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. This 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 dan TGF-β1 expression was done using the immunocytochemical technique to determine the effectiveness of propolis extracts from East Java Indonesia against proinflammatory cytokines. Data were analyzed using Anova test (p= 0,05). Propolis extract can inhibit the expression of TNFα and increase the expression of TGF -β1 on odontoblast like cell in human dental pulp. Anti-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. 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 membran, consisting peptidoglycan layer with 80 nanometer thickness. Lactobacillus acidophilus LTA stimulates odontoblastto express TLR2 and induct chemokine secretiom (CCL2 dan CXCL2). 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 stomatognathi function. During the clinical procedure the teeth pulp will sometimes undergo inflammation, like in the caries removal process. In this situation, pulp undergoes a process called reparative 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 stomatognathi 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 constituents 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-β1 excretion..

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 Lactobacillus 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 oclusal 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).
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°C for 5 minutes.  

Effective dose of bacteria exposure determination was carried by comparing cells : bacteria 1 : 25, by incubating them for 24 hours in incubator with 5% CO₂ at temperatures 37°C. The effective dose was used for inducting *odontoblast like cells* to express proinflammatory cytokines and not causing broken cell.  

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 and TGF-β1 expression 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.

![Odontoblast structure of mandibular third molar tooth](image)

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.
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.

**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 inflamed 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. 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. 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 daughter 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 daughter 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.

*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. This research proved that *Lactobacillus 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 IkBs that leads to IKKβ ubiquitination IkB and proteosomal degradation, that had been secreted from NF-κβ dimer, then it entered the core and inducted target gen’s transcription pro-inflammation cytokine that had been secreted by TNF-α, IL-1, IL-6, IL-12.

Human teeth’s dentin contains TGF-β1 that has double role in forming and repairing dentin pulp complex. Cytokine is ulosit, to control initiation and inflammation resolution respond. *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. 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.

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-β1 expression. 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-β1 secretion. The increasing of NF-κβp65 expression caused the decreasing of TGF-β1 expression. 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 odontoblast like cells in human teeth’s pulp. Anti-inflammation’s effect from East Java propolis extract was related to cytokine modulation.

REFERENCES


