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*Short Communication*

# Histological analyses of orthodontic force in *Cavia porcellus*: Comparison between immunohistochemistry and hematoxylin-eosin

[Análisis histológicos de la fuerza ortodóncica en *Cavia porcellus*: Comparación entre inmunohistoquímica y hematoxilina-eosina]

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**Abstract**

*Context*: Histological quantification of osteoclasts and osteoblasts can evaluate biological responses to orthodontic tooth movement. Histological analysis of bone samples can be technically challenging.

*Aims*: To evaluate the differences between hematoxylin and eosin (HE) staining and immunohistochemistry (IHC) in quantifying osteoblast and osteoclast cells following the application of static orthodontic force.

*Methods*: Orthodontic force was applied using a rubber separator around the maxilla incisor of *Cavia porcellus*. Tooth samples were taken at 0, 4, 8, 14, 21, and 28 days after applying orthodontic force. HE and IHC staining quantify osteoblast and osteoclast cells in the alveolar bone. IHC staining, i.e., Tartrate-resistant acid phosphatase (TRAP) staining, was used to identify osteoclasts, and osteocalcin (OCN) staining was used to identify osteoblasts.

*Results*: Significantly higher numbers of osteoclasts and osteoblasts were observed with IHC compared to HE staining ($p<0.05$). Significant positive linear correlations in the numbers of osteoclasts ($r = 0.757$) and osteoblasts ($r = 0.622$) identified were observed between IHC and HE staining.

*Conclusions*: The results of this study indicate HE staining may represent an acceptable alternative method of quantifying osteoclasts and osteoblasts in the preliminary research of orthodontic tooth movement (OTM).

*Keywords*: hematoxylin; immunohistochemistry; orthodontic; osteocalcin; tartrate-resistant acid phosphatase.

**Resumen**

*Contexto*: La cuantificación histológica de osteoclastos y osteoblastos puede evaluar las respuestas biológicas al movimiento dental ortodóncico. El análisis histológico de muestras de hueso puede ser técnicamente desafiante.

*Objetivos*: Evaluar las diferencias entre la tinción con hematoxilina y eosina (HE) y la inmunohistoquímica (IHC) en la cuantificación de células de osteoblastos y osteoclastos después de la aplicación de fuerza ortodóncica estática.

*Métodos:* Se aplicó fuerza de ortodoncia utilizando un separador de goma alrededor del incisivo maxilar de *Cavia porcellus*. Se tomaron muestras de dientes a los 0, 4, 8, 14, 21 y 28 días después de aplicar la fuerza de ortodoncia. La tinción con HE e IHC cuantifica las células de osteoblastos y osteoclastos en el hueso alveolar. Se usó tinción IHC, es decir, tinción con fosfatasa ácida resistente a tartrato (TRAP), para identificar osteoclastos, y tinción con osteocalcina (OCN) para identificar osteoblastos.

*Resultados*: Se observaron números significativamente más altos de osteoclastos y osteoblastos con IHC en comparación con la tinción con HE (valor de $p<0,05$). Se observaron correlaciones lineales positivas significativas en el número de osteoclastos ($r = 0,757$) y osteoblastos ($r = 0,622$) identificados entre la tinción IHC y HE.

*Conclusiones*: Los resultados de este estudio indican que la tinción HE puede representar un método alternativo aceptable para cuantificar osteoclastos y osteoblastos en la investigación preliminar del movimiento dental ortodóncico (OTM).

*Palabras Clave*: fosfatasa ácida tartrato resistente; hematoxilina; inmunohistoquímica; ortodoncia; osteocalcina.

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## INTRODUCTION

Based on the pressure-tension theory of the orthodontic tooth movement (OTM), irregular fibers are located on the pressure side, causing vascular constriction and the cell replication process to slow down and initiate bone resorption by osteoclast. In contrast, on the tension side, the cell replication process increased and caused bone deposition by osteoblast (Ariffin et al., 2011; Jonsdottir et al., 2012).

Various methods or techniques could assess the cellular analysis in orthodontic tooth movement (OTM) research. Enzyme-linked immunosorbent assay (ELISA) was used to analyze cytokines, neuropeptides (IL-1ß, calcitonin gene-related peptide), growth factors (TGF-ß, VEGF), enzymes, and hormones (Sufarnap et al., 2020; Vandevska-Radunovic and Murison, 2010). Different techniques like microarray and Real-Time–PCR were analyzed mainly for genes-related expression profiles connected to OTM; i.e., Runt-related transcription factor 2 (RUNX2), Osteocalcin (OCN) (de Araujo et al., 2007; Taddei et al., 2012). The most common methods to analyze the cellular numbers were histological analysis. Histology staining with Hematoxylin-eosin (HE) was the most basic method used to differentiate staining of the cytoplasm, cell nuclei, and other cell organelles. Hematoxylin (blue) was stained for the cell nuclei, and eosin (magenta-red) was stained for a cytoplasmic (Ruifrok and Johnston, 2001).

Immunohistochemistry (IHC) is an advanced histological method that stains a range of markers of cell lineages, tissue types, cytokines, and growth factors (Ruifrok and Johnston, 2001; Taylor and Levenson, 2006). The IHC-stained became an adjuvant to HE-stained primarily to diagnose the pathological condition (Taylor and Levenson, 2006), while in OTM research, a quantitative assessment of cellulars or cytokines were needed, so it was no doubt that IHC-stained showed a better feature for those purposes in histology research (Taylor and Levenson, 2006).

However, the HE-stained method was not to be ignored either. The ideal result of histological IHC-stained still had some limitations and difficulties due to many processes with risk of failures, i.e., specimen fixation, tissue processing, and antigen retrieval at some histological laboratories, especially for the bone specimen. In addition, the process becomes costly (Matos et al., 2010). Nevertheless, HE-stained has been used for at least a century. It also has many advantages; i.e., the stain remained unchanged for years, it could be used with various fixatives, and it is still essential to recognize various types of morphologic tissue's changes that showed a broad range of cytoplasmic, nuclei and extracellular matrix (Fischer et al., 2017).

Many new techniques for histological analysis have been developed. The essential histology with HE-stained orthodontic tooth movement analysis was abandoned and rejected, especially for research purposes. This study hypothesized that the histological HE-stained could still be used compared to the IHC-stained. This research aimed to compare and correlate between hematoxylin-eosin (HE) stained and immunohistochemistry (IHC) stained for the quantity of osteoblast and osteoclast cells due to the static orthodontic force in *Cavia porcellus*. *Cavia porcellus*, generally known as a guinea pig (*Cavia porcellus*), is commonly used in animal studies for various biological researches (immunology purposes) (Dang et al., 2008).

## MATERIAL AND METHODS

### Animal studies and study design

The research was *in-vivo* experimental and followed the ARRIVE guideline. The study had approval from the Animal Research Ethics Committee (AREC), Faculty of Mathematics and Natural Science - Universitas Sumatera Utara (USU), No.0022/KEPH-FMIPA/2018).

*Cavia porcellus* (n = 24), two to four months old, weighing 250-400 g, were chosen by random sampling. Animals were housed, and their husbandry was followed by the ARRP Guidelines No. 22 (Fawcett, 2012) and adapted seven days before the experiment. Each polycarbonate cage contained 2-3 animals that had wood saving on the floor and were fed with carrots and tap water. Low light and temperature of 20-25°C were maintained for a minimum of 12 hours.

A separator insertion surrounded one of the maxillary incisors and served as an orthodontic force. Inserting a separator did not need any anesthesia because the *Cavia porcellus* behavior was cuddled, gentle, and not prone to biting (Fawcett, 2012). At the end of each observation time, animals were euthanized with 75 mg/kg of ketamine (Germany) and 10 mg/kg Xyla (Holland) (Dang et al., 2008).

### Histological analysis

The alveolar bone of premaxillae of *Cavia porcellus* was fixed in 10% neutral formaldehyde for 24 hours. The tissue was decalcified with 10% EDTA. The processing proceeded with embedding, sectioning, and staining. Sectioning was sliced 5 µm by Leica microtome. Histological analysis was stained with hematoxylin-eosin (HE), tartrate-resistant acid phosphatase

(TRAP/Biossusa®; bs-634R/Bioss Inc.) was represented for osteoclast analysis, and osteocalcin (Biossusa®; bs-4917R/Bioss Inc.) were represented to osteoblast analysis. Osteoclast in HE-stained was grouped as OC-HE, and osteoblast was grouped as OB-HE. Osteoclasts in TRAP stained were grouped as OC-TRAP, and osteoblast in osteocalcin was grouped as OB-OCN.

### Observation outcome

Histological of osteoclasts and osteoblasts cell amounts were visualized using Olympus CX23 light microscope with 400× magnification by two observers, an orthodontist, and an anatomical pathologist. They were blinded to analyze each sample within five fields of view. The osteoclast characteristic is a giant multinucleated (3-20 nucleus) in a cytoplasmic. It has an oval until flattened shapes were shown at various sites along the bony surface, especially in the bone lacunae (Fig. 1A,C) (Downey and Siegel, 2006). The osteoblast characteristic is basophilic cuboidal or polygonal mononucleated cells shown in a bone matrix (Fig. 1B,D) (Florencio-Silva et al., 2015; Phan et al., 2004). The region of interest (ROI) for osteoclast was observed at the Howship's lacunae of the pressure side of the tooth. At the same time, the osteoblast was measured at the superficial of the alveolar bone, which represented the tension site of the teeth.

### Statistical analysis

Statistical analysis was done with the Statistical Package for Social Sciences-IBM Version 26.0. All data and variables were not distributed normally ($p<0.05$; Shapiro-Wilk test). The cellular observation comparison between orthodontists and anatomical pathologists (inter-rater reliability/IRR) was analyzed with Cronbach's alpha level. Comparison between HE and IHC for both osteoblast and osteoclast numbers at each observation time (i.e., 0,4, 8, 14, 21, and 28 days) was analyzed with Mann Whitney test. The correlation coefficient between HE and IHC was analyzed with Spearman's rank correlation coefficient test to see the strength and direction of the linear relationship between HE and IHC measurements at each cell (Mukaka, 2012).

## RESULTS

Osteoclast numbers (Fig. 2A) of OC-TRAP groups showed significantly higher than OC-HE groups ($p<0.05$), at all-time observation except day 14 by Mann Whitney's test. Spearman's rank correlation coefficient showed a solid and positive or linear correlation between OC-HE and OC-TRAP numbers of the statement ($r=0.757$; $p<0.001$). The osteoclast numbers gradually increased from day-0, and the highest numbers were found at day-8 for both OC-HE and OC-TRAP. The osteoclast numbers decreased afterward until the last day of observation was found linearly for both groups.

The osteoblast numbers (Fig. 2B) were also found to be significantly higher in OB-OCN groups than OB-HE groups but only in three subgroups. OB-HE and OB-OCN also had a linear correlation with moderate strength ($r=0.622$; $p<0.001$). The osteoblast numbers were highest in the control group (day-0). Immediate after an orthodontic force was applied (day-4), the amount of osteoblast reduced and continued with disorderly increased and reduced resulted after (days 8 and 14) and began gradually increase after day-21.

Cronbach's alpha level was interpreted for IRR analysis at the OB-HE group for six observations between 2 observers. The alpha level for OB-HE numbers was 0.048 with a $p = 0.144$ (Friedman test); it was concluded that it had low reliability.

## DISCUSSION

Physiologically, bone was remodeled through the action of osteoclast initiating bone resorption (7-10 days) and bone formation by osteoblast (2-3 months). It was explained that the numbers of osteoclast and osteoblast existed in the control group (day-0) (Florencio-Silva et al., 2015; Watts, 1999).

Osteoclast increased gradually since the orthodontic force was inserted, and it had the highest numbers on day-8; this result closely had the same effect as Holland et al. (2019) and Wahab et al. (2011) studies, which had increased the amount of TRAP at day 7 and continued at the peak amount at weak-5 while in this study only at week-1 (day-8). Different mechanicals, i.e., coil spring vs. separator, was made the differences. This became the limitation of the study; the short duration and the static force from the elastic separator have limited force decay, which could be achieved for a maximum of 7 days, while the NiTi coil spring took at least 12 days. But the separator insertion gave a better adaptive response to the animal than the NiTi coil spring mechanic (Kirschneck et al., 2020).

Osteoclast had various appearances; the numbers of osteoclast in OC-TRAP group showed significantly higher numbers of cells ($p<0.05$) at five out of the six-time point of observation groups. TRAP stained as an osteoclast biomarker appeared as a "ruffled border" osteoclast's lineage in the compartment (Howship's lacunae) (Blumer et al., 2012; Downey and Siegel, 2006; Phan et al., 2004). The osteoclasts were visible due to the specific color that appeared (light brown

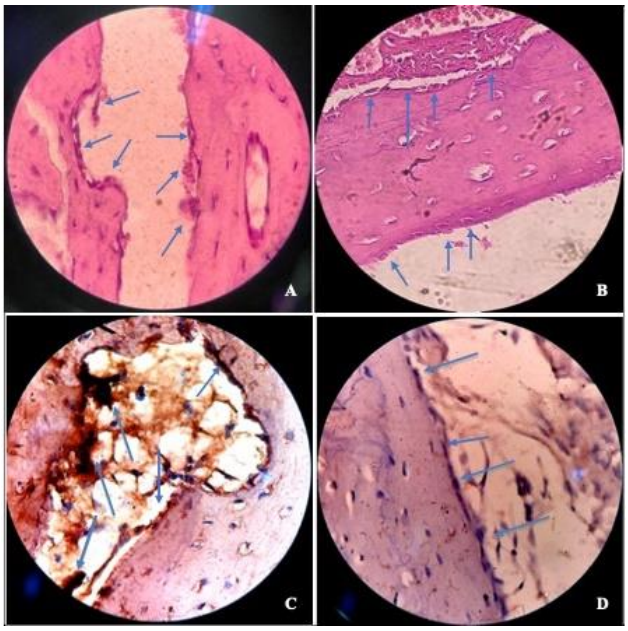


**Figure 1. A** and **B:** Hematoxylin eosin (HE) staining; **C** and **D:** Immunohistochemistry staining (TRAP and Osteocalcin); **A** and **C;** Osteoclast. **B** and **D:** Osteoblast.

Captured under 400× magnification.

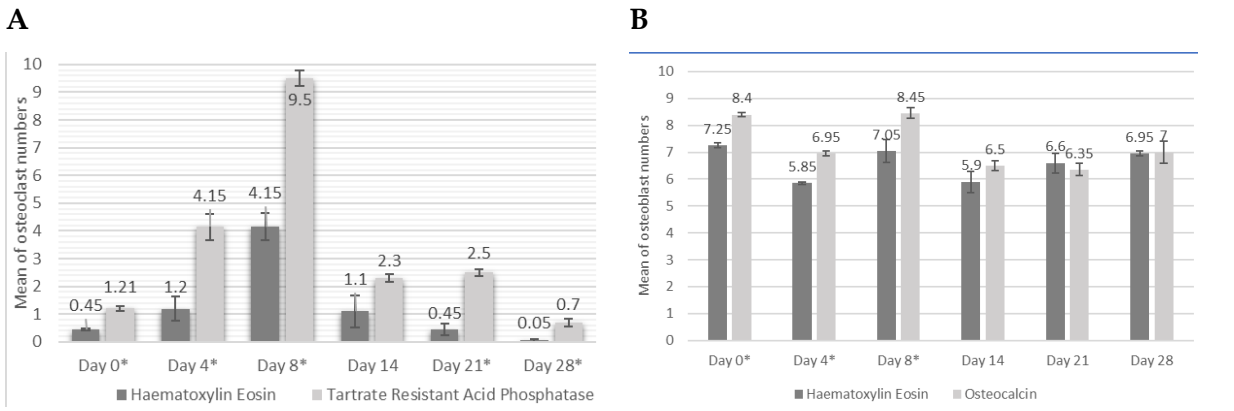


**Figure 2**. Mean values of cell numbers.

(**A)** Osteoclast for OC-HE group and OC-TRAP group. (**B)** Osteoblast for OB-HE group and OB-OCN group.

Data are represented as mean ± standard deviation (n = 4). Results were analyzed with the Mann-Whitney test. An asterisk denotes significant differences between groups ($p<0.05$). OC-HE: Osteoclast-Hematoxylin Eosin; OC-TRAP: Osteoclast-Tartrate Resistant Acid Phosphatase; OB-HE: Osteoblast-Hematoxylin Eosin; OB-OCN: Osteoblast-Osteocalcin.

for the cytoplasm and dark brown for the nucleus) (Fig. 1C), while in OC-HE group showed that osteoclast's cytoplasm in magenta color and nucleus in blue color) (Ruifrok and Johnston, 2001). The magenta osteoclast's cytoplasm color seemed to have blended with the color of alveolar bone, which became an interference during the observation (Fig. 1A).

The osteoblast cell numbers in this study were decreased after the orthodontic force had been given. It could be due to the osteoblast's apoptosis, a process related to the hyalinization within the PDL (Meikle, 2006). Another author described that a-SMA, osteocalcin, and bone sialoprotein (BSP) in IHC analysis had increased expression since day two and decreased after 2-3 days (Holland et al., 2019). This was a coincidence to the study results.

The osteoblast numbers between the OB-OCN and the OB-HE groups were significantly different at 3 out of 6-time points of the observation groups. The osteoblast quantification in this study was observed at the surface of the alveolar bone since the morphology of the osteoblast position was parallel lined to the synthesized bone and with the cuboid-shaped- mononucleated cells, so the osteoblast morphology became quickly to be observed (Fig. 1B,D) (Phan et al., 2004). Osteocalcin as a biomarker was expressed at a late-stage marker of a mature osteoblast more than a developed stage and as one of the first non-collagen proteins that formed an organic matrix to induce the mineralization of the bone (Bennett et al., 2001; Florencio-Silva et al., 2015).

Pros and contras found in histological studies due to some diversity of approaches from the beginning until the interpretation and reporting of the results. A standardized protocol to study the alveolar bone for OTM should be developed conclusively, paralleled and simplified for several types of research, i.e., mechanical experiment model (Kirschneck et al., 2020), methods of observation (histological/immunological assays/RT-PCR/micro-arrays) (de Araujo et al., 2007; Holland et al., 2019; Taddei et al., 2012; Vandevska-Radunovic and Murison, 2010), biomarkers decisions (proteins/enzyme/gene/hormone/growth factor) (Matos et al., 2010; Watts, 1999), interpretation and reporting the analysis (description of a morphological cells/quantification or scoring the stained cells/structures at the ROI's) (Fedchenko and Reifenrath, 2014). The lack of a standard scoring system for bone tissue at each different marker and method made the comparison of the results to other studies became difficult to be compared (Fedchenko and Reifenrath, 2014).

Despite the evident differences between HE and IHC stained analysis, the Spearman's rank correlation coefficient between HE stained and IHC stained in OTM research had a significant positive or linear correlation; strong correlation for osteoclast observation and moderate-strong for osteoblast observation. The hypothesis had been answered that HE-stained could still be justified as a reliable method to analyze the cellular quantification with a careful discreet of the morphology and the ROI's of each cell.

The IHC-stained were exclusively provided more accurate morphological analysis and diagnosis with their specific proteins or genes expression; by counterstained the HE prior IHC-stained were given a far more precise result (Grosset et al., 2019), i.e., TRAP+HE-stained in histological slide changed the TRAP-stained color into a red-stained to osteoclast (Blumer et al., 2012).

The IHC methods are costly and require specific skills from the operator to make the histological slide. Some histological anatomy of the bone tissues failed to attach to the slide. A few of the slides had to be excluded and reprocessed again for a new one, which added to the cost. Furthermore, the operators' experience and the reliability of the interpreter observation were also a limitation of the histological analysis (Matos et al., 2010). The Cronbach alpha level for inter-rater reliability (IRR) result was weak, considering the difference between expertise, perceptual, and cognitive processes. Microscopic observation is a complex process involving human sensory, perception, and cognition from the observer (Hamilton et al., 2009).

## CONCLUSION

The histological IHC-stained analysis showed a significant difference to HE-stained quantification, but both had a vital significance linear coefficient correlation. We can conclude that the HE-stained could still be justified and acceptable as one of the methods to observe the cells as a preliminary study in OTM research.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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## REFERENCES

Ariffin S, Yamamoto Z, Abidin I, Wahab R, Ariffin Z (2011) Cellular and molecular changes in orthodontic tooth movement. Sci World J 11: 1788–1803.

Bennett JH, Moffatt S, Horton M (2001) Cell adhesion molecules in human osteoblasts: Structure and function. Histol Histopathol 16: 603–611.

Blumer MJF, Hausott B, Schwarzer C, Hayman AR, Stempel J, Fritsch H (2012) Role of tartrate-resistant acid phosphatase (TRAP) in long bone development. Mech Dev 129: 162–176.

Dang V, Bao S, Ault A, Murray C, McFarlane-Mills J, Chiedi C, Dillon M, Todd JP, DeTolla L, Rao S (2008) Efficacy and safety of five injectable anesthetic regimens for chronic blood collection from the anterior vena cava of guinea pigs. J Am Assoc Lab Anim Sci 47: 56–60.

de Araujo RMS, Oba Y, Moriyama K (2007) Identification of genes related to mechanical stress in human periodontal ligament cells using microarray analysis. J Periodontal Res 42: 15–22.

Downey PA, Siegel MI (2006) Bone biology and the clinical implications for osteoporosis. Phys Ther 86: 77–91.

Fawcett A (2012) Guideline 22: Guidelines for the Housing of Mice in Scientific Institutions. Orange NSW: Animal Welfare Branch, pp. 1–143.

Fedchenko N, Reifenrath J (2014) Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - a review. Diagn Pathol 9: 221.

Fischer AH, Jacobson KA, Rose J, Rolf Z (2017) Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc 2008: pdb.prot4986.

Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simões MJ, Cerri PS (2015) Biology of bone tissue: structure, function, and factors that influence bone cells. Biomed Res Int 2015: 421746.

Grosset AA, Loayza-Vega K, Adam-Granger É, Birlea M, Gilks B, Nguyen B, Soucy G, Tran-Thanh D, Albadine R, Trudel D (2019) Hematoxylin and eosin counterstaining protocol for immunohistochemistry interpretation and diagnosis. Appl Immunohistochem Mol Morphol 27: 558–563.

Hamilton PW, van Diest PJ, Williams R, Gallagher AG (2019) Do we see what we think we see? The complexities of morphological assessment. J Pathol 218(3): 285-291.

Holland R, Bain C, Utreja A (2019) Osteoblast differentiation during orthodontic tooth movement. Orthod Craniofac Res 22: 177–182.

Jonsdottir SH, Giesen EBW, Maltha JC (2012) The biomechanical behaviour of the hyalinized periodontal ligament in dogs during experimental orthodontic tooth movement. Eur J Orthod 34: 542–546.

Kirschneck C, Bauer M, Gubernator J, Proff P, Schröder A (2020) Comparative assessment of mouse models for experimental orthodontic tooth movement. Sci Rep 10: 12154.

Matos LL, Trufelli DC, de Matos MG, da Silva Pinhal MA (2010) Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomark Insights 5: 9-20.

Meikle MC (2006) The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. Eur J Orthod 28: 221-240.

Mukaka (2012) Statistics corner: A guide to appropriate use of correlation coefficient in medical research. Malawi Med J 24: 69–71.

Phan TCAA, Xu J, Zheng MH (2004) Interaction between osteoblast and osteoclast: impact in bone disease. Histol Histopathol 19: 1325–1344.

Ruifrok AC, Johnston DA (2001) Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol 23: 291–299.

Sufarnap E, Siregar D, Lindawati Y (2020) Effect of vitamin E supplementation on orthodontic tooth movement in Wistar rats: A prelimary study. F1000Res 9: 1093.

Taddei SR, Moura AP, Andrade I Jr, Garlet GP, Garlet TP, Teixeira MM, da Silva TA (2012) Experimental model of tooth movement in mice: A standardized protocol for studying bone remodeling under compression and tensile strains. J Biomech 45: 2729–2735.

Taylor CR, Levenson RM (2006) Quantification of immunohistochemistry? Issues concerning methods, utility and semiquantitative assessment II. Histopathology 49: 411–424.

Vandevska-Radunovic V, Murison R (2010) Emotional stress and orthodontic tooth movement: Effects on apical root resorption, tooth movement, and dental tissue expression of interleukin-1 alpha and calcitonin gene-related peptide immunoreactive nerve fibres in rats. Eur J Orthod 32: 329–335.

Wahab RMA, Dasor MM, Senafi S, Abdullah AAA, Jemain AA, Kasim NA, Yamamoto Z, Ariffin SHZ (2011) Crevicular tartrate resistant acid phosphatase activity and rate of tooth movement under different continuous force applications. African J Pharm Pharmacol 5: 2213–2219.

Watts NB (1999) Clinical utility of biochemical markers of bone remodeling. Clin Chem 45: 1359–1368.

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| Contribution | Sufarnap E | Ilyas S | Nazruddin N | Putra DP | Rachmawati A |
|---|---|---|---|---|---|
| Concepts or ideas | x | x | x | x | |
| Design | x | x | | | |
| Definition of intellectual content | | x | 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 | | | | x |
| Manuscript review | x | x | x | x | x |

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