The expressions of NF-κβ and TGFβ-1 on odontoblast-like cells of human dental pulp injected with propolis extracts

by Ira Widjiastuti

Research Report

The expressions of NF-κβ and TGFβ-1 on odontoblast-like cells of human dental pulp injected with propolis extracts

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ABSTRACT

Background: *Propolis is known to have beneficial effects, namely anti- bacterial, anti-viral, anti-inflammatory, antioxidant, and immunomodulatory. Propolis extracts with anti-inflammatory properties are expected to be useful in treating inflamed pulp tissue with a diagnosis of reversible pulpitis. The inflammation of pulp tissue is caused by bacteria, namely Lactobacillus acidophilus. This research used odontoblast like cells derived from pulp tissue of human third molars. Odontoblast like cells exposed to Lactobacillus achidophilus were used as a model of proinflammatory cytokine signaling. This research examined the effects of propolis extracts on odontoblast like cells exposed to Lactobacillus acidophilus.* ***Purpose***: *This research aimed to determine the effectiveness of propolis extracts on the activities of odontoblast-like cells exposed to Lactobacillus acidophillus by measuring the expressions of NF-κβ and TGF- β1.* ***Methods***: *First, pulp odontoblast cultures were derived from human dental pulp tissues of impacted third molars removed by using digestion method. Next, odontoblast-like cells exposed to inactive Lactobacillus acidophilus bacteria were given propolis extract. Finally, the activities of odontoblast-like cells were monitored by measuring the expressions of NF-kβ and TGFβ-1 with immunocytochemistry technique.* ***Results***: *A decline NF-kβ expression and on increase of TGFβ-1 expression on odontoblast like cells exposed to inactive Lactobacillus acidophilus.* ***Conclusion***: *Propolis extracts inhibit the expression of NF-kβ, and increase the expression of TGF-β1 in pulp odontoblast-like cells exposed to inactive Lactobacillus acidophillus.*

Key words: *Propolis extracts, odontoblast-like cells, Lactobacillus acidophillus, NF-kβ, TGFβ-1*

ABSTRAK

Latar belakang: *Propolis dilaporkan mempunyai efek menguntungkan yaitu bersifat anti bakteri, anti virus, anti inflamasi, anti oksidan, dan imunomodulator. Ekstrak propolis dengan sifat anti inflamasi diharapkan bermanfaat untuk mengobati jaringan pulpa yang mengalami inflamasi dengan diagnosis pulpitis reversibel. Inflamasi jaringan pulpa disebabkan oleh bakteri diantaranya adalah Lactobacillus acidophilus. Pada penelitian ini digunakan Odontoblast like cells yang berasal dari jaringan pulpa dari gigi molar ke tiga manusia. Odontoblast like cells dipapar Lactobacillus acidophilus digunakan sebagai model signaling sitokin proinflamasi. Studi ini, meneliti pengaruh pemberian ekstrak propolis pada odontoblast like cells yang dipajan Lactobacillus acidophilus.* ***Tujuan***: *Penelitian untuk mengetahui efektifitas ekstrak propolis terhadap aktifitas odontoblast like cells yang dipajan Lactobacillus acidophillus dengan mengukur ekspresi NF-κβ dan TGF-β1.* ***Metode:*** *pembuatan kultur odontoblas pulpa berasal dari jaringan*

pulpa gigi Molar ke tiga impaksi yang dicabut menggunakan metode digesti. Odontoblast like cells dipajan bakteri Lactobacillus acidophilus inaktif, diberi ekstrak propolis dan aktifitas dari odontoblast like cells diukur melalui ekspresi NF-kβ dan TGFβ-1 secara imunositokimia. ***Hasil****: Terjadi penurunan ekspresi NF-κβ, dan peningkatan ekspresi TGFβ-1 pada kultur odontoblas yang dipapar bakteri Lactobacillus acidophilus inaktif.* ***Simpulan****: Ekstrak propolis menghambat ekspresi NF-kβ, dan meningkatkan ekspresi TGF-β1 pada odontoblast like cells pulpa yang dipajan bakteri Lactobacillus acidophillus inaktif.*

Kata kunci*: Propolis extract, odontoblast like cells, Lactobacillus acidophillus, NF-kβ, TGFβ-1*

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INTRODUCTION

In enamel and dentin caries will likely to stimulate, the innate immune response of dental pulp through the diffusion of bacterial products into the dentinal tubules. This excessive pathogen invasion will cause irreversible inflammation, destruct either immune response or pulp tissue necrosis.[1] Odontoblasts located in peripheral area of the dentine is the first cells received bacterial injury and also can be considered as the first dental defense process.[2] It is because odontoblasts have a role in dental immune response. This is supported by a research explaining that odontoblasts consistently produce both innate immunity components and adaptive immunity components,[3,4] and they can also be induced to express cytokines and chemokin.[3]

In dentin caries, moreover, *Lactobacillus acidophilus* as Gram-positive bacteria with a virulence component, namely lipoteichoic acid (LTA), are commonly found. As a result, odontoblasts with the presence of Gram-positive bacteria will initiate, develop, maintain, and terminate the dental pulp immune response. Detector components of Gram-positive bacteria, including lipoteichoic acid (LTA) and diacylated/triacylated lipopeptides primarily are produced through Toll-like receptor 2 (TLR2). The involvement of odontoblast cells in vitro can make LTA trigger TLR2 to stimulate the regulation and translocation of NF-κB producing proinflammatory of chemokines and cytokines. Thus, this is a potential target to interfere cascade signal ultimately leading to the excessive inflammation of the pulp. Actually, the bacteria can stimulate bioactive molecules, namely transformasi growth factor-beta (TGF-β1) or bone morphogenic protein (BMP) that will induce dentin formation on the surface of the pulp tissue. However, the formation will be disrupted if there is inflammation of the pulp.

Several strategies should be conducted to inhibit both inflammation via TLR2 and pro-inflammatory of intracellular signal transduction and chemokine. Consequently, a biocompatible material that can prevent or treat inflammation of the pulp tissue is needed. Material that has been used is calcium hydroxide, but this material has disadvantages, such as causing both tunnel defects in dentin formation and necrosis of the pulp tissue surface. As an alternative, propolis can be chosen since it has anti-inflammatory, anti-bacterial, anti-viral, anti-oxidant, and immunomodulatory properties.

Propolis is also contain a lot of resin and bioactive ingredients, such as bioflavonoids, artepilin, apigenin, and caffeic acid phenethil esther (CAPE) participating in body's immune response to inflammatory, can be used as antioxidants, antibacteria, and antivirus and can be considered as immunomodulator, as well as can stimulate the healing process of tissue.[5] Thus, propolis extracts are expected to be useful as a pulp capping medicine used as pulp protection. Pulp capping is used to protect the pulp by stimulating the formation of reparative dentin, and to maintain the vitality of the pulp tissue. Therefore, this study was aimed to determine the effectiveness of propolis extracts on the activities of odontoblast-like cells due to *Lactobacillus acidophillus* by measuring the expressions of NF-κβ and TGF-β1.

MATERIALS AND METHODS

All procedures performed in this research have been legalized with ethical clearance issued by Ethics Committee of the Faculty of Dentistry, Universitas Airlangga. The procedures consisted of culturing *Lactobacillus acidophilus*, making pulp cell culture, and conducting immunocytochemistry examination by using monoclonal antibodies to determine the expressions of NF-κβ and TGF-β1.

Pulp cell culture was made from dental pulp tissues of impacted third molars that was taken from patients aged 14-19 years. The teeth were disinfected by using 0.3% chlorhexidine gel put into 30% hydrogen peroxide for 30 to 120 seconds. The pulp was opened by conducting preparation using a sterile fissure bur and pulp cell cultures were made by using digestion method.

Differentiation of pulp fibroblast was conducted by doing supplementation of 10 nM dexamethasone, 50 mg/ml ascorbic acid and 10 mM-glycerophosphate (100-200 ng/ml) on prolifresi medium (DMEM + 10% FBS + penicillin/ streptomycin) to create odontoblast-like cells. During differentiation process, odontoblasts secreted specific matrix, ie dentin matrix protein 1 (DMP-1). The identification of DMP1 was conducted by using immunocytochemistry and anti-DMP1 (SantaCruz) based on Immunostaining assay kit (Biocare) instructions. Then, characterization of odontoblast-like phenotype was conducted.

Before odntoblast-like cells were induced with *Lactobacillus acidophilus, Lactobacillus acidophilus* had been inactivated by heat killed method. In this process, *Lactobacillus acidophilus* was heated at 121° C for 5 minutes. The determination of an effective dose of bacterial exposure based a certain ratio of cells and bacteria, namely 1: 25, incubated for 24 hours (an incubator at 37° C).[6] Finally, after *Lactobacillus acidophilus* was inactvated, propolis extracts derived from raw propolis produced by *Apis meliferra* bees of Lawang, East Java, Indonesia was taken. Propolis extracts were made by using maceration method using 70% ethanol.

RESULTS

The expression of NF-κβ was determined by using immunocytochemical examination. The result can be seen in Table 1.

Tabel 1. The mean and standard deviation of NF-κβ expressions in odontoblast culture by using ANOVA test

Group	$\bar{X}$ (%)	SD	ANOVA
Control (-)	4.2[a]	2.39	F=31.751
Control (+)	23.60[b]	3.84	p= 000
1,5µg/ml of Propolis	25.20[c]	4.14	
3µg/ml of Propolis	19[e]	3.16	
6 µg/ml of Propolis	12.40[d]	3.36	

Note: The different *Superscripts* indicate that there was significant difference among the groups (p<0.005)

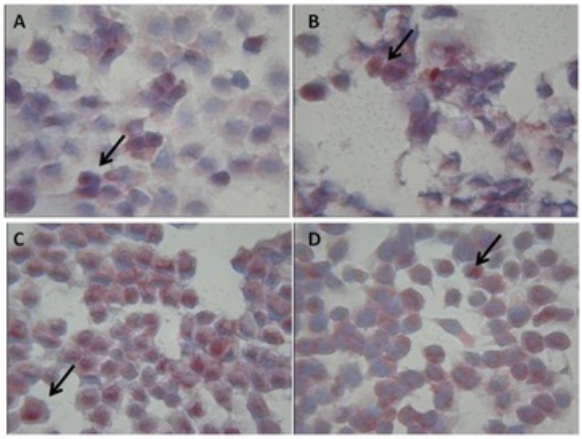

Figure 1. Odontoblast culture (AEC staining with magnification 400x). Cells expressing NFκβ (arrow) distributed in cell nucleus (red color). A) odontoblasts induced with inactive *Lactobacillus acidopilus*; B) odontoblasts induced with inactive *Lactobacillus acidopilus* and propolis extracts 1,5µg/ml; C) odontoblasts induced with inactive *Lactobacillus acidopilus* and propolis extracts 3µg/ml; D) odontoblasts induced with inactive *Lactobacillus acidopilus* and propolis extracts 6 µg/ml.

Immunocytochemistry examination was conducted to determine the number of NF-κβ expressions by immunostaining method using antibody, i.e. anti-NF-κβ. The results showed that

NF-κβ expressions (red) were distributed in the cell nucleus as shown in Figure 1. It is also known that the distribution of cells expressing NF-κβ was decreased. Next, TGF-β1 expressions in odontoblast cultured induced with inactive *Lactobacillus acidophilus* were identified by using immunocytochemistry examination as seen in Table 2.

Table 2. The mean and standard deviation of TGF-β1 expressions in odontoblast culture by using ANOVA test

Group	(%)	SD	ANOVA
Control (-)	21.4[a]	5.86	F= 10.731
Control (+)	12.20[b]	3.27	p=0.001
1,5µg/ml of Propolis	10.20[b,c]	2.38	
3µg/ml of Propolis	15.40[a,b,c]	3.64	
6 µg/ml of Propolis	23.4	3.50	

Note: The different *Superscripts* indicate that there was significant difference among the groups (p<0.005)

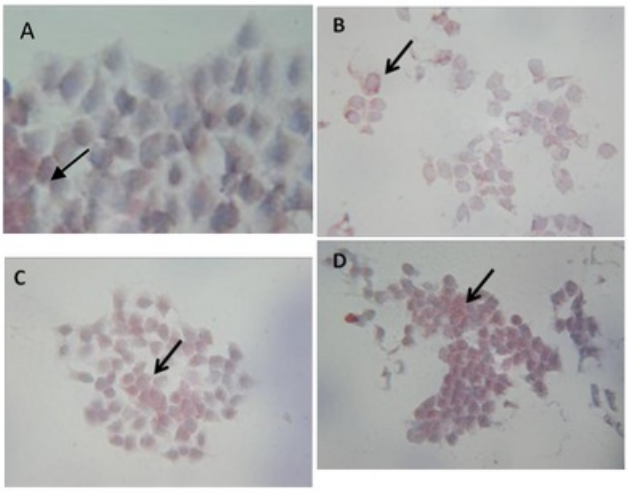

Figure 2. Odontoblast culture (AEC staining with magnification 200x). Cells expressing TGF-β1 (arrow) distributed in the cytoplasm of odontoblasts (red color). A) odontoblasts induced with inactive *Lactobacillus acidopilus*; B) odontoblasts induced with inactive *Lactobacillus acidopilus* and propolis extracts 1,5µg/ml; C) odontoblasts induced with inactive *Lactobacillus acidopilus* and propolis extracts 3µg/ml; D) odontoblasts induced with inactive *Lactobacillus acidopilus* and propolis extracts 6 µg/ml.

DISCUSSION

In this research, odontoblast like cell culture was induced with *Lactobacillus acidophilus* inactive. The induction of inactive *Lactobacillus acidophilus* into the odontoblast cultures has induced TLR2 receptors as trans-membrane receptors, the receptors of gram-positive bacteria binding to LTA, then has passed the transduction signal into cells, and later has induced NF-κβ to become activated and go into the cell nucleus. As a result, the transcription of the target genes

induces into TNF-α and TGF-β1. It means that odontoblasts as the main cells that form the peripheral layer of the pulp tissue in vitro have typical cellular morphology, and can be induced to express cytokines and chemokin.[3] An in vitro research on odontoblasts exposed to LTA binding TLR2 even shows that the increasing of the odontoblasts can activate the transcription factor of NF-κβ, so it will diffuse from the cytoplasm to the nucleus and then secrete proinflamatory cytokines. [10] Thus, all of these conditions then potentially lead to the inflammation of the pulp.

NF-κβ is a transcription factor, which will diffuse into the nucleus, and then activate the transcription of various target genes. The activation of NF-κβ protein caused by bacterial products can secrete a variety of cytokines, including proinflammatory cytokines that cause cell damage,[8,9] leading to the damage of the odontoblast-like cells. Next, phosphorylation of the serine residues usually occurs on the responsive signal (SRR) of classical IκBs by leading to IKKβ of IκB ubiquitination and proteosomal degradation secreted from NF-κβ dimers, which then diffuses into the nucleus, induces gene target transcription,[11,12] and secretes proinflammatory cytokines, such as TNF-α, IL-1, IL-6 and IL-12.[13] Similarly, in this research, NF-κβ expressions were increased after the odontoblast cultures were exposed with inactive *Lactobacillus acidophilus*. It is because NF-κβ p65 as a part of NF-κβ inhibitor with canonical line secreted proinflammatory cytokines and mediated signal transduction, or due to the induction of TNF-α, IL-1, LPS or LTA and the use of a variety of adapters to signals involved IKK activities.

Human dentin contains TGF-β1, which has a dual role in the formation and repairing of dentin-pulp complexes. These cytokines also act as a regulator for the activation of immunocompetent cells, such as lymphocytes, macrophages and granulocytes, to control the initiation and resolution of inflammatory response.[14] Therefore, pulp capping treatment with TGF-β1 can increase and accelerate the synthesis of collagen type 1, as a result, mineralization occurs, then perforation is closed, and reparative dentinogenesis occurs.[15]

Several strategies can be applied to heal pulp inflammation, including blocking or inhibiting the transduction of intracellular signal through TLR2 and pro-inflammatory cytokine/chemokine in odontoblasts. Therefore, with a better understanding of molecular mechanisms in odontoblast-like cells exposed to the bacteria, the effective therapeutic components that modulate pulp cell can also be designed to contribute in healing and repairing processes through the formation of reparative dentin.[10] The formation of reparative dentin is usually started with the binding of progenitor cells into the pulp cells differentiated into odontoblast-like cells and migrated to the injury area.[1] TGF-β1 has a role in regulating cell cycle. TGF-β synthesizes proteins, p15 and p21, that inhibit cyclin-CDK complexes in G1 phase of the cell cycle. TGF-β then regulates the proliferation process, so differentiation process occurs.[30]

The propolis extracts were then chosen as a treatment for inflamed dental pulp tissue since it has anti-inflammatory properties. Propolis extract can also be considered as a complex substance that has an anti-inflammatory factor.[18] Propolis extracts, furthermore, contain various ingredients depending on the type of bees and plants, but the main component contained in the extracts are bioactive, namely phenolics and flavonoids. The other components contained in propolis extract are anti-inflammatory, such as caffeic acid, quercetin, naringenin, and CAPE. [19] The main compositions of propolis extracts are phenolic acids and esters, flavonoids (flavones, flavonones, flavonols, dihydroflavonols, chalcones), terpenes, β steroids, aromatic aldehydes, alcohols, sesquiterpenes, naphthalene, stilbene derivatives of benzopyran, benzophenone, caffeic acid, cinnamic acid derivatives and benzoic acid.[19] For these reasons, in this research propolis extracts were used together with ethanol solvent containing polyphenols, proleinetin, flavonoids, tertepenoid, galangin, quercetin, minecetin, oligotinperginetin, nikobaleen A and B as well as CAPE.

Anti-inflammatory activities of propolis seem to be associated with flavonoid, especially galangin and quercetin. Flavonoids inhibit the activities of cyclooxygenase and lipoxygenase, as well as reduce and release of PGE2 and COX-2 isoform (COX-2) expressions.[22] CAPE can also be considered as an active component in propolis that inhibit the production of cytokines and chemokines, the proliferation of T cells and the production of lymphokine resulting in the decreasing of inflammatory process. Its mechanism is through signaling pathways of NF-κβ.[21] It means CAPE is a potent inhibitor of NF-κβ.[23]

Ansorge *et al*.[24] studied the effects and functions of some of the components of propolis that can activate the immunity in human blood cells, synthesize DNA and cytokine production in vitro by detecting the production of IL-1ß and IL-12 by macrophages, as well as produce IL-2, IL-4 , IL-10 and TGF-β growth factor. Propolis contains flavonoids and caffeic acid that have anti-inflammatory properties by inhibiting lipoksiginase line by arakidonik acid. It is also known that propolis affects immune system by stimulating both the activities of phagocytosis and cellular immunity and the formation of collagen, which will affect dentin bridge formation. Propolis also contains compounds arginine, vitamin C, provitaminA, B complex, trace minerals and bioflavonoids as well as antibacterial properties, which can accelerate healing process.[25,26]

Propolis extracts can suppress the expressions of proinflammatory cytokines better than quercetin, hesperidin, and CAPE since the propolis extracts have a synergistic effect in inhibiting proinflammatory cytokines.[24] Like the previous result, the results of this research also showed that the expressions of NF-κβ in the odontoblast cultures induced with inactive *Lactobacillus acidophilus* and exposed to the propolis extracts were decreased, meanwhile the expressions of TGF-β1 were increased. It means that the change of propolis extracts also altered the expressions of

NF-κβ and TGF-β1. In other words, the induction of the propolis extracts inhibits the activations of NF-κβ and TNF-α and also induce the secretion of TGF-β1.[6]

Like previous researches, this research also shows that propolis extracts could inhibit NF-κβp65 expressions on odontoblast culture induced with inactive *Lactobacillus acidophilus*. This is supported by the results of several previous researches that the active ingredient, namely CAPE, contained in propolis can significantly inhibit the constitutive expressions of COX-2. In other words, CAPE is a potent and specific inhibitor that inhibits the activation of NF-κβ. Histopathological examination conducted in this research also showed that CAPE significantly suppressed inflammation. Caffeic acid phenethil esther specifically blocked the activation of NF-κβ caused by various inflammatory agents, including TNF-α and H_2O_2, inflammatory cytokines (IL-1, TNF-α), bacterial products, and oxidative stress.

Caffeic acid phenethil esther, furthermore, does not only inhibit transcription factors, but also reduces the production of IL-8 and chemotactic monosit proteins.[9] Thus, propolis containing CAPE can inhibit phosphorylation of IκBα and activation of NF-κβ, but not through phosphorylation of mitogen-activated protein kinase (MAPK) in human monocyte-derived dendritic cells (MoDCs).[20] The results of this research were also supported by the results of a research conducted by Aviello *et al.*[27] showing that caffeic acid, quercetin, hesperidin and flavonoids contained in propolis can inhibit DNA synthesis and inflammatory cytokine production depended on the concentration of propolis. But, the production of TGF-β, a mediator of immunosuppression, was increased. These findings indicate that certain components contained in the propolis extracts could give direct effects on immune cell function settings, and could also be used as alternative natural ingredients that have anti-inflammatory effects. Similarly, a research on the biological activity of propolis shows that CAPE and artepillin C can be isolated from propolis, which can potentially be used as medicine.

In this research, it is known that the higher the dosage of propolis extracts, the more NF-κβ expressions will be inhibited and the more TGF-β expression of will be increased. This indicates that the propolis extracts can stimulate odontoblasts in the dental pulp to secrete TGF-β1, which can stimulate proliferation and differentiation. Propolis has an ability to stimulate TGF-β1 considered an important factor in the differentiation of odontoblasts in human dental pulp.[25] Based on the results of the research, it can be concluded that the propolis extracts not only can inhibit NF-kβ expressions, but can also increase the expression of TGF-β1 in odontoblast culture induced with inactive *Lactobacillus acidophillus*.

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