East Java Propolis Inhibits cytokine Pro-inflammatory in *Odontoblast like cells* Human Pulp

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ABSTRACT

Inflammation in pulp tissue is caused by caries bacteria. Most bacteria found are *Lactobacillus acidophilus* . Propolis is a sticky resin material that is derived from the bees and the surrounding plants, which are reported to have several biological effects including anti-bacterial and anti-inflammatory. This study will prove the effectiveness of propolis against proinflammatory cytokines on *odontoblast like cells* in human dental pulp. **T**his study was performed on cultured *odontoblast like cells* in pulp. Cell culture was derived from the pulp tissue of human M3 teeth that had been extracted. Odontoblast pulp culture was divided in 3 groups, group 1 was cultured cells with propolis; group 2, cultured cells induced with *Lactobacillus acidophilus* ; group 3, cultured cells induced with inactive *Lactobacillus acidophilus* and exposed to 3μg/ml propolis. A measurement of TNF-α danTGF-β1 expression was done using the immunocytochemical technique to determine the effectiveness of propolis extracts from East Java Indonesia against proinfalmmatory cytokines. Data were analyzed using *Anova* test (p= 0,05) . Propolis extract can inhibit the expression of TNFα and increase the expression of TGF-β1on *odontoblast like cell* in human dental pulp. **A**nti-inflammatory effects of east Java propolis extract are associated with cytokine modulation.

Keywords : propolis extract, TNF-α, TGF-β1, *odontoblast like cells*

INTRODUCTION

Caries prevalence in Indonesia is 90,05% and most have already reached dentine or even caused pulp perforation. On dentine caries, the most found bacteria is *Lactobacillus acidophilus*.[1] *Lactobacillus acidophilus* is a gram positive bacteria and has lipoteichoic acid (LTA) on the bacteria cell membrane. LTA causes inflammation in pulp tissue. Caries which reached the dentine will firstly be received by odontoblast. LTA is a primary component of gram positive bacteria cell membrane with cytoplasm membranr, consisting *peptidogoglycan* layer with 80 nanometer thickness. *Lactobacillus acidophilus* LTA stimulates odontoblastbto express TLR2 and induct chemokine secretiom (CCL2 dan CXCL2) **.**[2] Caries on dentine which will be firstly hit is odontoblast and will respond to dentine caries bacteria through TLR2 and TLR4 receptors. LTA inducts TNF-α through

TLR2. On dentine caries odontoblast is the first cell to receive the signal transduction of TGF-β1 released by dentine and inhibits TLR2, TLR4, and cytokine IL-8 also TNF-α.[3]

On dentine teeth caries with deep cavity leaves a thin layer of dentine or even pulp roof perforation so treatment is needed to maintain teeth vitality so that it can function in stomatognati function. During the clinical peocedure the teeth pulp will sometimes undergo inflammation, like in the caries removal process. In this situation, pulp undergoes a process called reparaytive dentinogenesis, where some cells form a new matrix deposit in parts undergoing lesion.[4] Adult teeth pulp respond to signal and contains precursor cell and form *odontoblast like cell.* [5]. *Direct pulp capping* treatment fix the pulp tissue so that the tissue could remain vital, healthy and be able to function in stomatognati system.

Pulpcapping's character is infection control, handling, micro leakage prevention, and to start hard tissue's forming. [6] During reparative process in the pulp, the damaged prime odontoblast is replaced with the new one, that is *odontoblast like cell.* This process is known to follow some consecutive step, namely: proliferation, migration, and differentiation progenitor cell or the main cell. [7] The new formed cells are pulp cells and mesenchymal cells. Various materials were used in *pulpcapping* procedure. Calcium Hydroxide is a material that have been used extensively and regularly for *pulpcapping* in dentistry. As it's known in dentistry, this material has potential role in order to push the hard tissue's fixation, this material has been used for the pulp that exposed to injury and it's expected to form a new dentin above the pulp. Calcium Hydroxide has antimicrobials character due to its high pH (12,5), so that it broke the membrane cells and protein structures. Calcium Hydroxide effectiveness depends on its dissociation and the release of hydroxyl ion (OH), that diffuses into other tissues and causes necrotic layer's forming. [8] Dentine reparative that formed by Calcium Hydroxide is porous so that the dentine forming is not perfect, therefore it's necessary to find alternative materials from nature that biocompatible and not toxic, one of the alternatives is propolis.

Propolis has been recognized as useful material for human health, which is has antimicrobial and anti-inflammation character. Honey bees collected resins from tree bark's cracks and leaf buds. [9] Generally, Propolis consist of 50% resin and vegetables balsam, 30% waxes, 10% essential and aromatic oils, 5% pollens, 5% other materials, including organic wastes depend on the location and collection time. [8] Propolis constituen is varies because of climate, season, and capping materials those are important factors to make the best treatment.

The research about nature substance propolis has been widely conducted, especially as *pulp capping,* but the mechanism of molecular stimulation of propolis extract towards dentin reparative forming process still unclear so that it is still needed to do a research about propolis extract stimulation towards dentin reparative forming process. In this research, problem solving about the

impact of propolis extract towards odontoblast development (on the bigger case is teeth caries) was done by laboratory testing from a model that had been chosen that based on introductory studies, consist of exploration and synthesis that is related to separated empirical facts that already exist. *odontoblast like cells* culture *in vitro* model selection was based on simplicity of the model, cells homogeneity, and the abundance of tissue's source for doing prime culture. In this research, as the parameter will be held by measuring TNF-α and TGF-β1excretion..

MATERIALS & METHODS

Every procedure that was done in this research is ethical worthy, this research was proposed to Ethics Committee of The Faculty of Dentistry, University of Airlangga beforehand. The research procedures are consist of *Lac*to*bacillus acidophilus* bacteria breeding, the making of pulp cell culture, and immunocytochemistry examination by using monoclonal antibody for TNF-α and TGF-β1.

Preparation of *odontoblast like cells* culture

Cell culture was isolated from mandibular third molar teeth's pulp tissues that is impacted and had been extracted from 14-19 years old patients. Tooth surface was cleaned with chlorhexidine 0.3% gel, rubbed with 70% (v/v) alcohol or be carefully soaked in hydrogen peroxide 30% for 30 till 120 seconds. Pulp was opened by doing preparation using sterilized fissure drill at occlusal area and bifurcated so that the pulp space was opened. And then, the pulp tissue was isolated and cultivated by digestion methods. [10]

Odontoblast like cells is pulp fibroblast that had been differentiate. The pulp fibroblast was differentiated to be *Odontoblast like cells* by doing supplementation with 10 nM dexamethasone, 50 µg/ml *ascorbic-acid* and 10 mM glycerophosphate or by adding BMP-2 (100-200 ng/ml) to proliferation medium (DMEM + 10% FBS + penicillin/streptomycin). After that, then it proceed with *odontoblast-like phenotype* characterization. Dentine's matrix formation at the process of differentiation to be odontoblast would secreting specific matrix, including dentine matrix protein 1 (DMP-1). DMP-1 identification was carried by immunocytochemistry technique, using anti-DMP1 (Santacruz), with procedure as it was said in Immunostaining Kit guidelines of assay (Biocare).

Lactobacillus acidophilus inactive exposure to odontoblast culture

Before *Lactobacillus acidophilus* bacteria was exposure to odontoblast culture, it was conducted the making of *Lactobacillus acidophilus* inactive by heating the bacteria. *Lactobacillus acidophilus* bacteria heating was done by heating the bacteria at temperatures 121^{o}C for 5 minutes. [11] effective dose of bacteria exposure determination was carried by comparing cells : bacteria 1 : 25, by incubating them for 24 hours in incubator with 5% CO_2 at temperatures 37^{o}C. The effective dose was used for inducting *odontoblast like cells* to express proinflammatory cytokines and not causing broken cell. [12] And then, it was continued by giving propolis extract. Propolis extract was taken from raw propolis that is produced by *Apis meliferra* bees at Lawang, East Java, Indonesia. Propolis extract was made by maceration method using ethanol solvent 70%.

Propolis extract allocation

Propolis extract allocation was using 3 μg/ml, carried towards odontoblast culture that had been inducted by *Lactobacillus acidophilus* inactive bacteria. The purpose of the act was to understand about propolis extract effectiveness to obstruct proinflammation cytokine secretion through TNF-α and TGF-β1 expression.

Observation of TNFα andTGF-β1expression toward *odontoblast like cells*

TNF-α and TGF-β1 identification was carried by immunocytochemistry technique, using anti-DMP1 (Santacruz), with procedure as it was said in Immunostaining Kit guidelines of assay (Biocare).

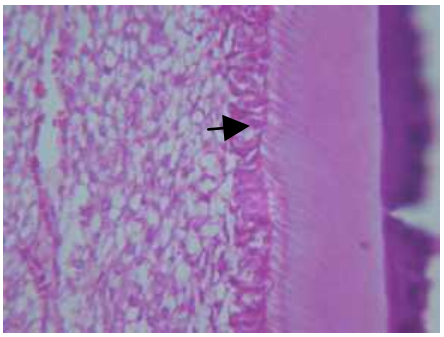
Statistical Analyses

The data were analyzed by One-Way Anova test and for knowing the difference among treatment group, data were analyzed by Tukey HSD test. Results were considered statistically significant when the p-value was less than 0.05.

RESULTS

Pulp odontoblast characteristic

This research used *odontoblast like cells* culture and fibroblast from mandibular third molar teeth's pulp tissues. Odontoblast structure of mandibular third molar tooth (picture 1), it is shown that the odontoblast formed a layer at peripheral area.

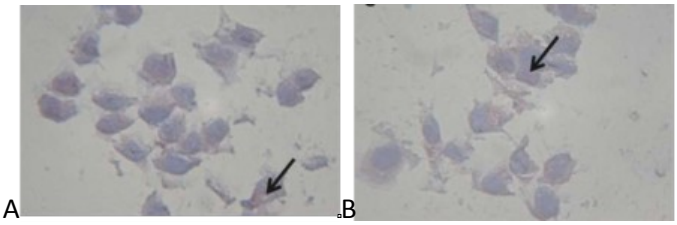


Picture 1. Odontoblast structure of mandibular third molar tooth (arrow line).

Pulp odontoblast structure with Hematoxilin-Eosin (HE) coloration (400x zooming). Picture 1 shows that odontoblast peripheral arrayed on cuboids dentin and extended into pulp tissue. This research was using cell culture of human's teeth pulp tissue, that is using *odontoblast like cells* from fibroblast so that it would have odontoblast characteristic.

The results of immunocytochemistry TNF-α expression

Cells that expressing TNF-α at odontoblast culture cytoplasm, that was inducted by *Lactobacillus acidophilus* inactive bacteria and exposed by propolis extract, was done using immunocytochemical examination.



Picture 2 odontoblast culture (AEC coloration, 400x zooming).

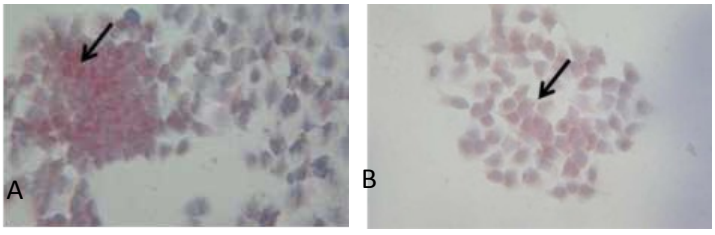
A. Odontoblast that had been inducted by *Lactobacillus acidophilus* inactive

B.Odontoblast that had been inducted by *Lactobacillus acidophilus* inactive dan propolis extract 3 μg/ml

Cells that expressed TNF-α (arrow line) was distributed to odontoblast cytoplasm (red color). A decline of cells percentage was occurred at odontoblast culture that had been inducted by *Lactobacillus acidophilus* inactive and propolis.

The results of immunocytochemistry TGF-β1 expression

Cells that expressing TGF-β1 at odontoblast culture cytoplasm, that was inducted by *Lactobacillus acidophilus* inactive bacteria and exposed by propolis extract, was done using immunocytochemical examination.



Picture 3 odontoblast culture (AEC coloration, 200x zooming).

A. Odontoblast that had been inducted by *Lactobacillus acidophilus* inactive

B.Odontoblast that had been inducted by *Lactobacillus acidophilus* inactive dan propolis extract 3 μg/ml

Cells that expressed TGF-β1 (red color) was distributed to cytoplasm. An escalation of cells percentage that expressed TGF-β1 was occurred at odontoblast culture that had been inducted by *Lactobacillus acidophilus* inactive and propolis.

DISCUSSION

This research was carried by an approach using *odontoblast like cells* culture model that had been inducted by *Lactobacillus acidophilus* inactive to find out about molecular mechanism from signal transduction of pro-inflammatory cytokines. The effect from chosen therapy of propolis extract as a material that has an anti-inflammation character, so that it will be useful for inflammated dental pulp tissue therapy. Odontoblast is prime cell and form a peripheral layer of pulp tissue by *in vitro* that has unique cellular morphology and could be inducted to express cytokine and chemokine. [13] In this research, odontoblast and fibroblast culture was obtained from third molar of mandibular teeth impacted that had been extracted from 14-19 years old patients. [14] This selection was based on teeth growth process only needs minimum amount of progenitor odontoblast cells that undergo mitosis before differentiating to form odontoblast. In the end of mitosis process will produced 2 *doughter* cells: 1. Cell that located close to basalt membrane which is functioned to receive signal so that it will induct a differentiation to be odontoblast contributing for Hohl layer; 2. The other *doughter* cell is progenitor cell that is functioned as cell that will replace odontoblast in the healing process when it forms reparative dentin and dentin bridge if odontoblast is broken. [15]

In vitro research towards odontoblast shown that LTA exposure, bond with TLR2 reseptor and if it increased it will activate transcription factor of NF-κβ so that it will inducted from cytoplasm to the core and secreting pro-inflammation cytokine. [14] This research proved that *Lactobacilus acidophilus* inactive exposure towards odontoblast culture will increase TNF-α expression through TLR2 signal. This was matched with the clausal that NF-κβp65 is belong to NF-κβ inhibitor *canonical* lane, that secreting pro-inflammation cytokine and signal transduction due to TNF-α, IL-1, LPS or LTA induction and using various adapter signal to involved in IKK activities. Fosforilation of serin residue towards responsive signal (SRR) from classic IκBs that leads to IKKβ ubiquitination IκB and proteosomal degradation, that had been secreted from NF-κβ dimer, then it entered the core and inducted target gen's transcription [16,18] pro-inflammation cytokine that had been secreted by TNF-α, IL-1, IL-6, IL-12.[19]

Human teeth's dentin contains TGF-β1 that has double role in forming and repairing dentin pulp complex. [17] Cytokine is ulosit, to control initiation and inflammation resolution respond. [20] *Pulp capping* treatment with TGF-β1 is increasing and accelerating collagen type 1 synthesis, mineralization so that closing the perforation and dentinogenesis reparative is occured.[21] Odontoblast is outermost layer dentin that will be the first to receive any injury. Odontoblast expresses TGF-β1 for defense mechanism an TLRs that played a major role to identify microba. [12]

This research's result shown that at odontoblas culture which is inducted by *Lactobacillus acidophilus* inactive was inducting TLR2 expression and activating transcription factor NF-κβp65 so

that it was inducting TNF-α and TGF-β1expression. It was shown by using examination with immunocytochemistry techniques. The result indicated if TLR2 expression increased, NFκβ expression will increased too. If NFκβ expression increased, TNF-α expression will increased too, and if NF-κβ expression increased, TGF-β1 expression will decreased, vice versa. This analysis shown that NF-κβ and TGF-β1 expression are inversely related, that indicating there was *Lactobacillus acidophilus* inactive induction to activated TLR2 receptor so that it pass on the signal transduction to odontoblast and to activated transcription factor NF-κβp65 so that it entered the cell core, activating gen transcription so that it inducted TNF-α and TGF-β1secretion. The increasing of NF-κβp65 expression caused the decreasing of TGF-β1expression. This was supported with a research [3], which told that odontoblast received stimuli gram positive and negative bacteria from dental caries through LTA and LPS that inducting signal activation through TLR2 and TLR4. Dentin with caries released TGF-β1 as anti-inflammation cytokine and obstructed TLR2, TLR4, pro-inflammation cytokine IL8, and TNF-α. That condition could be inferred as severity of pulp inflammation or pulpitis that could be associated with an equilibrium between TLR, which starts the inflammation signal and TGF-β1 as anti-inflammation.

In *in vitro* research, the LTA's inducted odontoblast would trigger TLR2 expression and activate NFκβ so that it would enter the cell core which eventually produced chemokine and cytokine. All of these events had the potential for targeted end to lead to a pulp inflammation. Some strategies could be considered to achieve the healing of pulp inflammation, including by blocking or obstructing the intracellular transduction signal through TLR2 and cytokine/chemokine pro-inflammation on odontoblast. Furthermore, to generated a better understanding of molecular mechanism of *odontoblast like cells* that was exposed by bacteria so that it would opened a way for designing therapy materials that was effectively modulating pulp cells in order to enable healing and repairs through the formation of reparative dentin.[22] Conclusion of the research was propolis extract could obstructs TNF-α expression and increases TGF-β1 expression of *odontoblastlike cells* in human teeth's pulp. Anti-inflammation's effect from East Java propolis extract was related to cytokine modulation.

REFERENCES

1. Martin FE, Nadkarni MA, Jaques NA, and Hunter N,. Quantitative Microbiological Study of Human Carious Dentine by Culture and Real-Time PCR: Association of Anaerobes with Histopathological Changes in Chronic Pulpitis. J. Clin. Microbiol. 2002. 40: 1698-1704

2. Hahn C and Liewebr FR. Update on the Adaptive Immune Responses of the Dental Pulp. J Endod. 2007. 33: 773-781.

3. Horst KA, Tompkins SR, Coats, P.H. Braham R.P , Darveaul and Dale BA. TGF-β1 Inhibits TLR-mediated Odontoblast Responses to Oral Bacteria. J Dent Res. 2009.88: 333-338.

4. Ahangari Z, NaserM, Jalili M, Mansouri Y, Masshhadiabbas F, Torkaman A. Effect propolis in dentine regeneration and the potential role of dental pulp stemcell in Guinea pigs. Cell J.2009.13. 223-28.

5. Bluteau G, Luder HU, De Bari C, Mitsiadis TA. Stem for tooth engineering.Eur cell Mater.2008.31. 1-9.

6. Schroder U. Effect of calcium hydroxide containing pulp -capping agent on pulp cell migration, proliferation and differentitation. J Dent Res. 19885.64.541-48.

7. Ji YM, Jeon SH, Park JY, Chung JH, Choung YH, Choung PH. Dental stem cell therapy with calcium hydroxide in dental pulp capping. Tissue Eng Part A. 2010.16. 1823-33.

8. Molan P. Why honey is effective as a medicine. Part 2 The scientific explainatation of its effect bee world. 2001. 82.22-40.

9. Olsen H, Peterson K, Rohlin M. Formation of a hard tissue barrier afther pulpcapping in humans .A systemic review. Int Endod J. 2006. 39.429-42.

10. Mauth C, .Huwig A, -Hausner GU and .Roulet JF, 2007. Restorative Applications for Dental Pulp Therapy. In (.Ashammakhi EN, Reis R and Chiellini E, Eds). Topics in Tissue Engineering. 3, E-Book, pp 1-22

11. Neumann E.,. Ramos,M.G, Santos, L.M.,. Rodrigues, A.C.PVieira, E.C.,. Afonso, L.C.C, Nicoli, J.R. , Vieira, L.Q. Lactobacillus delbrueckii UFV-H2b20 induces type 1 cytokine production by mouse cells in vitro and in vivo. Braz J Med Biol Res .2009 .42: 358-367.

12. Widjiastuti I, Mekanisme molekuler stimulasi ekstrak propolis pada *odontoblas likecells* yang dipapar *Lactobacillus acidophilus* inaktif dalam menginduksidiferensiasi fibroblas pulpa. Disertasi. Fakultas Kedokteran Universitas Airlangga Surabaya. 2012.

13. Veerayutthwilai O, Byers MR, Pham TT, Darveau RP & Dale BA . Differential regulation of immune responses by odontoblasts. Oral Microbiol Immunol. 2007.22: 5– 13.

14. Alliot LB, Hurtrel, D. and Gregoire M. Characterization of a-smooth muscle actin positive cells in mineralized human dental pulp cultures. Arch Oral Biol. 2001.46: 221-228.

15. Fitzgeral DMJ, Chieg JR and. Heys DR.. Autoradiographic analysis of odontoblast replacement following pulp exposure in primate teeth, Archs oral Bid. 1979. 35: 707-715.

16. Wong ET and Tergaonkar V. Roles of NF-*κ*B in health and disease: mechanisms and therapeutic potential.Clinical Science . 2009 .116. 451–465

17. Hayden MS, West, AP and Ghosh S. NF-*κ*B and the immune response. 2006. Oncogene 25: 6758–6780

18. (Oeckinghaus A and Ghosh S. The NF-kB family of transcription factors and oral bacteria. J Dent Res. 2009.88. 333-338.

19. Smith AJ. Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. J Dent Educ. 2003. 67: 678-689.

20. Li, LL, Wang, ZY, Bai, Z. C, Mao, Y, Gao, B, Xin H T, Zhou B, Zhang Y, Liu,B. Three dimensional finite element analysis of weakened roots restored with different cements in combination with titanium alloy posts. Chin. Med. J. 2006.119:305-311.

21. Sri kunarti. Stimulasi aktivitas fibroblas pulpa dengan pemberian TGF-β1 sebagai bahan perawatan *direct pulp caping*. Disertasi, Universitas Airlangga, Surabaya, Indonesia. 2005

22. Farges JC,. Understanding dental pulp innate immunity - a basis for identifying new targets for therapeutic agents that dampen inflammation. J. Appl. Oral Sci. 2009. 17: 1-5.