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Research Report

The role of inducible Nitric Oxide Synthase in teeth periapical lesions immunopathogenesis caused by *Enterococcus faecalis*

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ABSTRACT

Background: *Periapical lesions, are characterized by an immune response to the invading bacteria consequences periapical bone destruction. In root canal treatment failure was found Enterococcus faecalis (E. faecalis) as most species. iNOS found an important role in protection against infection, plays vital roles in fighting pathogens and contributing to disease pathology.* ***Purpose:*** *This study was to observed the role of iNOS in teeth periapical lessions immunopathogenesis caused by E. faecalis.* ***Methods:*** *The randomized post-only control group design used in this study, This study used 24 Wistar rats, were divided into three groups (each group consisted of 8 rats), as negative controls group is a normal teeth, in the positive controls group was made by drilling the upper right first molar to penetrate the dental pulp and was induced with 10µl BHI-b then filled with Glass Ionomer Cement (GIC) and the treatment group, after drilling the teeth, then inoculated with E. faecalis ATCC 29212 10^6 CFU into 10µl BHI-b then filled with GIC to prevent contamination. It takes 21 days to get periapical lesions and rat were sacrificed, and then the expression of iNOS was measured.* ***Results:*** *Statistical analysis using ANOVA found a significant differenced between control and treatment groups ($p<0.05$).* ***Conclusion:*** *This study concluded that iNOS role in teeth periapical lesions immunopathogenesis caused by E. faecalis.*

Key words: *Enterococcus faecalis, inducible Nitric Oxide Synthase, periapical lesions*

ABSTRAK

Latar belakang: *Lesi periapikal merupakan hasil suatu respon imun untuk melawan invasi bakteri yang mengakibatkan destruksi tulang periapikal. Pada perawatan saluran akar yang mengalami kegagalan ditemukan Enterococcus faecalis sebagai spesies terbanyak. iNOS berperan penting untuk proteksi terhadap bakteri, mempunyai peran yang vital untuk melawan patogen dan berkonstribusi secara patologik untuk menyebabkan suatu penyakit.* ***Tujuan:*** *Penelitian ini bertujuan untuk mengobservasi peran iNOS secara imunohistokimia pada lesi periapikal tikus Wistar.* ***Metode:*** *Penelitian ini menggunakan disain randomized post-only control group, digunakan 24 ekor tikus Wistar yang dibagi menjadi tiga kelompok yang masing-masing terdiri dari 8 ekor tikus, sebagai kelompok kontrol negatif adalah gigi normal, pada kelompok kontrol positif dilakukan pengeboran pada gigi molar pertama rahang atas sampai menembus pulpa kemudian diinduksi 10µl BHI-b kemudian ditumpat (Glass Ionomer Cement) GIC dan pada kelompok perlakuan, setelah dilakukan pengeboran dilakukan induksi E. faecalis ATCC 29212 sebanyak 10^6 CFU ke dalam 10 µl BHI-b kemudian ditumpat GIC untuk mencegah kontaminasi. Diperlukan waktu 21 hari untuk mendapatkan lesi periapikal pasca perlakuan kemudian tikus dikorbankan lalu dihitung sel-sel yang mengekspresikan iNOS.* ***Hasil:*** *Analisis menggunakan ANAVA membuktikan bahwa ada perbedaan yang bermakna antara kelompok kontrol dan kelompok perlakuan ($p<0,05$).* ***Kesimpulan:*** *iNOS berperan pada imunopatogenesis lesi periapikal gigi akibat E. faecalis.*

Kata kunci: *Enterococcus faecalis, inducible Nitric Oxide Synthase, lesi periapikal*

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INTRODUCTION

Periapical lesions are formed due to an infection in the root canal by the bacteria that live and breed in the apical part of root canal. A persistent infection after the preparation of root canal is a principal etiology in the failure of root canal treatment. *Enterococci* exist in the gastrointestinal tract and oral cavity in humans as normal comensals. They can cause a wide variety of diseases in humans. *Enterococci* can withstand hars enviromental conditions, they grow at 10° C to 45° C at pH 9,6; in 6,5% NaOCl and survive at 60° C for 30 minutes. (*E. faecalis)* can adapt to adverse conditions. Periapical lesions as the indicator of canal root treatment failure sometimes without clinical symptoms so the radiographic is the only way to check the presence of periapical lesions.[1] Periapical lesions in chronic state is usually without symptoms which is classified as Apical Periodontitis (AP) may also be the result of a secondary infection in the root canal treatment procedure. Infection in the periapical region is caused by the lack of control at the time of intracanal endodontic treatment resulting in re-infection of root canal system caused by non hermetic obturation or inadequate manufacturing of dental restorations resulting in a leakage that can be entered by bacteria.[2]

E. faecalis bacteria is found in the root canal that has been obturated and has undergone a 77.2% periapical disorders.[3] *E. faecalis* bacteria can survive in post endodontic treatment condition because of its ability of forming biofilm to defend themselves in harsh conditions, able to increase calcium ion (Ca^{2+}) in biofilm structure in anaerobic environments. The ability of this bacteria to enter and survive in the root canal system in a long term remains unclear, its ability to survive in stressful conditions including intracellular survival in macrophages and can survive for 4 months in urban water (tap water). *E. faecalis* is identified as a species which is able to survive during the root canal treatment process and persistent as pathogens on dentin tubuli because it has a broad spectrum of genetic polymorphisms and can hold a bond to dentin because it has serine protease, gelatinase, and gelatin binding protein.[4]

Nitric Oxide (NO) is a short lived free radicals known to cause several different cellular processes. NO is an important messenger molecule involved in many physiological and pathological processes in mammal's body that can be beneficial or harmful.[4] NO is gaseous molecule that plays in nervous, cardiovascular and immune systems. NO acts as a regulators that also serves as an effector when there is inflammation and infection. One of the functions of this effector is to yield toxicity effect on bacteria found in the inflamed tissue. The appropriate level of NO production is protective but excessive NO will induce NF-κB which can lead to toxicity in tissues. Chronic expression of NO is associated with various carcinomas and inflammatory conditions.[5]

NO is produced from amino acid L-arginin by the Nitric Oxide Synthase (NOS) enzymatic reaction.[6] Human and rat have three genomes containing three different genes that encode different synthesis NOS, they are neuronal NOS (nNOS or NOS-1), cytokine-inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). NO products from iNOS synthesize are very different from eNOS and nNOS. iNOS enzyme produces very much products and can last a long time which can lead to toxicity. NO was obtained from iNOS plays an important role on host defense.[6] NO in bone loss induced apical periodontitis with deficiency of iNOS produce inflammatory cells and increase osteolytic lesions.[7]

The objective of this study was to observe increase of iNOS expressions in the immunopathogenesis of periapical lesions of Wistar rat by induction of *E. faecalis*.

MATERIALS AND METHODS

Type of the research was laboratory experimental, post only control group design. Twenty four Wistar rats aged 12 weeks was divided by random technique into 3 groups, each groups consisted of 8 rats. Negative control group was the normal teeth, positive control group was the maxillary first molar tooth which was drilled until penetrating the pulp. Ten µl of BHI-b was put in then filled with GIC and the treatment group, after drilling then 10^6 CFU of *E. faecalis* ATCC 29212 was inoculated into the 10 µl BHI-b then filled with GIC to prevent contamination. It takes 21 days to get periapical lesions after pulp infections. Rats were sacrificed then immunohistochemical study was done to measure the iNOS expression by counting the number of cells that gave positive reactions to anti iNOS monoclonal antibody in the specimen under a microscope with 400 times magnification by 10 times the field of view then averaged.[8] Statistical analysis done with ANOVA to discover the differences among each group.

RESULTS

iNOS expression data obtained from observation of the number of cells on the periapical tissue that gave positive reactions to anti-iNOS monoclonal antibody using immunohistochemical method in the negative control group, positive control, and treatment group as shown in Figure 1. Statistical test was done using ANOVA to discover the differences among each group (Table 1). Tukey HSD test was done to discover the differences of iNOS among each research group as shown in Table 2. The result showed that there ware significant differences among the negative control group, positive control group, and treatment group as shown in Figure 2. Data analysis result showed that there were significant differences for iNOS variable among three research groups.

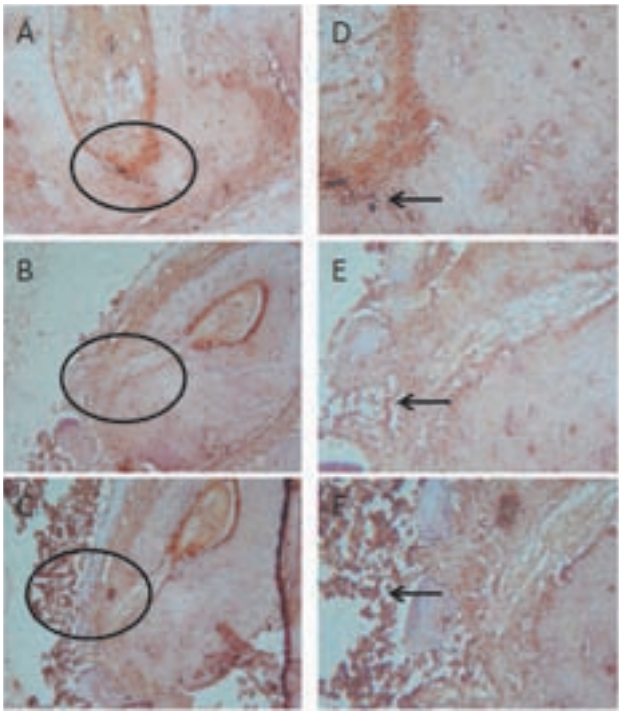

Figure 1. iNOS expression on Wistar rat periapical tissue with 400 times magnification. A: negative control, B: positive control, C: treatment. positive iNOS expressions are marked with arrows (D, E, F).

Table 1. ANOVA test result for iNOS variable

Treatment group	n	Σ INOS positive cells		
		Average	SD	p
Negative control	8	14.5	1.19	0.001*
Positive control	8	18.50	4.37	
Treatment	8	32.62	1.40	

Explanation:

* : Significant ($p < 0.05$); n: Amount of sample; SD: Standard Deviation

Table 2. Tukey HSD test result

	Negative control	Positive control
Negative control	–	p = 0.021*
Positive control	p = 0.021*	–
Treatment	p = 0.001*	p = 0.001*

* = Significant ($p < 0.05$)

DISCUSSION

Periapical lesions is a chronic infectious disease, many kind of bacteria in the oral cavity are involved. Nitric Oxide (NO) is a key molecule for fighting pathogens, is a short lived free radicals produced by inflammatory

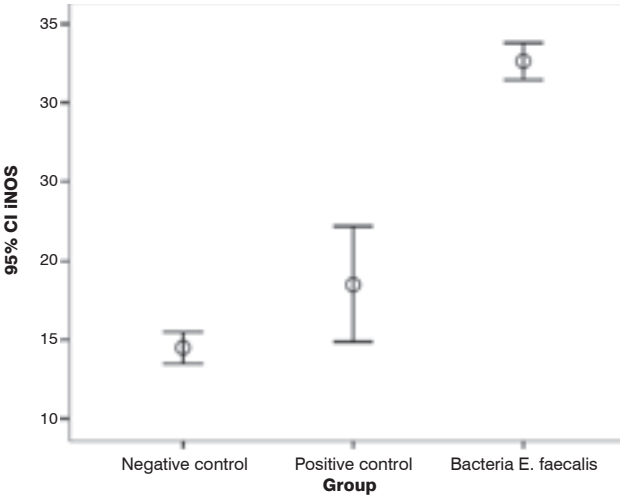

Figure 2. Three groups of iNOS expression on Wistar rat periapical tissue.

macrophages. NO plays an important role on protection against bacteria that causes infection but also cause tissue damage. Production of NO is thought to have an important role in the development of periapical lesions. In periapical lesions, macrophages and polymorphonuclear leukocytes are the major source of NO.[9]

NO is produced by phagocytes (monocyte, macrophage, and neutrophil) which is part of the immunity response. NO is synthesized inside the cell by NOS enzyme. NO product by iNOS synthesis is very different from eNOS and nNOS because iNOS produces so much and can last long which will lead to tissue toxicity and plays an important role in host defense. iNOS is activated by Interferon-gamma (IFN-γ) as a single signal or by Tumor Necrosis factor (TNF) as a secondary signal. NO can activate NF-κB which is an important transcription factor on iNOS gene expression in the reaction against inflammation. iNOS enzyme produces so much and can last for a long time which will lead to toxicity.[10]

The role of NO generated by iNOS is very complex. NO has an anti microbial effect and macro molecule nitrolyzation. In a few seconds NO will be oxidized into nitrite or nitrate produced by anion superoxide ($O2^-$) which can form peroxinitrite ($ONOO^-$) which has cytotoxic effect. Allegedly, an increase of iNOS is due to the increasing of NF-κB transcription factor resulting in an increase of the secreted genes which is iNOS that releases NO which can induce apoptosis in osteoblasts that are likely to increase the development of periapical lesions.[11]

In this study, it is proved that there is an increase of iNOS expression in the treatment group compared to the control groups ($p<0.05$) (Table 1 and 2). Large amount of increase in iNOS will lead to cell toxicity.[13] Increasing in iNOS is a response of IFNγ-activated macrophage as a single signal secreted through Th-1 cytokine.[12,13]

iNOS shows an opposite effect on the physiology of osteoclasts. NO has a different function during the

development and activation of osteoclasts. Expression iNOS for NO generation is stimulated by IFN or lipopolysaccharide iNOS expression and NO releasing cause an increasing of Receptor Activator of NF-kB Ligand (RANKL), and that response depends on the duration and dosage. Needs activation of NF-κB and protein synthesize that are specifically inhibited by osteoprotegerin (OPG) which is an angler receptor. This causes inhibition of NO-induced RANKL to increase the formation of osteoclast, this indicates that normal NO controls the osteoclastogenesis mediated by RANKL. iNOS deficiency accelerates the formation of osteoclast and resorption through in vivo and in vitro. RANKL induced in iNOS derived NO function is a negative signal to limit osteoclastogenesis which were jointly stimulated by RANKL.[14] NO donor will increase the production of OPG and inhibit osteoclastogenesis activities in stromal bone marrow cells on ovariectomy rat.[15] Increasing in bone resorption on rats with iNOS deficiency is correlated to the increasing of RANK expression and OPG reduction, so that can be concluded the NO deficiency causes disproportion of bone resorption modulation factor that stimulates bone loss.[6]

NO and superoxide are likely to react in vivo to produce peroxynitrite which molecularly will increase tissue damage. Production of extracellular superoxide and release of lytic enzymes gelatinase and hyaluronidase and the toxin cytolisin by *E. faecalis* can cause direct damage in dentinal as well as in periapical tissues. The role of NO in bone loss caused by bacteria infection which induces Apical Periodontitis, iNOS produce more inflammatory cells and osteolytic lesions than control rats. Tartrate resistant acid phosphatase positive ($TRAP^+$) osteoclasts significantly have a greater amount, this is correlated to the expression increasing of receptor activator *NF-κB* (RANK) stromal cell and decreasing in osteoprotegerin (OPG) expression. NO deficiency will result in disproportion of bone resorption modulation factor and aggravating bone loss.[7]

NO and superoxide are likely to react in vivo to produce peroxynitrite which molecularly will increase tissue damage. The role of iNOS and NO to control bone resorption progress in experimental rats in apical periodontitis.[10] iNOS deficiency is associated with the inbalance state in cytokine proinflammatory IL-1β and TNF-α, bone resorption modulator (RANK and RANKL) and MP1 chemokin. Interestingly, ROS production showed no involvement in the progress of periapical lesions that may mediate osteoclast differentiation. The conclusion of this study iNOS role in teeth periapical lesions immunopathogenesis caused by *Enterococcus faecalis.*

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