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BASIC RESEARCH:

# Analysis of RANKL Expression Following Ethanol Administration During Orthodontic Force Application: An *In Vivo* Study

## Análisis de la expresión de RANKL tras la administración de etanol durante la aplicación de fuerza ortodóntica: un estudio *in vivo*

Dwayne Daniel Fredrick Rehatta drg[1] https://orcid.org/0000-0001-7784-3039
Ardiansyah S. Pawinru Dr. drg. Sp.Ort(K)[2] https://orcid.org/0000-0002-4763-5439
Zulfiani Syachbaniah drg. Sp.Ort[3]
Baharuddin M. Ranggang drg. Sp.Ort(K)[4] https://orcid.org/0009-0002-6573-8922
Eddy Heriyanto Habar Dr drg. Sp.Ort(K)[2] https://orcid.org/0000-0002-4228-0624
Eka Erwansyah Dr. drg. M.Kes. Sp.Ort(K)[2] https://orcid.org/0000-0002-1760-2409

[1]Resident, Department of Orthodontics, Faculty of Dentistry, Hasanuddin University, Makassar, Indonesia.
[2]Associate Professor, Department of Orthodontics, Faculty of Dentistry, Hasanuddin University, Makassar, Indonesia.
[3]Lecturer Department of Orthodontics, Faculty of Dentistry, Hasanuddin University, Makassar, Indonesia.
[4]Assistant Professor, Department of Orthodontics, Faculty of Dentistry, Hasanuddin University, Makassar, Indonesia.

Correspondence to: Ardiansyah S. Pawinru - ardiansyah.pawinru@unhas.ac.id

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ABSTRACT: The prevalence of alcohol consumption is still quite high in worldwide. As more people become aware of malocclusion, it is also reasonable to expect that some orthodontic patients drink alcohol. Impacts of alcohol (ethanol) consumption on orthodontic force still unclear. Analyze the differences and effects of ethanol on RANKL expression in bone remodeling process during application of orthodontic force. This research was *in vivo* study, involving twenty-five Wistar rats, fitted with a closed coil spring and then divided into 5 groups; negative control group A (without force and ethanol) on day 0; group K1 (K=given orthodontic force) on day-3 and K2 on day-14, and group P1 (P=given force and 20% ethanol) on day-3 and P2 on day-14. Furthermore, animals were sacrificed, preparations were made and Immunohistochemistry (IHC) was examined to calculate RANKL expression, then data analysis using independent-t and regression test. Independent sample t-test showed, K and P group had significantly differences of RANKL expression on day 3 and 14 ($p<0,05$). Regression test results showed duration of ethanol administration significantly affected the level of RANKL expression ($p<0,05$). There were differences in RANKL expression after alcohol administration and there were effects of ethanol on RANKL expression in bone remodeling process during application of orthodontic force. It's speculated that ethanol increases osteoclastogenesis, which accelerates orthodontic movement, but more study required.

KEYWORDS: Orthodontic force; Ethanol; Alcohol; RANKL; Tooth movement; Bone remodellng.

RESUMEN: La prevalencia del consumo de alcohol sigue siendo considerablemente alta a nivel mundial. A medida que crece la conciencia sobre las maloclusiones, es razonable suponer que algunos pacientes con tratamiento ortodóntico consumen alcohol. Los efectos del consumo de alcohol (etanol) sobre la respuesta al tratamiento ortodóntico aún no están del todo claros. Analizar las diferencias y los efectos del etanol sobre la expresión de RANKL en el proceso de remodelación ósea durante la aplicación de fuerza ortodóntica. Se realizó un estudio *in vivo* con 25 ratas Wistar a las que se les colocó un resorte helicoidal cerrado. Los animales se dividieron en cinco grupos: grupo control negativo A (sin fuerza ni etanol) en el día 0; grupo K1 (K=fuerza ortodóntica) en el día 3 y K2 en el día 14; y grupo P1 (P=fuerza ortodóntica+etanol al 20%) en el día 3 y P2 en el día 14. Posteriormente, los animales fueron sacrificados, se realizaron las preparaciones correspondientes y se examinó la expresión de RANKL mediante inmunohistoquímica (IHQ). Los datos se analizaron mediante pruebas t independientes y regresión. La prueba t de muestras independientes mostró diferencias significativas en la expresión de RANKL entre los grupos K y P en los días 3 y 14 ($p<0,05$). El análisis de regresión indicó que la duración de la administración de etanol afectó significativamente el nivel de expresión de RANKL ($p<0,05$). Se observaron diferencias en la expresión de RANKL tras la administración de alcohol, así como efectos del etanol sobre la expresión de RANKL en el proceso de remodelación ósea durante la aplicación de fuerza ortodóntica. Se especula que el etanol incrementa la osteoclastogénesis, lo que podría acelerar el movimiento ortodóntico, aunque se requieren más estudios para confirmarlo.

PALABRAS CLAVE: Fuerza ortodóntica; Etanol; Alcohol; RANKL; Movimiento dentario; Remodelación ósea.

## INTRODUCTION

Orthodontic tooth movement are organic multistage process caused by biomechanical forces, with periodontal ligament (PDL) playing a key role. Presence of periodontal tissues allows tooth movement to occur within alveolar bone after prolonged application of forces to teeth. The fast development of compression and pressure zones within PDL during orthodontic treatment is a hallmark of tooth movement, which is reliant upon biological reaction of ligament and physical properties of applied force. During tooth movement, PDL reacts immediately to mechanical stress, which causes metabolic alterations that cause orthodontic tooth movement. Numerous inflammatory cytokines and enzymes that cause connective tissue remodeling mediate this reaction. Bone remodeling after tooth movement is initiated by activation of vascular changes, synthesis of cytokines, prostaglandins, and growth factors. Bone undergoes resorption in area of pressure parallel to the direction of force. Conversely, bone undergoes apposition in area of tension, or in direction opposite to orthodontic force. This event facilitates tooth movement in alveolar bone (1-4).

Tumor Necrosis Factor (TNF) family of cytokines and cytokine receptors has been found to be crucial for bone remodeling regulation. Among these receptors, Receptor Activator of Nuclear Factor-κB Ligand (RANKL) is the main inducer of osteoclastogenesis. It binds to Receptor Activator of Nuclear Factor-κB (RANK), which is found on the surface of osteoclasts and osteoclast precursors. This binding helps osteoclasts adhere to bone and increases their chances of surviving. Osteoprotegerin (OPG), a soluble decoy receptor produced by

osteoblasts, regulates RANKL activity. Osteoprotegerin inhibits RANKL activation in osteoclastogenesis while competing with RANK for binding to RANKL. In orthodontic tooth movement, RANKL binding to RANK and OPG production are crucial and intimately associated with bone remodeling (5-7).

Society frequently consume alcohol, a transparent liquid with chemical component hydroxyl group, -OH, often in the form of ethyl alcohol or ethanol C2H6O. In Indonesia, based on 2014 Global Status Report on Alcohol and Health, it was revealed that as many as 1,180,900 citizen were dependent on alcohol. Based on findings, ethanol is suspected to be a toxic substance not only for organs, but also for bones. Long-term alcohol use is strongly linked to pathological effects on tissue and bone integrity, which not only increases osteoblast apoptosis but also makes recovery from injury or surgery more difficult (8-10).

Nowadays, number of orthodontic patients has grown significantly over time and intense need to enhance dentofacial aesthetics, psychosocial functioning, and quality of life, as well as a high perception of malocclusion rates. There is a growing number of orthodontic patients and rising prevalence of alcohol use, which enhances possibility that some patients will drink alcohol while receiving treatment. Research related to ethanol on orthodontic tooth movement is still very limited (11-13). Research conducted by Bannach, *et al*. (2015) suggested that chronic alcohol consumption in Wistar rats can cause bone loss (14). In contrast, research by Araujo, *et al*. (2014) showed that effect of 20% ethanol consumption can cause slight bone resorption at the end of the movement, thus causing delayed tooth movement (15).

Based on several contradictory research results above, further exploration is needed regarding the use of ethanol in orthodontic tooth movement, and there has been no research that points to direct effect of ethanol on RANKL during bone remodeling process during orthodontic force application. Furthermore, our objective were to analyze the differences and effects of ethanol on RANKL expression in bone remodeling process during application of orthodontic force.

## MATERIAL AND METHODS

This research has obtained research ethics eligibility issued by Ethics and Advocacy Unit of Faculty of Dentistry, Hasanuddin University with number 020/KEPK FKG-RSGMP UH/EA/X/2024.

Samples- This study used 25 male Wistar rats of Rattus Norvegicus species weighing 224.8 ± 12.9 grams. Before started, rats were acclimatized and adapted to the new environment for 7 days. Temperature room of the experimental animals cage was at 19-22 °C with 12 hours of light/dark photoperiod. Rats were given crushed food and water ad libitum. All rats were randomly divided into 5 groups and each group consisted of 5 rats, consisting of:

- Five rats in group (A) were control without treatment on day 0.
- Five rats in group (K1) were treated with orthodontic force on day 3.
- Five rats in group (K2) were treated with orthodontic force on day 14.
- Five rats in group (P1) were treated with orthodontic force & ethanol on day 3.
- Five rats in group (P2) were treated with orthodontic force & ethanol on day 14.

Group P rats received a chronic ethanol dose of 20% ethanol (6), 2g/kg/BW (w/v) in distilled water daily using an oral gavage tube. Magnitude of orthodontic force applied was 50 grams measured using an Orthodontic Tension Gauge (Dentaurum, Germany) (16). Tooth movement was performed using a closed coil spring (CCS) (American Orthodontics, Washington, USA) which was fixed using a 0.10 mm SS ligature (Dentaurum, Newton,

Penn) and bonded using a flowable composite (Hexabond Orthobond Light-Cured, Indonesia) from maxillary molar to central incisor provide reciprocal movement. After initial activation, CCS was not reactivated, but position of CCS was observed daily (Figure 1) (17,18).

To investigate the effect of suspected ethanol intoxication on overall level of malnutrition that can directly affect bone development, animal body weight was controlled throughout entire treatment period. Rats were weighed daily using an electronic precision scale (Nagata CYT 12, Indonesia) for observation.

## IMMUNOHISTOCHEMISTRY (IHC)

After days 0, 3, and 14 were completed, rats were sacrificed by administering overdose of ketamine anesthesia 5.4 ml/kg intraperitoneally. Then, maxilla of each rats were taken and dissected. For a full day at room temperature, the right maxilla was preserved in 10% phosphate-buffered formalin after being removed. Then, for 30 days, specimens were demineralized at room temperature in 4.13% ethylenediaminetetraacetic acid (pH=7.2) after being cleaned for four hours under running water. The acid was changed weekly. Post demineralization, specimens were rinsed under running water for two hours, dehydrated using a dilution of ethanol, clarified with xylol, and then embedded in paraffin. Tissue in the paraffin block from embedding results was inserted into a microtome holder, adjusted to the surface parallelism, and cut with a microtome blade. Cutting was started by setting slice thickness above 10 µm. Good cutting will produce a ribbon-like cut shape. Slices were taken with an object glass in a perfect supine position. Selected pieces were dried and placed on a hotplate (38°-40°C) until dry. Furthermore, specimens were stored in an incubator at 39°C for 24 hours. Then deparaffinization were carried out with xylene 3 times for 3 minutes each, then rehydrated using alcohol. Slides were rinse in a 3% hydrogen peroxide solution (OneMed, Indonesia) for 10 minutes, then soaked in prediluted blocking serum at room temperature. Main antibody RANKL (Santa Cruz Biotechnology, Santa Cruz, USA) was used to incubate the slides at 4-8°C for 1 night, then rinsed with phosphate buffer saline, then incubated with secondary antibody biotin (Santa Cruz Biotechnology, Santa Cruz, USA) for 10 minutes at room temperature then rinsed with PBS. Slides were incubated with peroxide, then rinsed in PBS and incubated with diaminobenzinidase (DAB) solution (Sigma-Aldrich Corp., St. Louis, USA) (19).

## DATA ACQUISITION

Observation of slides were carried out using a light microscope (Leica ICC50W, Leica Camera, Germany) with a magnification of 200x to determine the calculation area, 400x magnification to calculate number of expressions, and 1000x magnification to determine shape of the staining results. Calculation of the number of expressions was carried out using the Immuno Ratio Scale (IRS) program. Expression can be calculated through the brown color image expressed in the preparation with a unit of quantity. Observations were made in three fields of view in pressure area; cervical third, middle third and apical third. Each specimens had 3 results of calculation of number of expressions which were then added up and divided by 3 to obtain average number of expressions in one number of specimen.

## STATISTICAL ANALYSIS

Analysis using JASP (University of Amsterdam, GNU Affero General Public License). Data were processed analytically and data normality tests were carried out using the Shapiro-Wilk test because number of samples were less than

50. Since data from each group were normally distributed, parametric test were conducted using independent sample T-test to find differences between variables and a linear regression test to asses the effect of duration of ethanol administration on RANKL expression, with confidence interval level 95% and p=0,05 were used.

## RESULT

Based on immunohistochemistry (IHC) image, amount of RANKL expression changed in force (K) and force + ethanol (P) groups on day 3 and 14 when compared to control group (A). At 1000x magnification, a black arrow can be seen indicating appearance of biomarker (RANKL) with a short cuboid or cylindrical shape. At 400x magnification, amount of RANKL expression was also greater in the P group than in the K group on days 3 and 14 (See Figure 2, Figure 3, and Figure 4).

Figure 5, shows that there was an increases in RANKL expression from days 0, 3, and 14 in each group. RANKL in force and 20% ethanol group (P) was higher than force group (K) on day 3, which was 9.00±1.58 and day 14 was 11.60±1.52.

Table 1 shows a comparison of RANKL expression in force group (K) and force + ethanol group (P). It can be seen that there was a significant differences in RANKL obtained between force group (K) and force + ethanol group (P), both on day 3 at p=0.017, and day 14 p=0.000475.

Table 2 demonstrates that duration of ethanol administration had a significant effects on RANKL expression on day 0, 3, and 14 ($p<0.05$). According to results of linear regression test, duration of ethanol administration had a 66.9% effect on RANKL expression from day 0 to day 14.

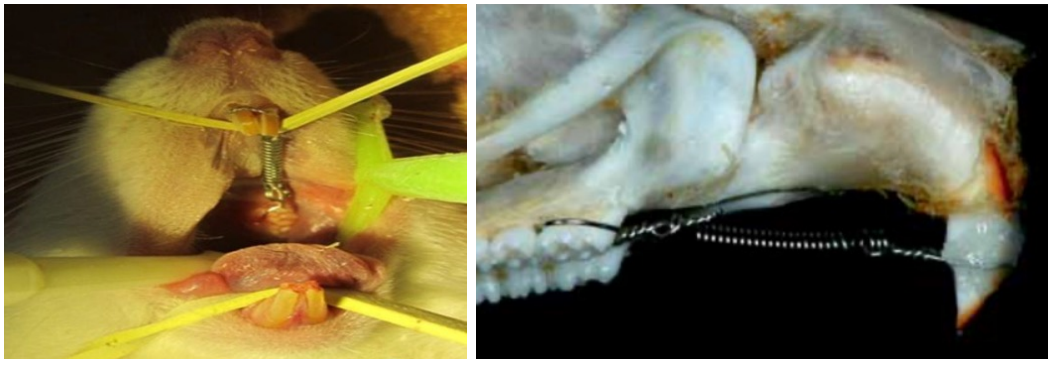

**Figure 1**. Experimental animal with mechanical force treatment using closed coil spring NiTi. Coil spring NiTi with force 50 gr (Inset: additional wire inserted into the end of coil spring) between the upper left first molar and upper incisor teeth for movement (10-11).

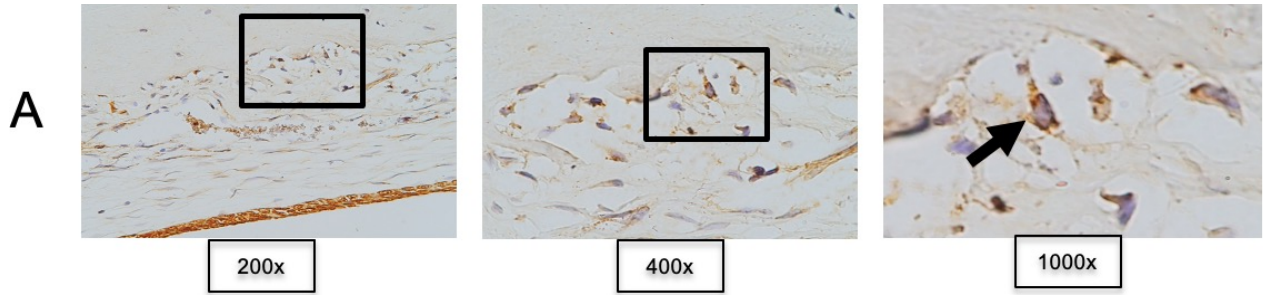


**Figure 2**. A = Negative control without orthodontic force and ethanol administration, with 200x, 400x, and 1000x magnification on day 0. Dark arrow shows RANKL expressions using Immunohistochemistry (IHC).

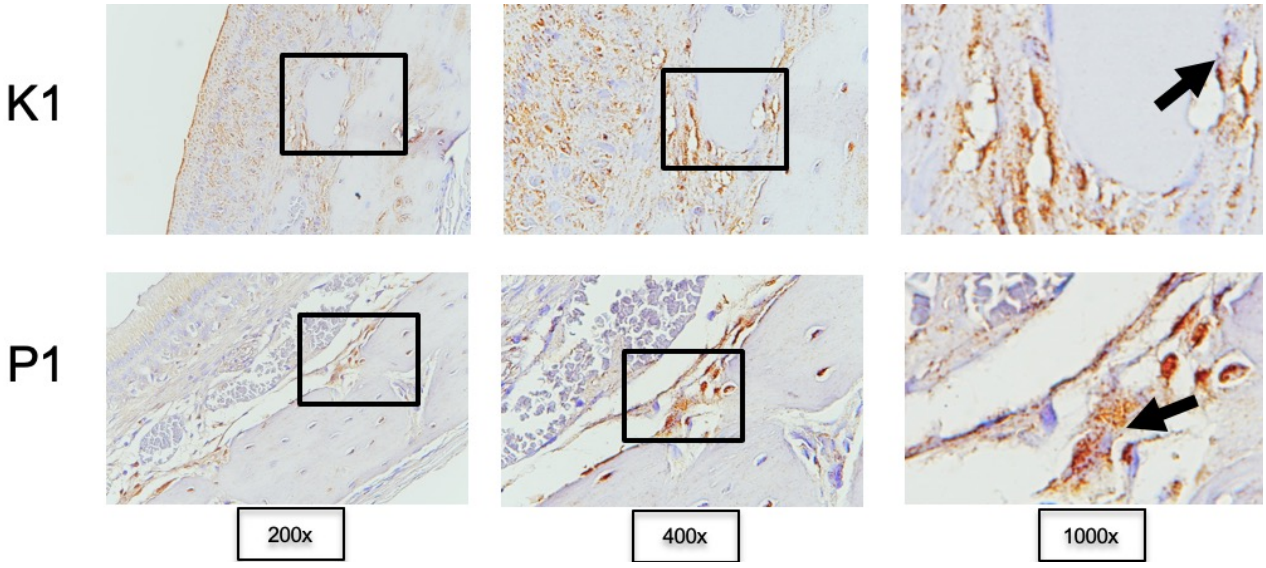


**Figure 3**. K=Positive Control (50gr force), P=Treatment Group (50gr force and ethanol), with 200x, 400x, and 1000x magnification on day 3. Dark arrows show RANKL expressions each group by immunohistochemistry (IHC).

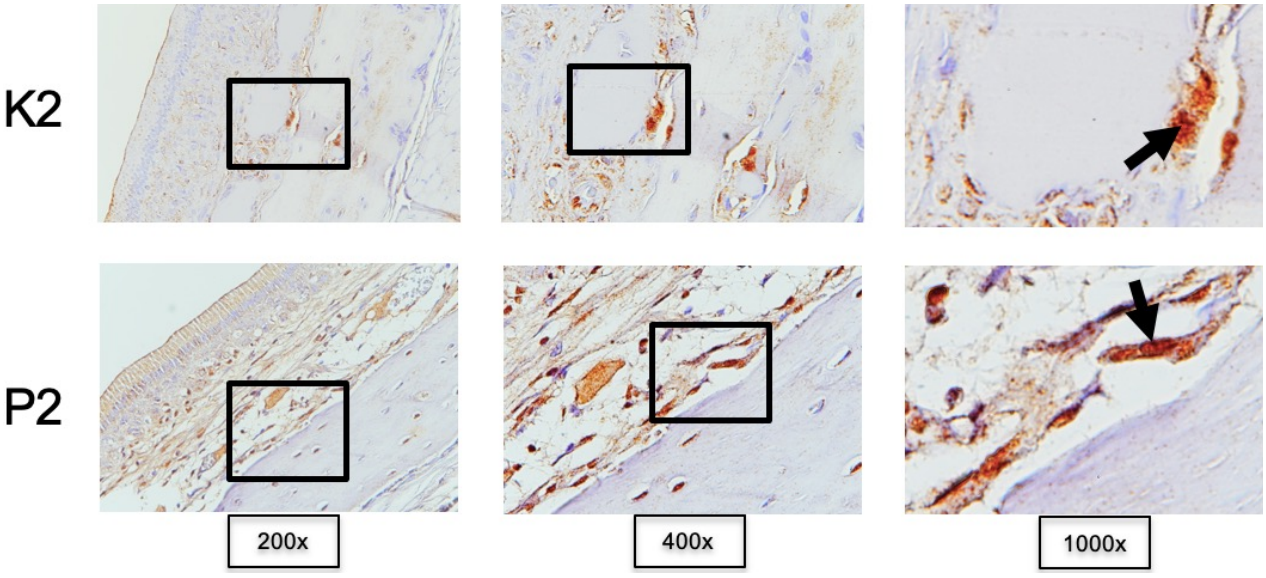


**Figure 4**. K=Positive Control (50gr force), P=Treatment Group (force and ethanol), with 200x, 400x, and 1000x magnification on day 14. Dark arrows show RANKL expressions each group by immunohistochemistry (IHC).

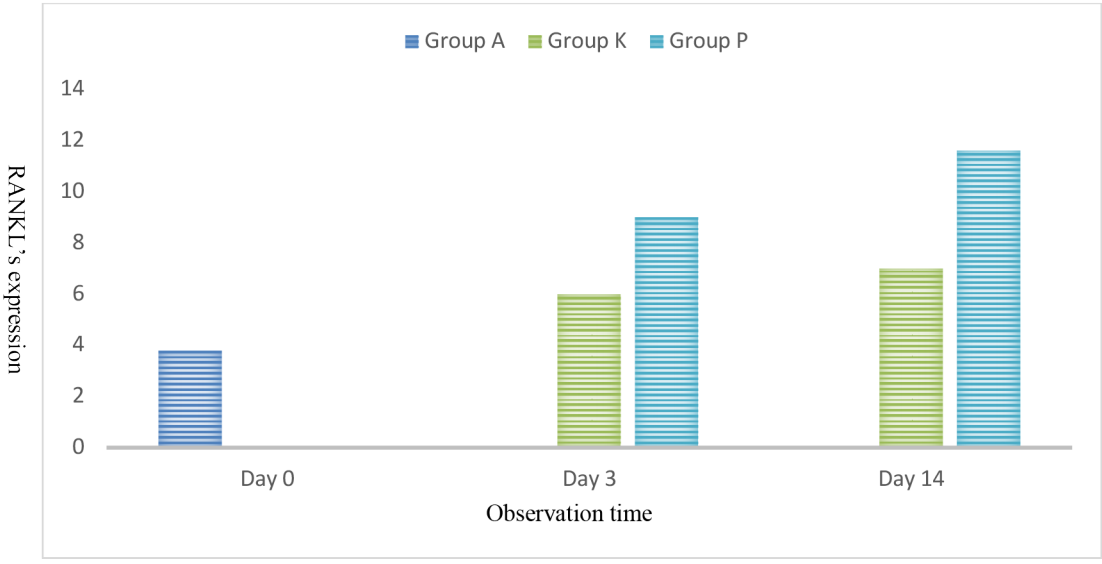


**Figure 5**. Distribution of RANKL expressions based on observation time.

**Table 1**. Comparison of RANKL expression on each day.

| Day | Group K (mean ± SD) | Group P (mean ± SD) | p-value |
|---|---|---|---|
| 3 | 6.00±1.58 | 9.00±1.58 | 0.017* |
| 14 | 7.00±1.00 | 11.60±1.52 | 0.000475* |

*Independent sample t-test, significant ($p<0.05$).

**Table 2**. Effect of duration of alcohol administration on RANKL expression.

| Duration of administration (day-) | RANKL (mean ± SD) | p-value | Coefficient | $R^2$ |
|---|---|---|---|---|
| 0 | 3.80±0.84 | | | |
| 3 | 9.00±1.58 | 0.000116* | 0.471 | 0.669 |
| 14 | 11.60±1.52 | | | |

*Linear regression test, significant ($p<0.05$).

## DISCUSSION

Orthodontic tooth movement is external result of force application on a complex biological system composed of bone, periodontal ligament, teeth, and gingiva. Direct effect of orthodontic forces is deformation or pressure, sometimes very small, on teeth and surrounding tissues. Under physiological conditions, this biological system adapts to constantly changing mechanical conditions caused by mastication. Application of orthodontic forces causes a series of reactions in extracellular matrix (ECM) and cells of dento-alveolar complex. The cells in these tissues detect pressure and respond to deformation of ECM or their own deformation by synthesizing and secreting various mediators, such as cytokines and growth factors. Ultimately, this leads to bone resorption on pressure side and bone apposition on tension side, as well as remodeling of PDL (20, 21).

In osteoclastogenesis, a variety of mediators produced from bone and PDL, including growth factors, colony-stimulating factors, and cytokines. Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) are two factors that essentials in osteoclastogenesis. RANKL is mainly a potent inducer of osteoclastic differentiation process, which results in bone remodeling (22, 23).

Figure 5 shows increases in distribution trend of RANKL expression from day 0, day 3, and day 14 each group. Reason for this event was due to application of orthodontic force stimulates osteoblast apoptosis temporarily through activity of apoptotic mediators, such as caspase-3, BCL-2-associated X protein, and B-cell lymphoma 2. Production of apoptotic mediators declines further following first three days of force application. Additionally, hypoxic environments and the subsequent stabilization of hypoxia-inducible factor-1 (HIF-1) promotes increases expression of vascular endothelial growth factor and RANKL in PDL fibroblasts and osteoblasts. When P38 MAPK pathway is activated due to hypoxia, cycloxygenase-2 levels rise, contributing in activation of prostaglandins production, mostly PGE2, from arachidonic acid (20, 22, 24).

PGE2 will subsequently suppress the expression of osteoprotegerin (OPG) and promote osteoblast differentiation, macrophage colony-stimulating factor (M-CSF), and RANKL. OPG inhibits osteoclast formation and bone resorption in addition to acting as a RANKL decoy receptor. Lastly, TNF-α expressed in greater quantities by PDL cells on pressure side, which directly contributes to bone resorption by stimulating MMP formation and raising RANKL levels (21, 23, 25).

Table 1 shows significant differences in RANKL between force group (K) and force+ethanol group (P), on day 3 ($p=0.017$) and day 14 ($p=0.000475$). From these results, it can be seen that RANKL expression after being given force and alcohol (P) increased compared to force only (K) group which implies an increased in RANKL concentration due to alcohol consumption. This result supported by Almeida *et al*. (2020) research demonstrates that alcohol intake will promote osteoclastogenesis by raising RANK expression, which is mediated by reactive oxygen species generation, extracellular signal-regulated kinases in precursor osteoclast cells, and elevated RANKL. In reaction to orthodontic force, RANKL produced will promote as main driving factor for tooth movement and osteoclastogenesis (26, 27).

Stimulating RANKL expression during orthodontic tooth movement can attract peripheral monocytes and macrophages, releasing inflammatory cytokines such interleukin (IL-1b), IL-6, IL-11, and tumor necrosis factor-alpha (TNF-α). These cytokines may promote bone remodeling. In response to mechanical stress, researchers have found that inflammatory cytokines in PDL regulate RANKL. Furthermore, alcohol reduces bone density, cancellous

bone, and cortical bone. Alcohol intake can also enhance osteocyte death and reduce osteoblast activity and differentiation. Hypothetically, ethanol exposure during orthodontic tooth movement can affect osteogenesis during remodeling, especially ethanol can cause inhibition of osteoblastogenesis. Ethanol can also induce IL-6/ROS (Reactive Oxygen Species) which mediates bone loss (17, 22, 26, 28).

Table 2 shows significant effect of duration of alcohol adminisitration on RANKL expression. This in line with research by Li *et al*. (2018) which stated that there was an increases in osteoclasts by RANKL in the control group and experimental group from day 0 to day 14. A meta-analysis study conducted by Makrygiannakis *et al.* (2023) found that effects on bone tissue depends on dose and duration of alcohol consumption. Light dosages of alcohol administration have no detrimental effects. Nevertheless, prolonged use or excessive consumption can exacerbate the effects of osteopenia on bone tissue, which is caused by activation of reduction osteoblastic activity. Lastly, we must acknowledge the need for additional study on the direct impact of ethanol on orthodontic movement stability and tooth movement (11, 29, 30).

## CONCLUSION

There were significant differences in RANKL expression after ethanol administration in bone remodeling process during orthodontic tooth movement on day 3 and 14. Also there were significant effects of duration of ethanol administration on RANKL expression in bone remodeling process during orthodontic tooth movement. RANKL expression rises in proportion of duration of ethanol administration. Ethanol has been shown to allegedly increases orthodontic tooth movement since it was thought promotes RANKL expression by 66.9%. However, movement's stability and systemic effects of alcohol must be taken into consideration.

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## CONFLICT OF INTEREST

There is no conflict of interest.

## AUTHOR CONTRIBUTIONS STATEMENT

Conceptualization: D.D.F.R. and A.S.P.
Investigation: D.D.F.R., B.M.R. and E.H.H.
Methodology: D.D.F.R., B.M.R., E.H.H. and E.E.
Resources: D.D.F.R., B.M.R. and E.H.H.
Project administration: D.D.F.R.
Supervision: D.D.F.R., A.S.P. and E.E.
Validation: D.D.F.R. and E.E.
Visualization: D.D.F.R., E.H.H.
Data curation: A.S.P., Z.S. and E.H.H.
Formal analysis: A.S.P. and Z.S., B.M.R. and E.E.
Writing-original draft and review & editing: D.D.F.R., A.S.P., Z.S., B.M.R., E.H.H. and E.E.
Software: E.H.H.
Funding acquisition: D.D.F.R. and A.S.P.

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