone and periodontal ligament were synchronized. Tooth loading causes local hypoxia and fluid flow, resulting in an aseptic inflammatory cascade with osteoclast resorption in compression zones and osteoblast deposition in tension zones, OIRR may occur from this syndrome (Li et al., 2018).

CAPE has been shown in animal studies to have anti-inflammatory, antioxidant, and anti-allergic properties (Zhang et al., 2014). This study was carried out on Wistar rats that had been given CAPE throughout OTM. For 4, 7, or 14 days, a force of up to 10 g/mm^2 was applied using a closed coil spring. In rats, the optimal and effective force for inducing experimental tooth movement was less than 10 g/mm^2 (Narmada et al., 2021; Nugraha et al., 2020; Sitasari et al., 2020) In the earlier study, it was also reported that systemic injection of CAPE is more effective than local application for bone repair (Kızıldağ et al., 2019). As a result, 20 mg/kg body weight CAPE orally was given in this trial.

From day 4 (K1) through day 14, the control group's TNF-α expression has been demonstrated to increase (K3). The treatment groups' TNF-α expression is at its greatest on day 4 (KP1), then declines on days 7 (KP2) and 14 (KP3) (KP3). The findings of Krishnan and Davidovitch (2021) showed that orthodontic tooth movement in animals significantly

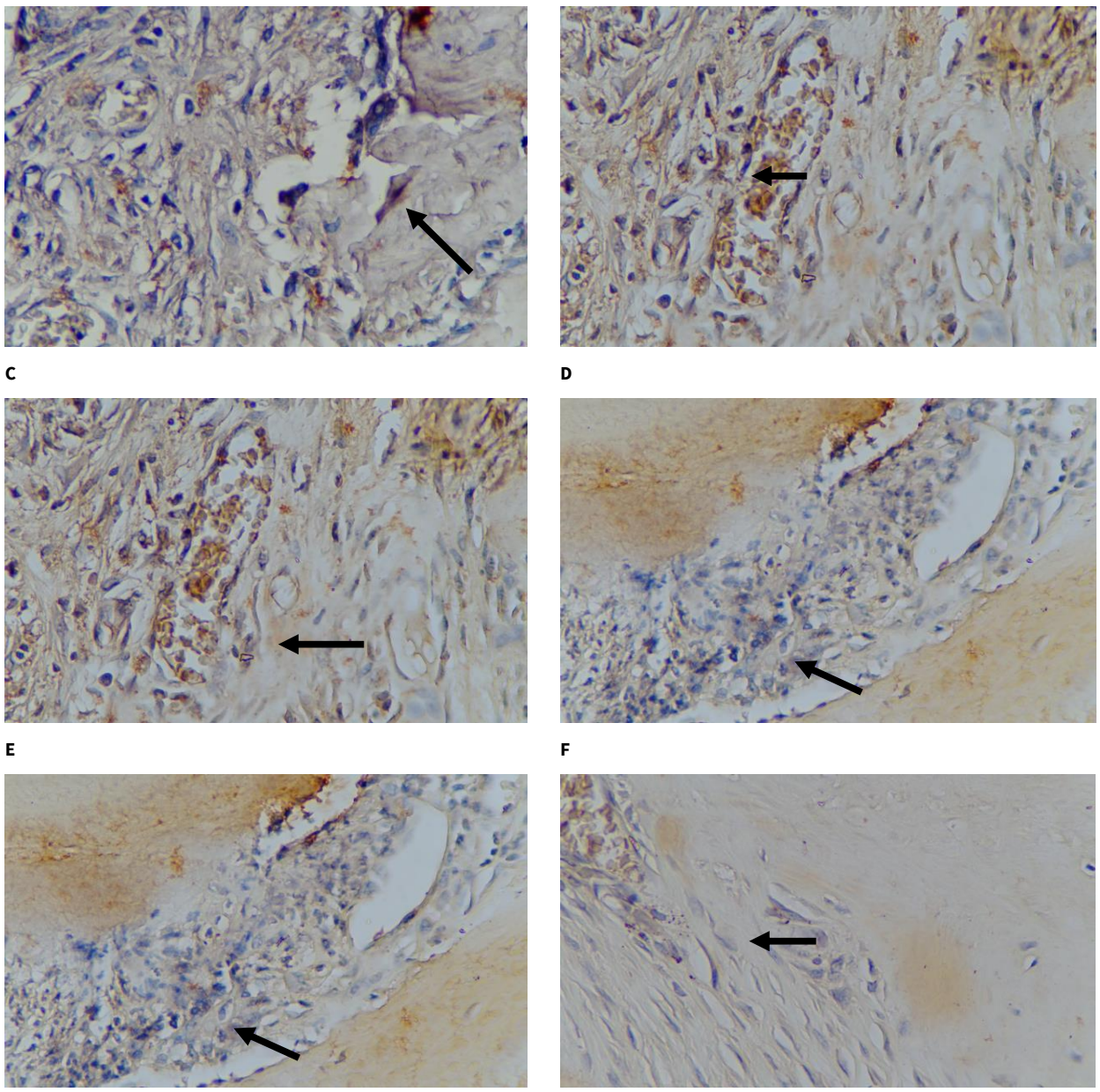

Figure 2. The immunohistochemistry results of NF-κB expression in the osteoclast of the Wistar rat's alveolar bone during OTM on the compression side.

The positive expression of NF-κB is revealed by a stained dark purple hue under an inverted light microscope at x400 magnification (black arrow). **(A)** K1: three days of OTM; **(B)** KP1: three days of OTM and CAPE; **(C)** K2: seven days of OTM; **(D)** KP2: seven days of OTM and CAPE; **(E)** K3: fourteen days of OTM; and **(F)** KP3: fourteen days of OTM and CAPE.

increased TNF-staining intensity in cells of the PDL and alveolar bone. According to Kook et al. (2011), compression forces *in vitro* increase the expression of the genes for TNF-α and osteoclastogenesis in human periodontal ligament (hPDL) cells. As a result of OTM tension, the periodontal ligament (PDL) experiences oxidative stress, which leads to the migration of inflammatory cytokines, including TNF-α. Almost all cell types experience NF-κB activation stimulation from TNF-α. When NF-κB moves from the cytoplasm to the nucleus, numerous genes involved in inflammation start to express themselves (Armutcu et al., 2015).

From day 4 (K1) through day 14 (K3) of this study, the control group's NF-κB expression increased, with day 14 having the greatest NF-κB expression (K3). In contrast, NF-κB expression decreased in the treatment groups from day 4 (KP1) to day 14 (KP3), with day 4 (KP1) being the greatest and day 14 the lowest (KP3). This is consistent with Zuo et al. (2007) finding, which showed that OTM stimuli raise NF-κB levels. A prior

study that showed CAPE therapy can lower NF-κB expression support this as well (Natarajan et al., 1996)

The activation of NF-κB caused by a variety of inflammatory stimuli, such as TNF-α, phorbol ester ceramide, okadaic acid, and H_2O_2, was completely inhibited by CAPE. The anti-inflammatory properties of CAPE are obtained during inflammation via the lipoxygenase route of arachidonic acid metabolism, and they help to lessen NF-κB activation brought on by reduced ROS (Armutcu et al., 2015). According to Toyoda et al. (2009), CAPE treatment inhibited IB breakdown and p65 phosphorylation, which in turn decreased *Helicobacter pylori*-induced NF-κB activation. Local CAPE treatment was found to increase leukocyte apoptosis and significantly reduce the number of neutrophils and monocytes in the exudate from the inflamed site (Al-Hariri et al., 2021).

One goal of OTM is to efficiently reposition and align the teeth with the least amount of damage to the teeth and surrounding tissue (Von Böhl et al., 2009). Alveolar bone remodeling balance must be carried out to attain optimal OTM. Anti-inflammatory and antioxidant properties of CAPE may promote bone formation and inhibit bone resorption (Murtaza et al., 2014; Narmada et al., 2021). Our research indicates that CAPE treatment can slow the OTM rate by suppressing TNF-α and NF-κB expression. Adjuvant herbal-based therapy that includes CAPE, which is present in propolis, maybe a possibility for avoiding recurrence after orthodontic treatment or OIRR brought on by excessive OTM force.

CONCLUSION

According to immunohistochemistry, administering CAPE throughout OTM can effectively diminish the quantity of TNF-α and NF-κB expression on the compression side. However, more research is needed to evaluate different inflammatory molecular markers *in vitro* and *in vivo* using diverse methodologies.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Salikha K	Narmada IB	Alida	Nugraha AP	Sari AF	Riawan W	Noor TNEBTA
Concepts or ideas	x	x	x	x	x		
Design	x	x	x	x			
Definition of intellectual content	x	x	x	x		x	
Literature search	x			x			
Experimental studies	x			x	x	x	x
Data acquisition	x			x	x	x	x
Data analysis	x			x	x	x	x
Statistical analysis	x	x	x	x	x	x	x
Manuscript preparation	x	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x

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