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Research Report

The expression of nuclear factor of activated T cell c1 and receptor activator of nuclear factor kappa β induced by *Enterococcus faecalis* in osteoclastogenesis (laboratory experiment on Wistar rats)

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ABSTRACT

Background: *Enterococcus faecalis (E. faecalis) is the most common bacteria species in persistent endodontic infection of teeth undergoing root canal treatment at a prevalence of 38%. The virulence factor of this bacterium is Lipoteichoic acid (LTA) which can be recognized by Toll-like receptors-4 (TLR-4) that produce a stimulus and provoke an immune response. Inflammation results in bone defects that feature multiple cytokines and interactions between different cell types. Bone loss within a periapical tooth is characterized by osteoclast formation (osteoclastogenesis) in the bone.* ***Purpose:*** *This study aimed to determine the expression of nuclear factor of activated T cell c1 (NFATc1) and receptor activator of nuclear factor kappa β (RANK) which played a role in osteoclastogenesis at different time intervals.* ***Methods:*** *36 upper molar teeth of the research subjects were induced with 10^6 CFU Enterococcus faecalis and subsequently observed for 7 and 21 days with the NFATc1 and RANK being counted microscopically at 1000X magnification across 20 viewing fields. Thereafter, the data was examined and analyzed by means of an independent T test using SPSS.* ***Results:*** *NFATc1 and RANK expression were higher in the group including E. faecalis on days 7 and 21 than in the control group. There were significant differences between the treatment group and control group with regard to NFATc1 and RANK expression ($p<0.05$).* ***Conclusion:*** *The study showed that the expression of NFATc1 and RANK, which plays a role in osteoclastogenesis, was higher in periapical bone defects in Wistar rats induced by E. faecalis than those which were not induced.*

Keywords: *Enterococcus faecalis; endodontic infection; Lipotheicholic acid; NFATc1; RANK, wistar rats*

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INTRODUCTION

Secondary root canal infections can occur due to inadequate management of primary root canal infection during treatment. The complex anatomy of root canal systems often results in incomplete root canal preparation. Cleansing and shaping steps involving the use of disinfectant cannot completely eliminate bacteria, especially in the apical area, rendering the site liable to re-infection.[1] Gijo *et al.* (2015)[2] proved that in cases of unsuccessful root canal treatment *Enterococcus faecalis (E. faecalis)* was confirmed as the most common bacterium causing persistent endodontic infection in teeth undergoing root canal treatment with a prevalence of 38%. *E. faecalis* was detected in obturated root canals and was responsible for 77% of periapical deformities.[3] *E. faecalis* represents one bacterium that causes periapical disease due to significant virulence factors, the main one being Lipoteichoic acid (LTA) which is a major constituent of the outer envelope of gram-positive bacteria.[2] LTA can be recognized by specific signaling molecules on the surface of host cells called Toll-like receptors-4 (TLR-4) that produce a stimulus and provoke an immune response.[4]

The interface between the pulp and periapical region may cause the remaining bacteria in the root canal to

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inflame periapical tissue which, in turn, can cause bone defects in the periapical region. Inflammation resulting in bone defects constitutes a complex regulatory process involving multiple cytokines and interactions between different cell types. The primary cells responsible for bone resorption are osteoclasts.[5] Bone loss in a periapical tooth is characterized by osteoclast formation (osteoclastogenesis) within the bone. During osteoclastogenesis, osteoclast differentiation factors produce a bonding reaction with the receptor.[6] Osteoclast differentiation and the subsequent bone resorption are initiated by the nuclear factor of activated T cells c1 (NFATc1). Activation of NFATc1 will induce TRAP + osteoclast formation which produce mature osteoclasts and, furthermore, form an active osteoclast. The higher production of active osteoclast promoted greater bone resorption.[7] receptor activator of nuclear factor kappa β (RANK) is also referred to as TNFRSF11A/TRANCE-R2 which forms part of the tumor necrosis factor (TNF) receptor. RANK can be found in osteoclasts and precursors, hematopoietic precursors, dendritic cells and mammary epithelial precursors as type I transmembrane receptors.[8] RANK can be activated when binding to RANKL.[6–8]

RANKL is detected in osteoblasts, T cells, dendritic cells and its precursor as type II transmembrane proteins. RANKL can activate RANK, while RANK can, in turn, activate RANKL. RANK and RANKL play a critical role in osteoclast formation. RANK activity provides signals that will activate NF-κB, NFATc1 and P38. If RANKL is blocked, RANK cannot be activated alone and osteoclastogenesis will not occur.[8] This study aims to determine the level of expression of RANK and NFATc1 that can activate osteoclastogenesis after induction of *E. faecalis* bacteria in the periapical teeth of Wistar rats at different time intervals.

MATERIALS AND METHODS

The study reported here was approved and supervised by the Universitas Airlangga Faculty of Dental Medicine Research Ethical Clearance Commission with number 38/KKEPK.FKG/III/2015 and constituted a laboratory-based experiment. The subjects were 8-12 weeks old, adult, male, *Rattus norvegicus*, weighing between 120 and150 grams, in good physical condition with fully developed molar teeth, supplied by the Faculty of Veterinary Medicine, Universitas Airlangga. Each sample consisted of the right upper molars of nine Wistar rats, the total sample comprising 36 subjects. Each subject was first secured to the jaw retractor board, prior to the pulp chamber roof of the maxillary molar being perforated with a nozzle bur (1/4) (Dica®, Austria). Subjects satisfying the requirements were injected with intra-peritoneal anesthesia consisting of 80 mg/kg body weight of ketamine and 10 mg/kg body weight of xylazine. (Onemed®, Indonesia).[9] After being induced with 10^6 CFU *E. faecalis* ATCC212, the cavity was sealed with GIC resin.

The research involved the participation of two study groups: treatment group A, as the control group, on whose members cavity preparation was performed until perforation of the pulp chamber roof had been effected, prior to sterile brain heart infusion broth (BHIB) being injected. In treatment group B, cavity preparation was undertaken until the pulp chamber roof had been perforated, with 10µl BHIB containing 10^6 CFU bacteria *E. faecalis* ATCC212 subsequently being injected by micropipette. Both Groups A and B were composed of two sub-groups, namely; A day 7 and A day 21, and B day 7 and B day 21. The subjects were then sacrificed by means of cervical dislocation. The jaw slices were separated and fixed with 10% neutral formaldehyde buffer for 24 hours, and decalcified with 4% EDTA for 30 days, before being made into paraffin block preparations. At that point, samples were made into HPA preparations, enabling periapical bone defects to be observed. Immunohistochemical imaging was performed using anti-NFATc1 antibodies (Biolegend # 649601, United States) and RANK, in order to facilitate observation from 20 fields of view with a light microscope at 1000x magnification and calculation of the number of osteoclast cells expressing NFATc1 and RANK in the periapical. For inferential purposes, a normality test was performed using a Kolmogorov-Smirnov test. A Levene's test of homogeneity and a one-way t-test to evaluate the effect of *E. faecalis* induction on the difference between NFATc1 and RANK cell numbers in control group (A) and treatment group (B). P value <0.05 indicated a significant difference.

RESULTS

NFATc1 and RANK expression data were obtained through observation of osteoclast cell numbers in the periapical bone, given that positive reactions to anti-NFATc1 and anti-RANK monoclonal antibodies with immunohistochemical methods were characterized by brown coloration of the cytoplasm in the control and treatment group (Figure 1 and 2). The number of NFATc1 and RANK expressions can be seen in Table 1. Following calculation of the NFATc1 expression number, it was discovered that NFATc1

Table 1. The average of NFATc1 and RANK expression results in the control and treatment groups

Group	N	X NFATc1	SD NFATc1	X RANK	SD RANK
K7 Control	9	8.56	2.297	4.22	2.386
K21 Control	9	12.22	1.922	11.78	2.489
K7 Treatment	9	15.56	2.449	12.56	1.236
K21 Treatment	9	19.89	1.537	19.67	2.291

Note: X: The average of NFATc-1 and RANK expression; SD: Standard Deviation; N: Number of samples per group

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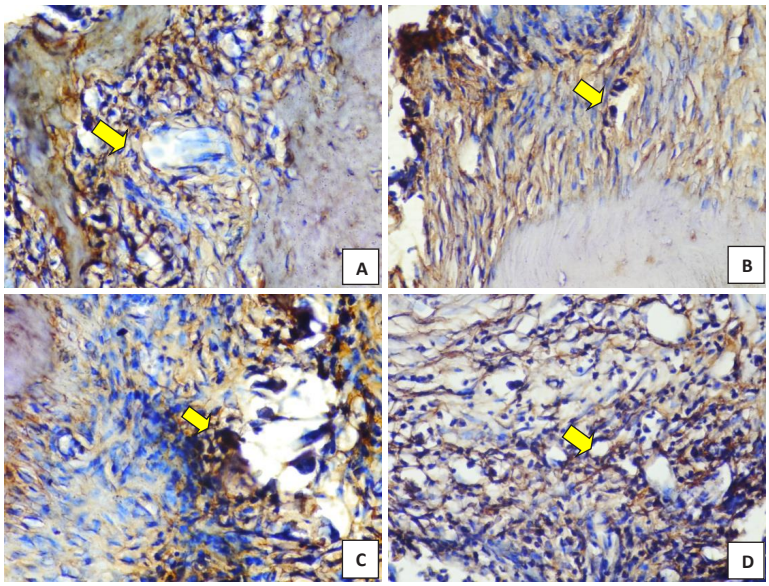

Figure 1. Imaging of NFATc1 expression (indicated by brown coloration and an arrow) in the immunohistochemical preparation of RA Wistar rat molars A) Control group at day 7; B) Control group at day 21; C) Treatment group at day 7; D) Treatment group in day 21; (1000x magnification per 20 field of view). The expression of NFATc1 becomes higher on day 21 than on day 7 in the control and treatment groups.

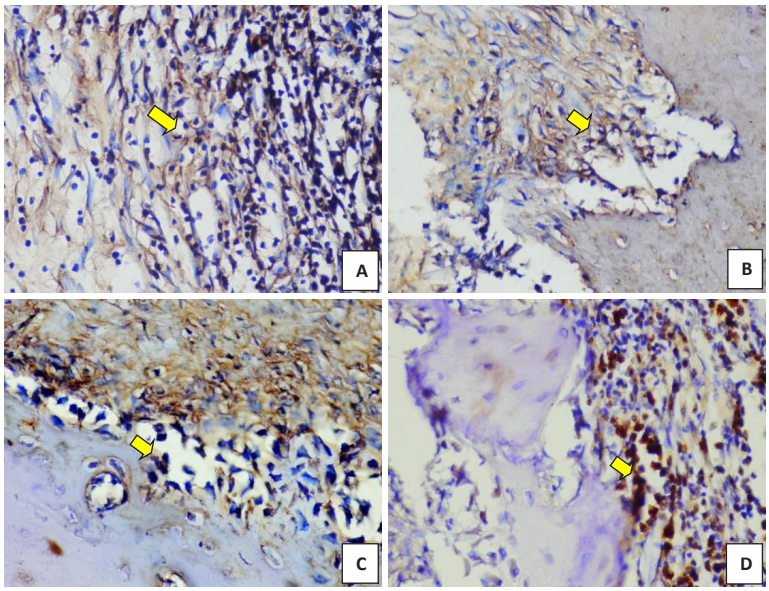

Figure 2. Imaging of RANK expression (indicated by brown coloration and an arrow) in the immunohistochemical preparation of RA Wistar rat molars A) Control group on day 7; B) Control group on day 21; C) Treatment group on day 7; D) Treatment group on day 21; (1000x magnification per 20 field of view). The expression of RANK became higher on day 21 than on day 7 in the control and treatment groups.

Table 2. Independent T test results for NFTAc1 and RANK expression in each treatment group

Group	p value	
	Expression of NFTAc1	Expression of RANK
Control day 7 Treatment day 7	0.000*	0.000*
Control day 21 Treatment day 21	0.000*	0.000*

*indicated a significant statistical difference ($p < 0.05$)

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expression in the treatment group was higher than in the control group. In the control and treatment groups, NFATc1 expression increased between day 7 and day 21.

Based on the calculation of the RANK expression number, it was discovered that RANK expression in the control group increased between days 7 and 21, while in the treatment group it also increased steadily during the same period. Normality test results using a Kolmogorov-Smirnoff test for NFATc1 and RANK expressions showed the research data to be normally distributed ($p > 0.05$). Data analysis was subsequently processed by means of an independent T-test to show whether any difference between the groups existed. The independent T-test results are contained in Table 2.

The results of an independent T-test on NFATc1 expression showed significant differences between the control group and the treatment group on day 7 and day 21. Similar results were also derived for RANK expression. The test results using the independent T-test of RANK expression also showed significant differences between the control group and the treatment group on days 7 and 21.

DISCUSSION

The remaining bacteria present during root canal treatment represent a major etiology of endodontic treatment failure since they will cause inflammation of the periapical tissue. *E. faecalis* is a bacterium that often leads to endodontic treatment failure and causes re-infection.[3] The connection between the root canal and apical causes inflammation of the periapical and high virulence of *E. faecalis*, with the presence of LTA, will lead to bone defects.[4] Such bone defects in the periapical are characterized by the formation of osteoclasts (osteoclastogenesis) within the periapical bone.

This study used Wistar rats as research subjects due to their possessing a genome similar or almost homologous to that of humans, while also representing an experimental model (animal) for periodontal disease. The time periods adopted for this research were day 7 and day 21. Their selection was based on the inflammation theory that posits days 0-7 as representing the recognition and activation phase, days 7-14 as constituting the activation and effector phase, while days 14-30 form the homeostasis phase during which restoration occurred.[10]

Osteoprotegerin (OPG) is a RANK and RANKL bonding inhibitor which enables the homeostasis phase to occur. If RANKL is larger than OPG it will bind to RANK, resulting in osteoclastogenesis and bone resorption. In the inflammatory phase, the RANKL ratio will increase in the periapical tissue and stimulate osteoclast activity, thereby initiating bone resorption.[8]

According to the research results, NFATc1 and RANK expression in the control group increased between days 7 and 21 due to the activation of body molecules secreted from the damaged tissue and damage-associated molecular pattern (DAMP) including heat shock protein (HSP70). Under normal conditions, HSP70 exists in small concentrations. However, environmental stimuli (ultraviolet radiation, heat, heavy metals and amino acids), pathological stimuli (viruses, bacteria, fever or parasitic infections and inflammation), or physiological stimuli (growth factor, cell differentiation, hormonal stimulation and tissue development) affect the increase in HSP70 synthesis.[11,12] In the positive control group, dental perforation and BHIB application of 10µl represented the stimuli in the formation of HSP70.

There was a significant increase in NFATc1 and RANK expression in the treatment group from day 7 to day 21. This indicates that *E. faecalis* possesses a strong virulence factor that cannot be compensated by a homeostasis mechanism. Therefore, the expression of NFATc1 and RANK is an important factor in the continuous increase in osteoclastogenesis. Statistically, there was a significant difference in the NFATc1 and RANK expression results between the control and treatment groups, where the treatment group expression was higher than that of the control group.[7,13]

E. Faecalis, with the presence of LTA, will activate monocyte/macrophage which releases pro-inflammatory cytokines such as IL-1α, IL-1β, TNFα, IL-10, and IL-6. These cytokines act as pro-osteoclastogenic factors inducing RANKL production that will activate NFATc1. The expressed NFATc1 will induce TRAP+ osteoclast formation resulting in osteoclast maturation which will further form the active osteoclast (ruffled border osteoclast). Higher production of active osteoclasts means greater bone resorption.[7,13]

The study results of NFATc1 and RANK expressions between the control and treatment groups confirmed a significant increase. This result is similar to that of the study by Park *et al*. (2015) which states that *E. faecalis* inhibits osteoblast formation. Moreover, it is also supported by the research of Tian *et al*. (2013) which proves that LTA from *E. faecalis* inhibits the proliferation of osteoblasts and induces apoptosis of human-osteoblast-like cells. Therefore, osteoblasts become undeveloped, resulting in high levels of osteoclasts and more extensive bone damage.[14,15]

The results of NFATc1 and RANK expressions indicated that *E. faecalis* bacteria cause damage to periapical bone. This finding is in accordance with that of the study conducted by Wang *et al.* (2015),[8] which posited that *E. faecalis* contributes to bone resorption in apical periodontitis by promoting osteoclastogenesis through an increase in the regulation of osteoclast-specific marker expression, one such marker being NFATc1 and RANK. It can be concluded that the number of NFATc1 and RANK expression cells in periapical bone defects is higher in Wistar rats induced by *E. faealis* than those not induced.

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