The Role of Salivary Receptor Activator NF-Kappa B Ligand (RANKL) On Persistent Primary Teeth in Minangkabau Children

Fuccy Utamy Syafitri[1]*, Nila Kasuma[2], Eti Yerizel[3], Roni Eka Sahputra[4], Fildzah Nurul Fajrin[2]

1. Departmen of Orthodontics, Faculty of Dentistry, University of Andalas.
2. Departmen of Oral Biology, Faculty of Dentistry, University of Andalas.
3. Department of Biochemistry, Faculty of Medicine, University of Andalas.
4. Department of Surgery, Faculty of Medicine, University of Andalas.

Abstract

Primary tooth persistence involves the processes of primary tooth root resorption, bone resorption on the occlusal surface, and basal bone formation around the roots of erupting teeth. The receptor activator of NF-kappaB ligand (RANKL) plays a role in regulating bone resorption in the primary to permanent tooth replacement process. RANKL levels are influenced by endogenous factors, one of which is ethnicity. This study aimed to analyze salivary RANKL concentration's effect on the persistence of primary teeth in Minangkabau children.

Cross-sectional research was conducted on 30 native Minangkabau children aged 6-13. Subjects were divided into two groups: the group with tooth persistence and the control group. Subject saliva was collected and tested for RANKL levels by ELISA method. Differences in RANKL concentrations between groups were statistically tested using the U-Mann-Withney test and logistic regression on SPSS software.

The results showed that the salivary RANKL concentration of the dental persistence and control groups was not significantly different ($p>0.05$). However, the mean RANKL concentration in the persistence group was lower than the control. Salivary RANKL's effect on primary teeth' persistence is minimal and not proven statistically significant ($p>0.05$).

From the study results, it can be concluded that the concentration of RANKL does not affect the incidence of persistence of primary teeth in children.

Clinical article (J Int Dent Med Res 2023; 16(4): 1652-1656)

Keywords: Persistent primary teeth, RANKL, tooth eruption, saliva.

Received date: 21 July 2023 **Accept date:** 15 August 2023

Introduction

Persistent primary teeth are the state of primary teeth still in position and do not even exfoliate when the permanent teeth erupt[1]. Primary teeth are also said to be persistent if they remain in position when the roots of the replacement permanent teeth have reached 75% of their final size, which can be seen through the supporting examination of panoramic radiographs. Primary tooth persistence is a condition that often occurs in the mixed dentition period[2].

Research states that the prevalence of primary tooth persistence cases is 4.5% of the Turkish population experiencing primary tooth persistence[2]. Research by Sanjith et al. states that the prevalence of primary tooth persistence cases in India is 11.5%[3]. In Indonesia, a retained (persistent) primary tooth is the most prevalent case in rural and suburban areas[4].

Deciduous tooth loss involves a resorption mechanism in the dental follicle region of the permanent tooth. The dental follicle plays a vital role because it accelerates the root resorption process of primary teeth and is where bone remodeling occurs. The requirements for tooth eruption are bone resorption on the occlusal surface to provide space for the crown to penetrate the alveolar bone, resorption of the roots of primary teeth, and bone formation on the basal part around the roots of erupting teeth [5,6,7].

Root resorption of primary teeth is also a physiological factor for primary tooth loss and permanent tooth eruption. Physiological root resorption is regulated by odontoclastic activity

Corresponding author:
Fuccy Utamy Syafitri,
Departmen of Orthodontics, Faculty of Dentistry, University of Andalas.
E-mail: fuccyutamy@dent.unand.ac.id

genetically, followed by alveolar bone remodeling. Odontoclasts have the same characteristics, enzymatic and metabolic properties as osteoclasts but are more involved in the resorption process of dental hard tissues such as dentin and cementum[7].

The development of jaw bone and muscle in children is associated with a significant increase in masticatory force over the periodontal ligament surface of the primary teeth. The masticatory forces induce cytokine production to stimulate periodontal ligament fibroblasts to secrete RANKL and recruit macrophages and monocytes to activate odontoclasts to resorb tooth roots. The process of primary tooth root resorption is regulated similarly to the bone remodeling process, which includes a ligand-receptor system known as RANKL Receptor activator of NF-kappaB ligand (RANKL) is a type II transmembrane protein expressed by osteoblasts, osteocytes, and immune cells that form bone tissue[8]. RANKL is also present in odontoblasts, pulp tissue, and periodontal ligament fibroblasts (cementoblasts)[9].

Osteoblast membrane-bound RANKL binds to RANK on the surface of osteoclast progenitor cells to stimulate osteoclastogenesis, bone remodeling, and calcium homeostasis[10]. Yang et al. proved that RANKL is produced in the periodontal ligament and surrounding bone cells, playing a role in tooth movement. RANKL is a major osteoclastogenesis regulator, so RANKL deficiency can cause osteopetrosis due to osteoclast deficiency[9].

Endogenous and exogenous factors such as parenting, ethnicity, breastfeeding patterns, and dietary habits affect the eruption cycle of permanent teeth [11]. Ethnic factors play a role in gene polymorphisms that cause dental eruption disorders. Several studies have conducted studies on certain ethnicities related to RANKL concentrations. In line with Arid's research in Brazil on Latin American races, it shows that RANKL gene polymorphisms cause the persistence of primary teeth[12]. Garmash et al.'s study in the Ukrainian population concluded that SNPs in the RANKL gene increase the risk of late tooth eruption[11]. Hart's study showed that RANKL concentrations in white and African Americans were not significantly different, but white Americans tended to show higher RANKL levels[13]. Currently, there is no research on the role of salivary RANKL in ethnic Minangkabau related to the persistence of primary teeth.

Saliva is a physiological oral fluid that functions as a buffer, facilitates flavor recognition, and antimicrobials that modulate oral flora[14]. Saliva has enormous diagnostic value because it contains inflammatory cytokines, enzymes, and contents that are manifestations of various diseases. Thus, their levels can be examined to help early diagnosis of certain diseases[15]. Research by Behfarnia et al. stated that there was no difference in the concentration of RANKL in saliva, serum, and GCF in serum, so the method of detecting RANKL in saliva can be an alternative detection besides blood serum[16].

Based on the above problems and theories, the author is interested in analyzing the concentration of RANKL in saliva in the case of the persistence of primary teeth in Minangkabau children.

Materials and methods

This cross-sectional study was conducted at the Oral Dental Hospital and Biomedical Laboratory of Andalas University, Indonesia. This study has passed the ethical test from the Faculty of Medicine, Andalas University. The research subjects were 30 children with at least one persistent primary tooth. Selected by consecutive sampling method, which was then divided into 2 groups of 15 people each, namely the group with persistent teeth and the control group. Subjects aged 60-13 years and native Minangkabau ethnicity of at least two descendants were included in the study. Subjects with agenesis, odontoma, and supernumerary teeth, with a history of congenital abnormalities, trauma or infection to the face, metabolic diseases, and drug consumption. Syndromic abnormalities and damaged research specimens that could not be assessed were excluded from the study.

Clinical Examination of Persistent Teeth

Respondents were examined using a mouth glass, disposable probe, and LED headlamp for good lighting. The examination was carried out on the upper and lower jaws, and the eruption status of each deciduous and permanent tooth was recorded. Teeth were then compared with the ideal eruption age according to the Permanent teeth chronologic age chart. If the teeth erupted appropriately, they were regular teeth, coded. Teeth erupted more than 12

months from the standard eruption time were classified as persistent teeth.

Saliva collection

Subjects rinsed their mouth with water for 30 seconds and expectorated before saliva collection. Subjects sat upright, head slightly downward, and were asked not to swallow or move the tongue and lips during the collection period. Saliva was collected from 8:00-11:00 am. Saliva was allowed to drip from the lower lip and collected with an Eppendorf tube. A total of 1.5 ml of saliva was collected. Specimens were stored in a freezer at -20o C in the Biomedical Laboratory of Andalas University.

Examination of Salivary RANKL levels

RANKL was measured by ELISA method, following previous protocol by Widayati. All samples and standards were analyzed twice17. Saliva specimens were centrifuged at 10,000 rpm for 10 minutes. 50 µL of the standard was inserted in the standard well, followed by 40 µL of specimen in the specimen well. Anti RANKL antibody and 50 µL Streptavidin-HRP into the specimen well and standard well. The plate was covered with sealer and then incubated for 60 minutes at 37℃. The sealer was removed and rinsed five times with wash buffer. Wells were soaked with wash buffer for 30 minutes at each rinse. The plate was dried with absorbent paper. 50 µl of substrate liquid A was dripped into each well and incubated for 10 min at 37℃. Stop solution was added as much as 50 µL in each well so that there was a color change from blue to yellow.

Optical Density (OD) was calculated using a microplate reader set at 450 nm within 30 minutes after adding the stop solution. The numbers that came out of the reader were the mean numbers and RANKL concentrations that were equalized with the help of a standard graph with units of ng/ml.

Group	Shapiro Wilk (W)	Df	*P*
Persistent primary teeth	0,92	36	<0,05
Healthy control	0,90	36	<0,05

Table 1. Normality test results of salivary RANKL concentration data.

Statistical analysis

The difference in RANKL concentration between the primary tooth persistence group and control tooth persistence groups was analyzed with SPSS software. The saphiro-Wilk normality test was performed to see if the data were normally distributed. Concentration differences between groups were tested with U-Mann Whitney.

Results

Salivary RANKL Concentration

Before the bivariate test is performed, it is necessary to test the normality of salivary RANKL concentration data using the Shapiro-Wilk test. Normality testing is carried out for each group (persistence and normal groups). Based on Table 1, it was found that the salivary RANKL concentration data in the persistence group did not follow a normal distribution ($W = 0.92$; $p < 0.05$). The same results are also shown in the normal group, where the data does not follow a normal distribution ($W = 0.90$; $p < 0.05$).

Because the Shapiro-Wilk test results showed that the salivary RANKL concentration data in the two groups did not meet the normality assumption, the difference test was performed using a non-parametric test, namely U-Mann Whitney. The results showed no difference in salivary RANKL concentration between the primary tooth persistence group and the normal group ($U = 543$; $z = -1.18$; $p = 0.24$).

Group	*Median (IQR)*	*Mean Rank*	*U*	*Z*	*P*
Persistent primary teeth	384,58 (320,29)	33,58	543,00	-1,18	0,24
Healthy control	401,07 (558,58)	39,42			

Table 2. U-Mann Whitney test results of salivary RANKL concentration.

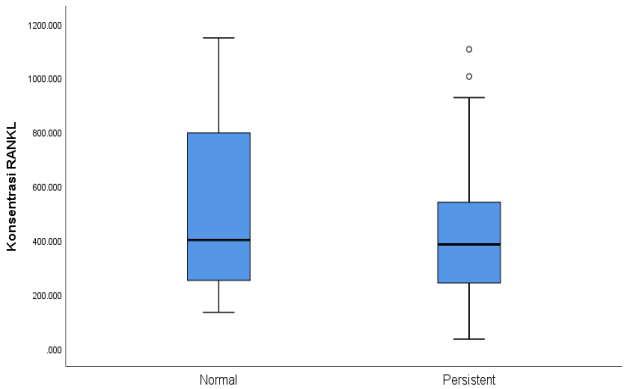

Figure 1. Comparison of Salivary RANKL Concentration of Tooth Persistence and Normal Groups.

Based on Figure 1, it can be seen that the RANKL concentration of the normal group is higher (Median = 401.07 ng/ml) than in the persistence group (Median = 384.58 ng/ml). However, the difference is not statistically significantly different, so the hypothesis in this study has yet to be proven by existing empirical evidence.

Effect of Salivary RANKL Concentration on Tooth Persistence

Logistic regression analysis was performed to see the effect of salivary RANKL concentration on the incidence of dental persistence. The dependent variable of this model is the incidence of dental persistence (Y = 1), with the control group as the control (Y = 0), while the independent variable is the salivary RANKL concentration. The logistic regression analysis results (Table 5.7) show that an increase in RANKL concentration can reduce the odds of dental persistence (OR = 0.99; 95%CI = 0.99 - 1.00). However, this effect is minimal and not statistically significant, so it can be concluded that the RANKL concentration factor does not influence the incidence of primary tooth persistence in children (p = 0.14).

Variables	B	SE	Wald	df	P	OR (95%CI)
Persistent primary teeth	0,60	0,47	1,64	1	0,20	1,82
Salivary RANKL level	-0,00	0,01	2,18	1	0,14	0,99 (0,99 – 1,00)

Table 3. Logistic regression test results of the effect of RANKL concentration.

Discussion

This study showed no significant difference between the concentration of salivary RANKL in the subject of persistence of primary teeth with the control group. Similar results were also obtained by Noumova et al., who reported no significant difference in the concentration of RANKL in saliva during the phases of tooth movement[18]. The slight difference between the two groups may be due to the dilution effect by saliva, or the sampling interval used in the study design is not likely to reflect the highest osteoclast activity during tooth movement, where RANKL levels are very different from normal conditions[19].

Nonetheless, judging from the average concentration, the concentration of RANKL in the control group was higher than in the persistence group. This condition is in line with the research of Gama et al., who conducted a study on patients with persistence of primary molars[20]. Osteopetrosis results from constitutive suppression of RANKL (Rankl-/-), which is accompanied by considerable growth retardation, changes to bone metabolism brought on by a decline in osteoclastic differentiation, disruptions of dental and bone cell communication, and changes to tooth eruption. This research is also supported by Brodetska et al. stating that the concentration of RANKL in non-impacted teeth is higher than in the alveolar bone of impacted teeth. In impacted teeth, there is a decrease in RANKL in both membrane-bound and soluble forms[21].

In the condition of deciduous tooth persistence, RANKL inhibition occurs temporarily. In this situation, environmental factors that disrupt RANKL signaling during growth may impact dental and craniofacial development, with the degree of variability depending on the intervention stage of these factors and their local or systemic application. As a result, in the case of systemic intervention, craniofacial growth and tooth eruption were interdependent processes that relied on osteoclastogenesis, which was genetically controlled in time and place by RANKL signaling [22, 23].

The temporary inhibition of RANKL might affect signaling pathways involved in root elongation and alveolar bone modeling, such as the (Tgfβ/Bmp) protein pathway, tumor growth factors/bone morphogenetic, fibroblast growth factor (FGF) pathway, Wingless/β-catenin (Wnt/β-catenin) pathway, insulin-like growth factor (IGF) pathway and Sonic hedgehog (Shh) pathway [24]. RANKL inhibition could still alter tooth eruption even though osteoclasts exist[20]

This cross-sectional study is the first in vivo study in humans using saliva sampling to monitor RANKL levels in Minangkabau ethnic children with persistent primary teeth. RANKL concentration did not affect the incidence of persistent primary teeth in children. However, RANKL levels in persistent teeth are lower than in normal conditions. Saliva sampling at the phase of highest osteoclast activity during tooth movement where RANKL levels are very different from normal conditions should be considered for

future research.

Conclusions

This first in vivo study in humans using saliva sampling to monitor RANKL levels in Minangkabau ethnic children with persistent primary showed that RANKL concentration did not affect the incidence of persistent primary teeth in children. Nonetheless, judging from the average concentration, the concentration of RANKL in the control group was higher than in the persistence group. stating that the concentration of RANKL in non-impacted teeth is higher than in the alveolar bone of impacted teeth.

Acknowledgements

We thank our colleagues from the University of Andalas, who provided insight and expertise that greatly assisted the research.

Declaration of Interest

The authors report no conflict of interest, and the article is not funded or supported by any research grant.

References

1. Taran PK, Ölmez A. Prevalence, distribution, and condition of persistent primary teeth in children and adolescents. Int J Pedod Rehabil 2019;4:50-4
2. Aktan AM, Kara I, Sener I, et al. An evaluation of factors associated with persistent primary teeth. *Eur J Orthod*. 2012;34(2):208-212.
3. Sanjith, A. Sai; Don, K. R.; Mohanraj, Karthik Ganesh. Knowledge and awareness of primary teeth and their importance among parents. Indian Journal of Forensic Medicine & Toxicology, 2020, 14.4: 5427-5436.
4. Dewanto I, Koontongkaew S, Widyanti N. Characteristics of Dental Services in Rural, Suburban, and Urban Areas Upon the Implementation of Indonesia National Health Insurance. *Front Public Health*. 2020;8:138.
5. Kjær, Inger. 2014. "Mechanism of Human Tooth Eruption: Review Article Including a New Theory for Future Studies on the Eruption Process." Scientifica 2014: 341905:1-13
6. Richman, Joy M. Shedding new light on the mysteries of tooth eruption. Proceedings of the National Academy of Sciences, 2019, 116.2: 353-355.
7. Wang Z, McCauley LK. Osteoclasts and odontoclasts: signaling pathways to development and disease. *Oral Dis*. 2011;17(2):129-142. doi:10.1111/j.1601-0825.2010.01718.x
8. Nelson CA, Warren JT, Wang MW, Teitelbaum SL, Fremont DH. RANKL employs distinct binding modes to engage RANK and the osteoprotegerin decoy receptor. *Structure*.2012;20(11):1971-1982. doi:10.1016/j.str.2012.08.030
9. Yang CY, Jeon HH, Alshabab A, Lee YJ, Chung CH, Graves DT. RANKL deletion in periodontal ligament and bone lining cells blocks orthodontic tooth movement. *Int J Oral Sci*. 2018;10(1): 1-9.
10. Li, J., Sarosi, I., Yan, X.-Q., Morony, S., Capparelli, C., Tan, H.-L., et al. (2000). RANK Is the Intrinsic Hematopoietic Cell Surface Receptor that Controls Osteoclastogenesis and Regulation of Bone Mass and Calcium Metabolism. Proc. Natl. Acad. Sci. U.S.A. 97 (4), 1566–1571.
11. Arid, Juliana, et al. RANKL is associated with persistent primary teeth and delayed permanent tooth emergence. International Journal of Paediatric Dentistry, 2019, 29.3: 294-300.
12. Hart, Katherine Ann. RANKL and Osteoprotegerin levels in response to orthodontic forces. UTHSC Digital Commons. 2012. https://dc.uthsc.edu/dissertations/107/.
13. Garmash, O. V. Single-Nucleotide Polymorphisms Of The Esr1 [Rs 2234693], Il-1 [Rs1143627], Rankl [Rs9594738] And [Rs9594759] Genes As Possible Risk Markers For Various Variants Of The Course Of Periodontal Disease In Patients Born Macrosomic. Ukrainian Dental Almanac, 2020, 1: 5-18.
14. Dawes C., Pedersen A.M., Villa A., Ekström J., Proctor G.B., Vissink A., Aframian D., McGowan R., Aliko A., Narayana N., et al. The functions of human saliva: A review sponsored by the World Workshop on Oral Medicine VI. Arch. Oral Biol. 2015;60:863–867.
15. Costantini E., Sinjari B., Piscopo F., Porreca A., Reale M., Caputi S., Murmura G. Evaluation of Salivary Cytokines and Vitamin D Levels in Periodontopathic Patients. Int. J. Mol. Sci. 2020;21:2669.
16. Behfarnia P, Saied-Moallemi Z, Javanmard SH, Naseri R. Serum, saliva, and GCF concentration of RANKL and osteoprotegerin in smokers versus nonsmokers with chronic periodontitis. *Adv Biomed Res*. 2016;5:80.
17. Widayati R, Sastradipura DFS, Purwaningsih EH, Iskandriati D. Root resorption and RANKL concentration in orthodontic tooth movement accompanied by topical PGE2 gel application. Journal of International Dental and Medical Research. 2016;9(3):228-232.
18. Naoumova J, Olofsson R, Almståhl A, Cevik-Aras H. Salivary levels of Osteoprotegerin and receptor activator of nuclear factor-kappa ligand during orthodontic tooth movement-A prospective pilot study [published online ahead of print, 2023 Jun 29]. *Orthod Craniofac Res*. 2023;10.1111/ocr.12687. doi:10.1111/ocr.12687
19. Flórez-Moreno GA, Isaza-Guzmán DM, Tobón-Arroyave SI. Time-related changes in salivary levels of the osteotropic factors sRANKL and OPG through orthodontic tooth movement. *Am J Orthod Dentofacial Orthop*. 2013;143(1):92-100. doi:10.1016/j.ajodo.2012.08.026
20. Gama A, Maman L, Vargas-Franco JW, Omar R, Royer BB, Yagita H, Babajko S, Berdal A, Acevedo AC, Heymann D, Lézot F, Castaneda B. Primary Retention of Molars and RANKL Signaling Alteration during Craniofacial Growth. J Clin Med. 2020 Mar 25;9(4):898. doi: 10.3390/jcm9040898.
21. Brodetska L, Natrus L, Lisakovska O, et al. The regulatory role of the RANKL/RANK/OPG signaling pathway in the mechanisms of tooth eruption in patients with impacted teeth. *BMC Oral Health*. 2020;20(1):261.
22. Berdal A, Castaneda B, Aïoub M, et al. Osteoclasts in the dental microenvironment: a delicate balance controls dental histogenesis. *Cells Tissues Organs*. 2011;194(2-4):238-243. doi:10.1159/000324787
23. Lézot F, Thomas BL, Blin-Wakkach C, et al. Dlx homeobox gene family expression in osteoclasts. *J Cell Physiol*. 2010;223(3):779-787. doi:10.1002/jcp.22095
24. Li A, Cong Q, Xia X, et al. Pharmacologic Calcitriol Inhibits Osteoclast Lineage Commitment via the BMP-Smad1 and IκB-NF-κB Pathways. J Bone Miner Res. 2017;32(7):1406-1420. doi:10.1002/jbmr.3146.