



East Java green tea methanolic extract can enhance RUNX2 and Osterix expression during orthodontic tooth movement *in vivo*

[Extracto metanólico de té verde de Java Oriental puede mejorar la expresión de RUNX2 y Osterix durante el movimiento de diente de ortodoncia *in vivo*]

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Abstract

Context: Alveolar bone remodeling is important to achieve an optimal Orthodontic Tooth Movement (OTM). Runt-related transcription factor 2 (RUNX2) and Osterix (OSX) expression are important for bone remodeling. Green tea (*Camelia sinensis*) can enhancing bone remodeling.

Aims: To investigate the effect of a methanolic extract of green tea (MEGT) on the OSX and RUNX2 expression during OTM in Wistar rats.

Methods: The experiment with post-test only and simple random sampling was conducted. The samples consisted of twenty-eight Wistar rats, which were then divided into 4 groups (n=7), negative control (CN) group, positive control (CP) group with OTM but without MEGT administration, group with OTM for 14 days and MEGT administration from day 7 to day 14 (T1), group with OTM with MEGT administration for 14 days (T2). Nickle-titanium coil spring with 10 g/mm² force was placed between the incisors and the maxillary molars. MEGT was collected from East Java and identified its principal metabolite by HPLC analysis. RUNX2 and OSX expression were analyzed by utilizing Immunohistochemical analysis. Analysis of Variance (ANOVA) was performed and then continued with least significant difference (p<0.05).

Results: The highest RUNX2 and OSX expression were found in the tension side of group T2 with significant difference between groups (p<0.05). The principal metabolite of the extract was identified as epigallocatechin-3-gallate (EGCG).

Conclusions: Post administration of a MEGT could increase RUNX2 and OSX expression in alveolar bone of OTM Wistar rats. Part of this action could be attributed to the presence of EGCG in the extract.

Keywords: alveolar bone remodeling; epigallocatechin-3-gallate; orthodontic tooth movement; Osterix; RUNX2.

Resumen

Contexto: La remodelación ósea alveolar es importante para lograr un movimiento óptimo de los dientes de ortodoncia (OTM). La expresión del factor de transcripción 2 relacionada con Runt (RUNX2) y Osterix (OSX) es importante para la remodelación ósea. El té verde (*Camelia sinensis*) puede mejorar la remodelación ósea.

Objetivos: Investigar el efecto de un extracto metanólico de té verde (MEGT) sobre la expresión de OSX y RUNX2 durante OTM en ratas Wistar.

Métodos: Se realizó el experimento con un muestreo aleatorio simple y una prueba *a posteriori*. Las muestras consistieron en veintiocho ratas Wistar que luego se dividieron en 4 grupos (n = 7), grupo de control negativo (CN), grupo de control positivo (CP) con OTM pero sin administración de MEGT, grupo con OTM durante 14 días y MEGT administración del día 7 al día 14 (T1), grupo con OTM con administración de MEGT durante 14 días (T2). Se colocó un resorte helicoidal de níquel-titanio con una fuerza de 10 g/mm² entre los incisivos y los molares maxilares. El té verde fue colectado en Java Oriental e identificado su metabolito principal por análisis de HPLC. La expresión de RUNX2 y OSX se analizó utilizando análisis inmunohistoquímico. El análisis de varianza (ANOVA) se realizó y luego continuó con diferencia significativa (p<0,05).

Resultados: La mayor expresión de RUNX2 y OSX se encontró en el lado de tensión del grupo T2 con una diferencia significativa entre los grupos (p<0,05). El metabolito principal en el extracto fue identificado como 3-galato de epigallocatequina (EGCG).

Conclusiones: La administración posterior de MEGT podría aumentar la expresión de RUNX2 y OSX en OTM del hueso alveolar de ratas Wistar. Parte de esta acción podría ser atribuida a la presencia de EGCG en el extracto.

Palabras Clave: epigallocatequina-3-galato; movimiento de dientes de ortodoncia; Osterix; remodelación ósea alveolar; RUNX2.

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INTRODUCTION

Orthodontic Tooth Movement (OTM) is a treatment that moves the teeth to obtain good teeth alignment and achieves the aesthetic and occlusion function. The teeth can move in the alveolar bone in orthodontic force due to the mechanical changes in the biological system that cause stretching and then stimulating the cellular response and the occurrence of remodeling in the periodontal ligament as well as the alveolar bone around the tooth, which receives the orthodontic force (Narmada et al., 2019a;b). OTM can only occur in the event of bone remodeling and periodontal tissue around the teeth. The movement of teeth will not happen if the bone remodeling does not occur (Krishnan and Davidovitch, 2006). Bone remodeling combines rapidly the process that begins with bone resorption and is followed by a bone apposition process, which is an important factor for the movement of teeth (Narmada et al., 2019a;b). The remodeling process is used to maintain the thickness of bones and the relationship between dental and alveolar bones in order to be relatively constant (Nugraha et al., 2018a;b). Factors that can affect the occurrence of relapse are bone resorptions, which experience the onset of teeth movement nine times greater than the apposition bones, thus, it enables a greater relapse. The bone apposition acceleration process can be done by increasing the osteoblast cell proliferation (Sutjiati et al., 2017).

In the adult patients with fixed orthodontic treatment, a marginal bone loss is often found. Orthodontic treatment plan should be considered local and general conditions of periodontal tissue (Alansari et al., 2015). An increasing number of adult patients leads many researchers now to focus on the discovery of methods to accelerate the movement of teeth, which provides shorter maintenance time. Metabolism in adults is much slower than in younger patients and the time required for treatment in adults is significantly greater than those taken in adolescents (Singh et al., 2011). The bone remodeling process used to

make bone and alveolar bone is relatively constant (Sutjiati et al., 2017). According to Indayani, the factors that influence relapses are related to the bone resorption, which is nine times greater than the relapse bone apposition. To improve the process of apposition, it can be done by increasing osteoblast cell proliferation. The prevalence of post-orthodontic relapse treatment is generally quite high, according to Sheibani et al. (2010), it was 52 of 200 cases (26%), a minimum of 500 patients were treated, and the prevalence of relapse was 61.5%. It was important to prevent relapse by increasing the bone apposition.

Green tea (*Camellia sinensis* (L.) Kuntze, family *Theaceae*) is one of the most popular beverages to be consumed on a regular basis and is strongly associated with high antioxidant effects. Many studies explain that the chemotherapy effect of green tea is the content of polyphenols called catechins. The richest catechins in green tea is epigallocatechin-3-gallate (EGCG) (Legeay et al., 2015). The effects of EGCG is on the bone density in osteoporosis. EGCG may increase the bone resorption by affecting the bone remodeling mechanisms (Shen et al., 2009). EGCG stimulates the bone density in the area around the orthodontic microimplants (Twafeeq et al., 2017).

The differentiation of osteoblasts and osteoclasts is controlled by signal transduction and complex gene transcription. Some key factors of transcription for osteoblasts are Runt-related transcription factor 2 (RUNX2) and Osterix (OSX) (Nugraha et al., 2018a; Chen et al., 2015). Several osteoblasts signaling pathways are found that transcriptional enhancement of RUNX2 and OSX expressions lead directly to the increased osteoblast formation (Komori, 2006).

The hypothesis of this study was a methanolic extract of green tea from East Java, rich in EGCG, could enhance the RUNX2 and OSX expression in OTM animal model. Thus, the aim of this study was to investigate the effect of a methanolic extract of green tea on the RUNX2 and OSX expression during OTM in Wistar rats (*Rattus norvegicus*).

MATERIAL AND METHODS

Ethical clearance

All experimental procedures involving animals were carried out in accordance with the Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines to ameliorate any suffering of the animals. Ethical clearance was obtained from the Research Ethics Committee of the Faculty of Dentistry, Airlangga University (Indonesia) with reference number 074/HRECC.FODM/III/2019.

Plant material

The green tea was purchased from Perkebunan Nusantara XII Company, Wonosari Agro Tourism, Malang, East Java, Indonesia.

Preparation of the green tea extract and the identification of EGCG

The maceration and reflux methods were used to obtain the methanolic extract of green tea (MEGT). Every 2 g dried green tea leaves that had been dried was refluxed separately in 60 mL methanol at 60°C. The green tea extract was filtered under vacuum while it was hot. The extract was then washed with 20 mL methanol while carried on the filtration. After filtration, it was used the rotary evaporator under vacuum to get rid the residue of methanol. This procedure was performed in duplicate. The identification of phenolic compounds was done using an HPLC system (Agilent 1260, Agilent Technologies, German) consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 400 bar. In this study was performed two methods (maceration and reflux) to compare in which of them could obtain higher percentage of EGCG.

Preparation of orthodontic tooth movement in animal study

All the rats were placed individually in polycarbonate cages (0.90 × 0.60 × 0.60 m) for a week on a 12-h light/dark cycle at a steady temperature of 25°C and humidity of 50% for the acclimatization to compensate for their various origins. Ani-

mals were fed with a standard pellet diet with tap water ad libitum and routinely inspected for food consumption and fecal characteristics. Sample size (n=3) was determine based on Lemeshow's formula. The sample was 28-healthy-male Wistar rats (n=7), aged between 16-20 weeks-old with weighted around 200-250 g and selected blind-randomly into control and treatment groups.

The animals were separated into four groups: 1) negative control (CN) without OTM and without MEGT administration, 2) positive control (CP) with OTM and PBS administration for 14 days, 3) treatment 1 (T1) with OTM for 14 days and MEGT administration from day 7 to day 14, 4) treatment 2 (T2) with OTM with MEGT administration for 14 days.

The orthodontic tooth movement was done by 8.0 mm-long Nickle-titanium coil spring (Ortho technology, China), which was placed between the maxillary central incisors to move the molar towards the mesial and was fixed using 0.07 stainless steel ligature wire around the 2-maxillary incisor with 10 g/mm force measured using a tension gauge (Ortho technology, China). Then the administration of 4.91% MEGT with dose of 150 mg/kg body weight (bw) of rat daily was performed peroral. All samples were then sacrificed by rodent anesthesia (60 mg/kg bw of ketamine and xylazine 3 mg/kg bw (Sigma-Aldrich, USA). Rat's premaxillae were dissected and placed in 10% formalin (OneMed, Indonesia) for four days. After fixation, the springs were removed, and the premaxillae were decalcified with 5% nitric acid (OneMed, Indonesia) for 1-month days. The decalcified premaxillae were fixed again in the same manner for another 1 month. The sample was then dehydrated in a graded series of ethanol and embedded in paraffin according to previous method (Narmada et al., 2019b).

Immunohistochemistry staining

Samples were then examined by immunohistochemistry staining by using a 3,3'-diaminobenzidine stain kit (Sigma-Aldrich, USA) with the counter staining of hematoxylin eosin (Sigma-Aldrich, USA). Monoclonal antibodies of RUNX2

and OSX were used in this study (Santa Cruz Biotechnology™, USA). The examination was carried in five different visual fields by using Nikon H600L light microscope (Japan) at 1000x magnification with a 300 megapixels Fi2 DS digital camera and image processing software of Nikon Image System (Nikon, Japan).

Statistical analysis

The data were analyzed by Statistical Package for the Social Sciences 20.0 software (SPSS for Windows, SPSS, Chicago, USA). The descriptive statistics were given as means \pm Standard Deviation (SD). Shapiro-Wilk and Levene's test ($p > 0.05$) was completed then continued with one-way analysis of variance (ANOVA) and least significant difference ($p < 0.05$) to compare the RUNX2 and OSX expression between groups.

RESULTS

RUNX2 and Osterix expression

The positive expression of RUNX2 in the pressure and tension side (Fig. 1A-B) and OSX in the pressure and tension side (Fig. 2A-B) were confirmed by using IHC staining in all groups. The data showed that the highest expression of RUNX2 (Fig. 1C-D) and OSX in T2 compared to other group in tension side (Fig. 2C-D). Meanwhile, in pressure side, the highest expression of RUNX2 and OSX was found in CP. There was a significant difference in tension side and pressure side between groups in RUNX2 and OSX expression ($p < 0.05$).

Identification of EGCG

In the Fig. 3 is showed the percentage of EGCG detected by HPLC in the methanolic extracts of green tea using maceration or reflux extract with 4.37 and 4.14%, respectively.

DISCUSSION

In this study we found that, on the pressure side, there is an increased expression of RUNX2 in the T1 and T2 groups compared to CP. This is consistent with previous study on the effects of Ro-

busta for orthodontic tooth movement, which stated that osteoblasts play an important role in bone formation to reshape the resorption area on the pressure side and to form new bone on the tension side (Uribea et al., 2011; Herniyati et al., 2018). The RUNX2 expression of the pressure area is increasing but lower than the CN group, because in the CN group there is no continuous pressure, which stimulates the inflammatory process.

There was an increase in OSX expression for CN and CP groups compared to the T1 and T2 groups. The increased expression of OSX will stimulate an increased RUNX2 expression because OSX is a co-factor of RUNX2. OSX and RUNX2 play an important role for DNA transcription in stromal cells to differentiate osteoblasts in both early and late phase (Nugraha et al., 2018a;b). RUNX2 and OSX phosphorylation by p38 and ERK signaling will then form the integration point of extracellular stimulation leading to the strong modulation of DNA transcription activity and controlling osteoblastic phenotype (Chen et al., 2009; Artigas et al., 2014). OSX is a derivative of RUNX2 in the transcriptional differentiation cascade of osteoblastogenesis (Mori et al., 2013). Consistently, OSX expression is positively regulated by the direct binding of RUNX2 to the responsive element in the OSX gene promoter. This is in line with previous study, which stated that an increased expression of RUNX2 and OSX in the pressure side is reasonable because it is a process for upregulation of bone formation through Wnt signaling (Mao et al., 2018). The increased expression of RUNX2 and OSX in T1 is lower than T2, this result is similar with the previous study conducted, which mentioned that OTM in mice can be seen after day 2 and decrease annually within 2 weeks (Ren et al., 2004). The lag phase of OTM in rats occurs from day 6 to day 15. In this phase, the secretion of cytokines for bone remodeling is increased (Ghajar et al., 2013).

Green tea leaves consist of 26% fiber, 15% protein, 2-7% lipids, 5% vitamins and minerals, secondary metabolites as 1-2% pigment, 30-40% polyphenols with at least 80% flavonoids and 3-4% methylxanthines (Legeay et al., 2015). More than 80% of green tea polyphenols are catechins

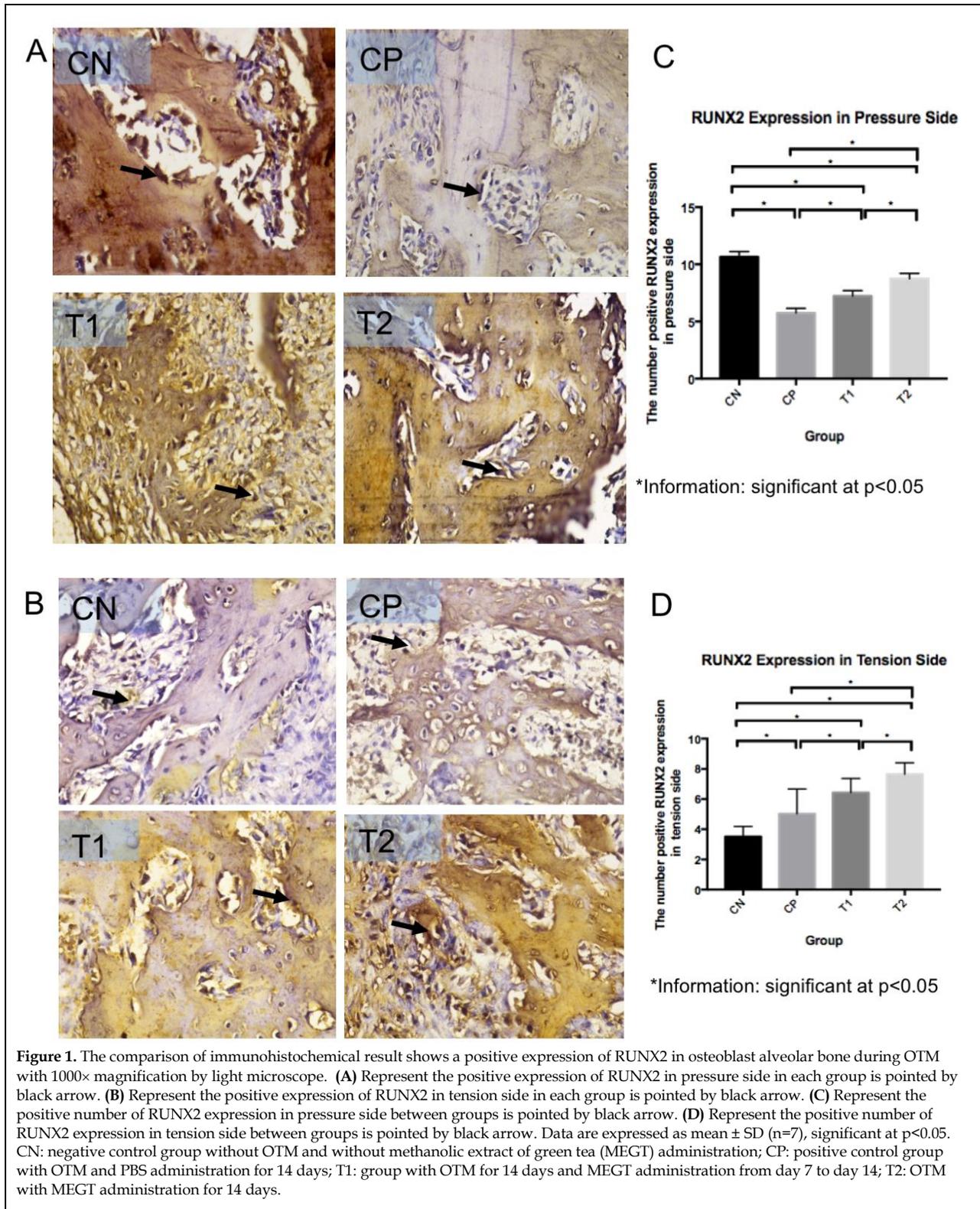
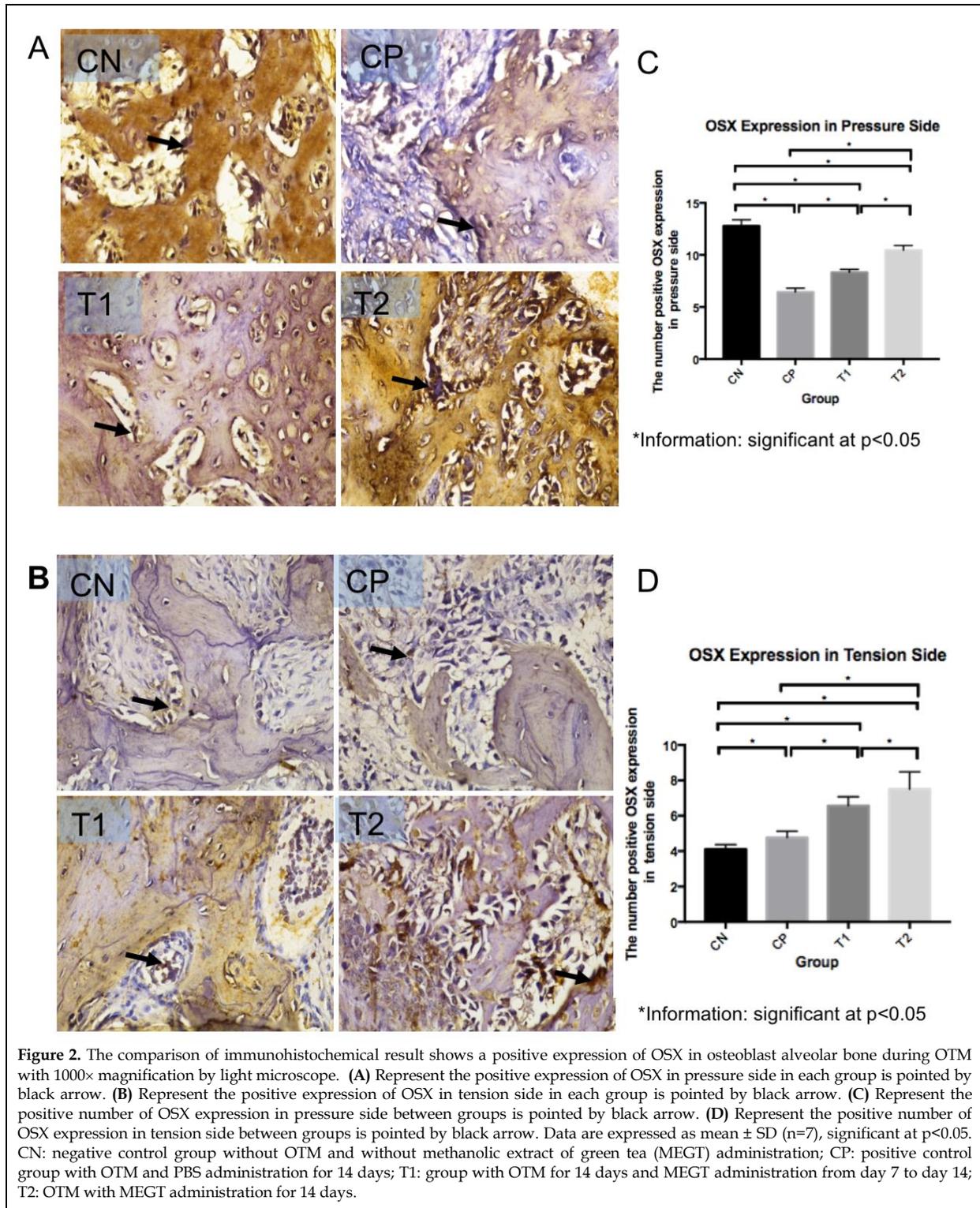


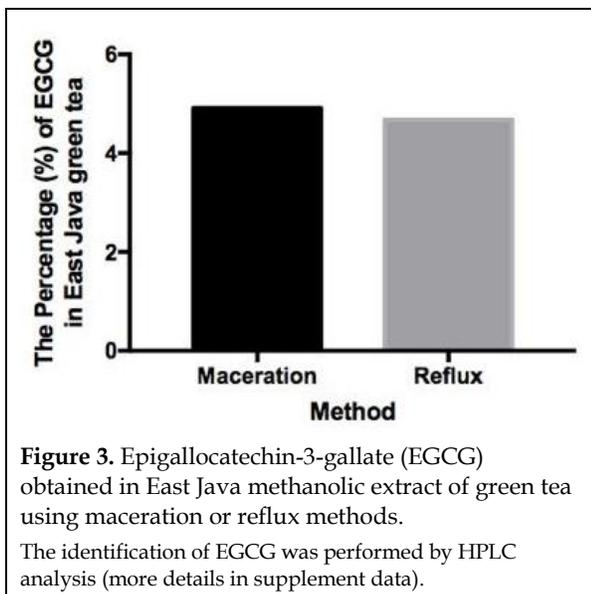
Figure 1. The comparison of immunohistochemical result shows a positive expression of RUNX2 in osteoblast alveolar bone during OTM with 1000× magnification by light microscope. **(A)** Represent the positive expression of RUNX2 in pressure side in each group is pointed by black arrow. **(B)** Represent the positive expression of RUNX2 in tension side in each group is pointed by black arrow. **(C)** Represent the positive number of RUNX2 expression in pressure side between groups is pointed by black arrow. **(D)** Represent the positive number of RUNX2 expression in tension side between groups is pointed by black arrow. Data are expressed as mean ± SD (n=7), significant at p<0.05. CN: negative control group without OTM and without methanolic extract of green tea (MEGT) administration; CP: positive control group with OTM and PBS administration for 14 days; T1: group with OTM for 14 days and MEGT administration from day 7 to day 14; T2: OTM with MEGT administration for 14 days.



(Chen et al., 2015). EGCG (epigallocatechin-3-gallate) is the content commonly found in green tea, which is around 50-80% of 200-300 mg green tea using a purifying method (Legeay et al., 2015).

In this study, we focused on the identification of EGCG as the most abundant active compound in green tea extract (about 4.3% in the extract) using a standard procedure. EGCG has beneficial role to

enhance the bone remodeling. EGCG is very useful to accelerate bone remodeling because it can reduce the function of osteoclasts while increase osteoblasts through the enhancement of RUNX2 expression (Vali et al., 2007; Wang et al., 2016). The results of this study are in accordance with the previous study on EGCG, which explains that the administration of EGCG will increase the osteoblastogenesis by the increased the osteogenic differentiation of mesenchymal stem cells (MSC) through the stimulation of RUNX2, OSX and alkaline phosphatase (ALP) expression. The post administration of EGCG in human bone marrow MSC *in vivo* increases the RUNX2 expression on the 3rd, 7th and 14th day (Zhang et al., 2019). The effect of EGCG administration is proven to be able to stimulate the bone density around orthodontic micro implants through RUNX2 expression stimulation (Twafeeq et al., 2017).



There are several main mechanisms of bone remodeling acceleration by EGCG in green tea extract; such by reducing the bone resorption through antioxidant pathway, anti-inflammatory pathway, acceleration of osteoblastogenesis through Wnt signaling (Singh et al., 2011). Tumor necrosis factor- α (TNF- α) is an inflammatory cytokine that initiates bone resorption in OTM, especially on the pressure side. TNF- α will stimulate monocytes and macrophages to start the osteoclas-

togenesis around the tooth area. TNF- α can induce osteoblast apoptosis and reduce the osteoblast differentiation *in vitro*. ALP, RUNX2, OSX and Wnt signaling, which are important marker for bone remodeling, which can be suppressed by TNF- α through oxidative stress or activation of nuclear factor *kappa*-B (Liu et al., 2016).

EGCG as an anti-inflammatory agent will increase the osteoblastogenesis to compensate bone resorption that occurs during OTM by suppressing the production of TNF- α and IL-6 (Lin et al., 2018). Low concentrations of TNF- α increase osteogenic differentiation through regulation of RUNX2, OSX, Osteocalcin, and ALP. TNF- α can inhibit osteoblastogenesis in the presence of pre-osteoblast. This inhibition may occur at a different level, first TNF- α induces Dickkopf WNT signaling pathway inhibitor 1 (DKK-1) expression, which leads to inhibition of Wnt pathway (Osta et al., 2014). The inflammatory process is very important for OTM, but an irregular or excessive inflammation is a burden. The excessive and pro-long duration of inflammation during OTM can lead to orthodontic-induced root resorption (OIRR) (Wauquier et al., 2009). The cellular mechanism of OIRR is similar to osteoclastic bone resorption and correlates with the increased RANKL expression and the reduced OPG expression in the periodontal ligament. EGCG can control and prevent the excessive inflammatory response during OTM (Li et al., 2018).

CONCLUSIONS

Based on its molecular aspect, the alveolar bone of OTM Wistar rats post administered with a methanolic extract of East Java green tea demonstrated increased expression of RUNX2 and Osterix compared to that without the extract administration. This action could be attributed, in part, to the presence of epigallocatechin-3-gallate within the extract.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found at http://jppres.com/jppres/pdf/vol8/jppres19.787_8.4.290.suppl.pdf

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AUTHOR CONTRIBUTION:

Contribution	Sitasari PI	Narmada IB	Hamid T	Triwardhani A	Nugraha AP	Rahmawati D
Concepts or ideas	x	x	x	x	x	x
Design	x	x	x	x	x	x
Definition of intellectual content	x	x	x	x	x	x
Literature search	x	x	x	x	x	x
Experimental studies	x	x	x	x	x	x
Data acquisition	x	x	x	x	x	x
Data analysis	x	x	x	x	x	x
Statistical analysis	x	x	x	x	x	x
Manuscript preparation	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x

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