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Original Article

Enterococcus faecalis-Induced Nuclear Factor-Kappa Beta and Osteocalcin Expressions in Rat's Periapical Tissue Damage

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Abstract

Background/Aims: Nuclear factor kappa beta (NF-κB) is a member of the transcription factor family, and it plays a key role in coordinating the expression of genes in many chronic inflammatory diseases. The main etiology of endodontic treatment failure is caused by bacteria in root canal, including *Enterococcus faecalis*. The aim of this study to determine NF-κβ and osteocalcin expressions during the periapical tissue damage due to induction of *E. faecalis*. **Materials and Methods:** Fifty-four male rats were randomly divided into two main groups, each of which had three subgroups. In Group C (control), every tooth was induced only by sterile brain–heart infusion broth (BHIb). Group C had three subgroups, namely Group C3 with 3 days inductionprocess, Group C10 with 10 days induction process, and Group C21 with 21 days induction process. In Group T, every tooth was induced by 10 µl of BHI-b *E. faecalis* ATCC212 (10^6 CFU). Similarly, Group T also had three subgroups, namely Group T3 with 3 days induction process, Group T10 with 10 days induction process, and Group T21 with 21 days induction process. The animals were sacrificed based on their group schedule and then prepared for histological and immunohistochemical examinations of periapical tissue. Afterward, the expressions of NF-κβ and osteocalcin were calculated on the light microscope. **Results:** The results revealed that the number of cells expressed of NF-κβ and osteocalcin increased significantly in all sub-groups of Group B induced by *E. faecalis* compared to the control group. **Conclusion:** It can be concluded that the number of cells expressed NF-κβ and osteocalcin increase during the periapical tissue damage induced by *E. faecalis*.

Keywords: *Enterococcus faecalis*, nuclear factor kappa beta, osteocalsin, periapical, tissue damage

Introduction

Root canal treatment failure is mostly caused by the elimination of bacteria and their products improperly. Besides, the root canal treatment failure can also be caused by both external objects, such as filling material and traumatic injury.[1]

E. faecalis are Gram-positive bacteria commonly found in the root canal of chronic apical periodontitis.[2] Lipoteichoic acid (LTA) is the main virulence component of *Enterococcus faecalis* which can induce inflammation and generate immune response by the host.[3]

The bond of Toll Likr receptor 2 and LTA then triggers a signaling for activation of the natural immune response through activation and transcription of nuclear factor-kappa beta (NF-κβ) factor, the main regulator of the inflammatory response.[4] NF-κβ is also considered as a complex protein controlling proinflammatory cytokines. NF-κβ can be activated in inflammatory and neoplastic conditions.[5] The expression of NF-κβ will result in stimulation of pro-inflammatory cytokines. These proinflammatory cytokines then can stimulate bone damage. The main cells that play a role in bone resorption are osteoclasts.[6]

Osteocalcin is a noncollagen protein derived from bone and dentine. Osteocalcin plays a role in calcium ionization and homeostasis. Osteocalcin is a biomarker of bone resorption

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that can be used as a parameter for diagnosing bone loss in periodontal tissues.[7]

Materials and Methods

This is the experimental laboratory study using Wistar rats model and has been received an ethical approval from Universitas Airlangga No. 31/KKEPK. FKG/III/2015. The male rats aged 8–12 weeks and weighed between 120 and 150 g provide by from the Faculty of Veterinary Medicine, Universitas Airlangga. Those criteria were applied since during the study changes in the body weight of those rats were expected to be relatively small since their molar teeth had already grown perfectly and their physical condition was healthy. The number of the rats used as the samples were 54 rats, divided into six subgroups each of which consisted of 9 Wistar rats.

There were two main groups, namely Group C and Group T. In Group C as a control group, tooth preparation was carried out until pulp roof perforation occurred. In this group, sterile brain–heart infusion broth (BHIb) injection was given. On the other hand, in Group B, tooth preparation was also conducted until pulp roof perforation occurred. However, in this group, 10 µl of BHI-b injection containing *E. faecalis* ATCC212 as much as 10^6 CFU was given with micropipette. Each of both groups, Group A and Group B had three sub-groups, namely Group A with 3 days induction process, Group A with 10 days induction process, and Group A with 21 days induction process, as well as Group B with 3 days induction process, Group B with 10 days induction process, and Group B with 21 days induction process. Then, the experimental animals were sacrificed according to the time length of each group determined, i.e., days 3, 10, and 21.

Subsequently, the rats were smeared on the jaw retraction board, and then, the pulp roof of their upper molar was perforated using a bur no.¼. Afterward, those who had met the requirements were intra-peritoneal anesthetized with an injection containing ketamine with a dose of 80 mg/kg and xylazine with a dose of 10 mg/kg.[8] After that their cavity was closed using the Glass Ionomer Cement resin after *E. faecalis* ATCC212 penetration as much as 10^6 CFU.

The next step was cutting the tissues with the following steps. First, euthanasia in those rats was performed with cervical dislocation. Second, those rats were fixated on the workbench, then decapitated, and separated cranium from the mandible. Each piece of jaws was fixated in 10% neutral formalin buffer for 24 h and then decalcified on 4% ethylenedinitrilo tetraacetic acid (Sigma Aldrich Singapore) for 30 days. Fourth, paraffin block preparations were made. The histopathological preparations were made to evaluate periapical bone damage.

After that damage was confirmed, immunohistochemical staining was carried out using anti-NF-κβ and osteocalcin antibodies. The number of cells expressed NF-κβ and osteocalcin were observed with a magnification of ×1000 in 20 fields of view using a light microscope. The number of cells

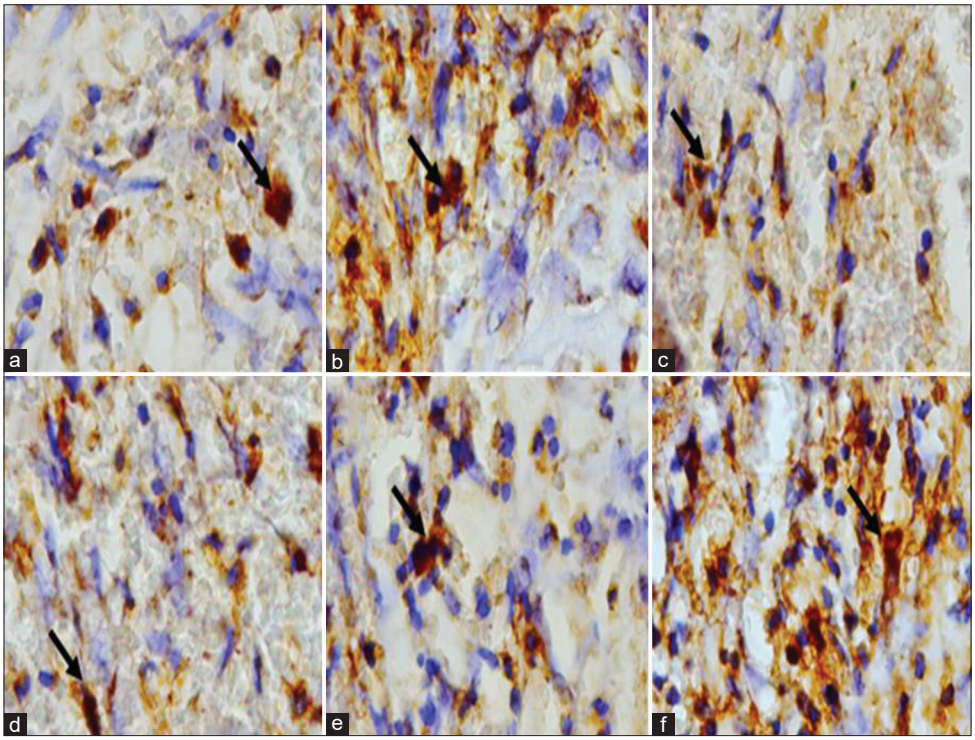

Figure 1: The nuclear factor kappa beta expressions (brown color) on immunohistochemical preparations of the Wistar rats' RA molar. (a) In the control group on days 3, (b) in the control group on days 10, (c) in the control group on days 21, (d) in the treatment group on days 3, (e) in the treatment group on days 10, (f) in the treatment group on days 21 (with × 20/1000 fields of view). The nuclear factor kappa beta expressions increased from days 3 to days 21 in the control and treatment groups

Table 1: The mean number of cells expressed nuclear factor kappa B in the control (C) and treatment groups (T)

Group	Number of cells expressed NF-κβ		
	n	Mean	SD
C 3	9	3.22	0.667
C 10	9	8.77	0.667
C 21	9	4.11	1.054
T 3	9	7.00	0.707
T 10	9	17.77	1.715
T 21	9	20.56	1.424

NF-κβ: Nuclear factor kappa B, SD: Standard deviation

Table 2: The mean number of cells expressed osteocalcin in the control (C) and treatment groups (T)

Group	Number of cells expressed osteocalcin		
	n	Mean	SD
C 3	9	2.33	0.707
C 10	9	7.77	1.201
C 21	9	4.22	0.971
T 3	9	5.56	0.726
T 10	9	15.22	1.201
T 21	9	22.33	1.581

SD: Standard deviation

Table 3: Results of the Kolmogorov-Smirnoff test of the number cells expressed Nuclear factor kappa B

Da group	NF-κβ	Significance of normality
C 3	0.404	0.736
C 10	0.404	0.043
C 21	0.828	0.073
T 3	0.491	0.736
T 10	0.726	0.043
T 21	0.439	0.073

NF-κβ: Nuclear factor kappa B

Table 4: Results of the Kolmogorof-Smirnoff test of the number cells expressed osteocalcin

Gr group	Osteokalsin	Significance of normality
C 3	0.500	0.000
C 10	0.591	0.446
C 21	0.591	0.020
T 3	0.318	0.000
T 10	0.678	0.446
T 21	0.653	0.020

expressed NF-κβ and osteocalcin in the periapical tissues then was manually calculated.

Statistic analysis

The normality test was done to evaluate the distribution of normality by using Kolmogorov–Smirnov test, then homogeneity test to see homogeneity of the data based on population, age, as well as weight by using Levene test, and one-Way *t*-test to see the effect of *E. faecalis* induction on differences in the number of cells expressing NF-κβ and osteocalcin in each control groups (C) and treatment groups (T). The data would be considered to be no different if *P* value was more than 0.05, or to be different if $P < 0.05$.

Results

The histopathological results of the research groups with *E. faecalis* induction showed lymphocyte and osteoclast cells in the periapical tissue areas [Figure 1]. The immunohistochemical results in the control groups and treatment groups showed of the number cells expressed NF-κβ and osteocalcin in the periapical tissue areas [Figure 2]. The increase in the number of cells expressed of NF-κβ and osteocalcin due to *E. faecalis* bacterial induction on periapical tissue damage in the control groups and the treatment groups can be seen in Tables 1 and 2.

Before the difference of the increasing of the number of osteoclasts between treatment groups was evaluated, the data distribution in each group was evaluated for its normality by using the Kolmogorov–Smirnov test and for its homogeneity by using the Levene test. The results of the Komlogorov–Smirnov test and Levene test can be seen in Tables 3 and 4. The Kolmogorov–Smirnov normality test shows that the data obtained in the entire research groups for osteocalcin expressions were normally distributed. Moreover, the results of the homogeneity test then demonstrate a significance level of 0.05. This means that all data were homogeneous.

There was an increase in the number of cells expressed of both NF-κβ and osteocalcin when compared between the control groups (C) and the treatment groups (T). To determine the differences in the number of cells expressed NF-κβ and osteocalcin, an independent *t*-test was carried out between each control group with treatment. The results of the independent *t*-test can be seen in Tables 5 and 6. Analysis from Tables 5 and 6 showed that there was an increase in the number of cells expressed of both NF-κβ and osteocalcin when compared between the control groups (C) and the treatment groups (T). It can be seen that there was an increase in the number of cells expressed of both NF-κβ and osteocalcin when compared between the control groups (C) and the treatment groups (T). Statistical analysis indicated the $P < 0.05$. It means that there was a significant difference in the number of cells expressing NF-κβ and osteocalcin between the control group and the treatment group according to the duration how long periapical bone damage occurred due to *E. faecalis* induction.

Discussion

E. faecalis is a bacterium that often causes endodontic treatment failure and reinfection.[9] The connection between the root canal and apical tissue causes inflammation in periapical and high virulence of *E. faecalis* with the presence of LTA, resulting

in bone damage Bacteria still left behind during root canal treatment are the main etiology for the failure of endodontic treatment. The remaining bacteria will cause inflammation of the periapical tissue. Bone damage in periapical teeth is the results of osteoclast formation (osteoclastogenesis) in the bone. Hence, we analyze the expressions of NF-κβ and osteocalcin

Table 5: Results of the independent *t*-test of number cells expressed nuclear factor kappa B

	Groups	*P*
NF-κβ	C 3	0.000
	T 3	
	C 10	0.000
	T 10	
	C 21	0.000
	T 21	

NF-κβ: Nuclear factor kappa B

Table 6: Results of the independent *t*-test of cells expressed osteocalcin

	Groups	*P*
Osteocalcin	C 3	0.000
	T 3	
	C 10	0.000
	T 10	
	C 21	0.000
	T 21	

which were play an important role in osteoclastogenesis in the periapical teeth lesion induced by *E. faecalis* infection. The duration of *E. faecalis* induction chosen for this research were 3, 10, and 21 days based on inflammation theory. On days 0–7, there is the recognition phase and activation phase. Next, on days 7–14, there will be an activation phase and effector phase. And then, on the days 14–30 homeostasis phase, a phase of improvement, occurs.[8]

Furthermore, the number of cells expressed NF-κβ in the control groups increased from days 3 to days 10, and then decreased on days 21. This corresponds to the pattern of normal body inflammation, in which on the 21st days the homeostasis phase occurs, i.e., the body can balance damage that occurs so that NF-κβ expression is the main key to decrease bone damage. On the other hand, in the treatment groups from days 3 to days 21, NF-κβ expressions significantly increased. This indicates that *E. faecalis* has a strong virulence factor so that the body is unable to do homeostasis. As a result, NF-κβ expression as an important factor of inflammation continues to increase. There were significant differences in the number of NF-κβ expressions between the control and treatment groups. The number of NF-κβ expressions in the treatment groups was higher than in the control groups.

Subsequently, NF-κβ expressed will produce proinflammatory cytokines which will cause periapical tissue damage, resulting in bone damage. In bone damage, osteoclasts are considered as cells that play an important role. The more active osteoclast

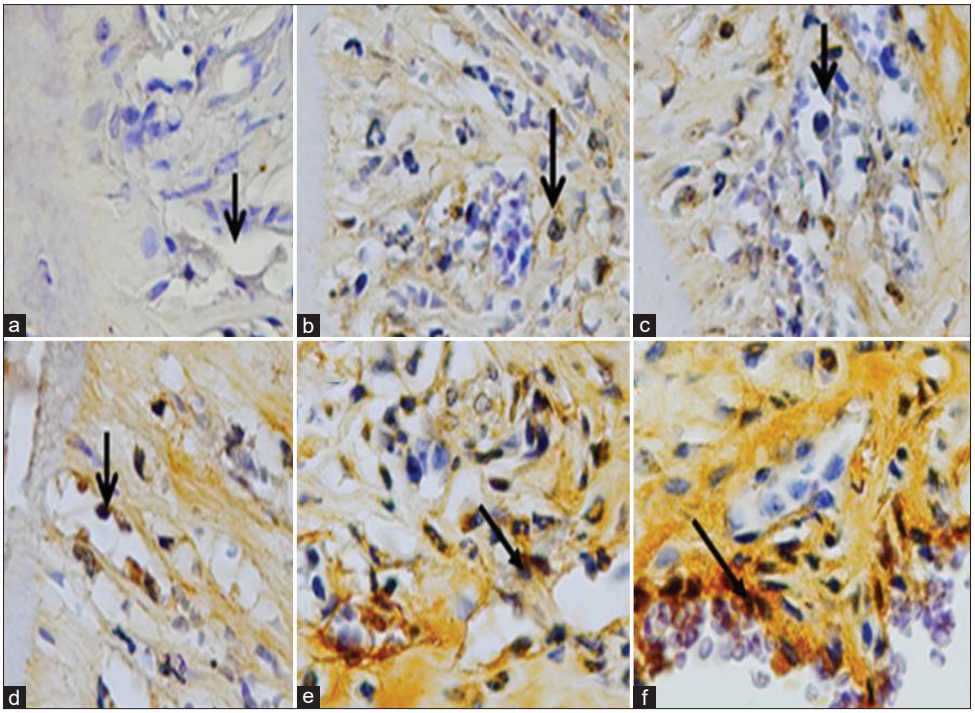

Figure 2: The osteocalcin expressions (brown color) on the immunohistochemical preparations of the Wistar rats' RA molars. (a) In the control group on days 3, (b) in the control group on days 10, (c) in the control group on days 21, (d) in the treatment group on days 3, (e) in the treatment group on days 10, (f) in the treatment group on days 21 (with × 20/1000 fields of view). The appearance of osteocalcin expressions increased from days 3 to days 21 in the control and treatment groups

production is, the more bone absorption will occur.[8] After osteoclasts are active and mature, osteocalcin will be produced. Osteocalcin is calcium which is related to protein in bone. Besides, osteocalcin is considered as a large and most important noncollagen protein in tissue mineralization. Osteocalcin is also known to have chemoactive activities of osteoclast and monocyte progenitor cells. *In vitro*, this synthesis is stimulated by 1,25-dihydroxyvitamin D3. This is also indicated by increasing bone resorption and stimulating differentiation of osteoclast progenitor cells. Increased osteocalcin in serum shows a period of rapid bone loss.[9] Osteocalcin in serum also shows a valid marker for bone loss when resorption and bone formation occur in the same time.[10]

The results show that in the control groups osteocalcin expressions increased from days 3 to days 10, and then decreased on days 21. This indicates a process of body homeostasis. Meanwhile, in the treatment groups, osteocalcin expressions continuously increased from days 3 to days 21. This indicates the occurrence of periapical bone damage in the teeth due to *E. faecalis* infection. There was a statistically significant difference in the expressions of osteocalcin between the control groups and the treatment groups ($P < 0.001$). The number of osteocalcin expressions in the treatment groups was higher than in the control groups.

In general, the expressions of NF-κβ and osteocalcin in the control groups have the same pattern, in which they increased from days 3 to 10 and then decreased on days 21, according to the concept of the pattern of body inflammation that reaches the homeostasis phase. The homeostasis phase is the body's effort to balance the damage that occurs with repairs. A damage to the periapical bone will be responded to by the body by removing osteoblasts playing an important role in bone remodeling.[11] Osteoblasts will produce osteoprotegerin (OPG) which is an anti osteoclastogenic because OPG is a bait receptor capable of binding to nuclear factor-κB ligand (RANKL) so that it can block the interaction between RANK and RANKL and prevent osteoclastogenesis.[12] It is actually possible that on days 21, the body produces osteoblasts producing OPG so that osteoclastogenesis is inhibited and the expression of NF-κβ and osteocalcin indicating osteoclastogenesis decreases.

Thus, NF-κβ and osteocalcin expressions in the control groups were significantly different from those in the treatment groups. In the treatment groups, the number of NF-κβ and osteocalcin expressions as bone damage parameters was higher than in the control groups due to *E. faecalis* induction. *E. faecalis* infection resulted the damage to the periapical tissues of the teeth as indicated by NF-κβ and osteocalcin expressions. Similar to the results of this study, a research conducted by Park *et al*. also reveals that *E. faecalis* can inhibit osteoblast formation, resulting in bone damage.[11] Similarly, a research conducted by Tjan also proves that LTA from *E. faecalis* can inhibit the proliferation of osteoblasts and induce apoptosis of human-osteoblast-like cells, so osteoblasts do not form, while osteoclasts are produced highly, resulting in bone damage.[12]

Conclusion

In conclusion, the number of cells expressing NF-κβ and osteocalcin in periapical bone damage of Wistar rats' teeth induced by *E. faecalis* bacteria is higher than the control group.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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