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Original article

Interleukin-6 expression on inflamed rat dental pulp tissue after capped with *Trigona* sp. propolis from south Sulawesi, Indonesia

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ABSTRACT

Background: Propolis is a natural product of plant resins collected by honeybees from various plant sources. It is used as a remedy in folk medicine since ancient times because of its several biological and pharmacological properties. Recently, propolis has been used by dentist to treat various oral diseases. It was always mentioned as an anti-inflammatory agent. Cytokines are proteins that provide communication between cells and play a critical role in a wide variety of processes. It released from cells in an inflammatory process that active, mediate or potential actions of other cells or tissues. When dental pulp has inflammation, several pro-inflammatory cytokines including Interleukin-6 (IL-6) was released by innate immune cells. *Objective:* To analyse the expression of IL-6 on inflamed rat dental pulp tissue following application of propolis. *Material and methods: Trigona* sp. propolis was obtained from Luwu Regency, south Sulawesi Province, Indonesia. Flavonoid and non-flavonoid extracts were purified from propolis using thin layer chromatography. The study was applied on 80 male *Sprague Dawley* rats, 10–12 weeks of age, divided randomly and equally into 5 groups. Group I, as negative control group was not conducted any treatment. At group II, III, IV and V. A Class I cavity (Black Classification) were made on the occlusal surface of right maxillary first molar. The dental pulp was perforated using dental explorer and allowed in the oral environment for 1 h, after that, Ethanolic Extract Propolis (EEP) (Group II), Extract Flavonoid-Propolis (EFP) (Group III), Extract Non-Flavonoid Propolis (ENFP) (Group IV), or Calcium Hydroxide ($Ca(OH)_2$) (Group V) were applied on dental pulp. All cavities were then filled with Glass Ionomer Cement as permanent filling. The rats being sacrificed in 6 h, 2 days, 4 days and 7 days. Sample biopsy were obtained, IL-6 expression was detected by using immunohistochemistry method. Data was analyzed statistically using Freidman and Kruskal Wallis tests with significance level of $P < 0.05$. *Results:* All agent showed IL-6 expression in inflamed rat dental pulp tissue, and this expression was decreased with the longer of observation time periods. EEP more stronger to decreased IL-6 expression on inflamed rat dental pulp tissue than other agent. There is significant difference ($P < 0.05$) of IL-6 expression between group I and other groups in 6 h and 2 days but not in 4 and 7 days time periods. *Conclusion: Trigona* sp. propolis from south Sulawesi, Indonesia could suppressed the expression of IL-6 on inflamed rat dental pulp tissue.

1. Introduction

Dental pulp is a connective tissue uniquely situated within the rigid encasemet of mineralized dentin. Inflammation of dental pulp is similar to that in other connective tissue in that it is mediated by cellular and molecular factors (Okiji, 2002; Fouad, 2002). The inflammatory response to dental pulp injury or infection has major clinical significance. Injury may be caused by dental caries, dental restorative procedures (iatrogenic), tooth fracture or attrition (Trowbridge, 2002).

Cytokines are soluble proteins that play an important role in the initiation and maintenance of inflammatory and immune responses as well as intercellular crosstalking. Interleukin-6 (IL-6) is a multifunctional cytokine synthesized in response to stimuli such as inflammation and trauma (Abbas et al., 2007) by a variety of cells such as macrophages, neutrophils, keratinocytes, fibrob-

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lasts, and endothelial cells (Nibali et al., 2012). Interleukin-6 cell signals are transmitted through a receptor expressed in a wide range of target cell types. In addition to this, a soluble IL-6 receptor (sIL-6R) enables to widen the repertoire of cells responsive to IL-6 (Jones et al., 2001). Interluekin-6 is able to stimulate a number of biologic processes including antibody-producing cells, activation of T cells, B cell differentiation, synthesis of acute-phase proteins B cells, hematopoiesis, induction of angiogenesis, vascular permeability, and osteoclast differentiation (Abbas et al., 2007). Interleukin-6 activity in inflammation is considered double-edged, acting both as anti-inflammatory (e.g., down regulation of neutrophil recruitment and proinflammatory cytokine expression) but also as proinflammatory in chronic diseases (Fouad, 2002).

Propolis is a resinous material bee honey product which collect from various plant mainly buds and leafs. Honey bee used propolis as antibiotic, seal hole or cracks of its combs, and also protect it from insects (Shakespeare and Henry, 2011). Propolis has been used since long time ago as traditional medicine such as antibacterial and anti-inflammatory drugs. The chemical composition of propolis is very complex, depends on the collecting location, time, and plant source (Toreti et al., 2013). However, the composition of propolis primarily consists of resinous (50%), wax (30%), essential and aromatics oils (10%), bee pollen (5%), and other substances (5%) (Bankova, 2009).

In recent years, propolis has been used in dentistry including in Conservative Dentistry and Endodontics to treat many tooth and pulp diseases such as a cariostatic agent to prevent caries (Libério et al., 2009), as a desensitizing agent to treat hypersensitivity dentin (Purra et al., 2014), intracanal irrigant (Bhardwaj et al., 2013) and medicament (Awawdeh et al., 2009) during root canal treatment and also as direct pulp capping agent to stimulate reparative dentin barrier (Parolia et al., 2010). Previous studies have demonstrated that propolis is toxic to dental pulp fibroblasts at 2 mg or above (Al-Shaher et al., 2004) and not reduced the viability of dental pulp fibroblasts at 1 mg/mL (Jahromi et al., 2014).

One of honeybee species that we can found in south Sulawesi province, Indonesia was *Trigona sp*. This honeybee species is stingless and can produce a lot of propolis. Therefore, the aim of the present study was to analyse the expression of IL-6 on inflamed rat dental pulp tissue following application of *Trigona* sp. propolis from south Sulawesi province, Indonesia.

2. Material and methods

Propolis (*Trigona* sp.) was collected from honeycomb in Luwu Regency, south Sulawesi province, Indonesia in the early monsoon season. Dry propolis was subjected to exhaustive maceration, filtered using aqueous ethanol, and concentrated using a rotary evaporator. The residue was separated using toluene solution to yield flavonoid and non-flavonoid fractions, which were then subjected to silica gel thin layer chromatography. Examination under ultraviolet light showed that the flavonoids group from propolis contain flavones, flavonols, flavanols, and chalcone (Sabir et al., 2015). The study was conducted at The Animal Research Development Center, Faculty of Veterinary and Department of Pathology, Faculty of Medicine, Gadjah Mada University, Yogyakarta.

Eighty male 10–12-week-old *Sprague-Dawley* rats (weight 200–250 g) were divided into 5 groups, each consisting of 16 animals. Group I, as negative control group was not conducted any treatment. At group II, III, IV and V. The rats were anesthetized intramuscularly with ketamine (Ketalar, Warner Lambert, Ireland) (65 mg kg^{-1} body weight) and xylazine-HCl (Xyla, Interchemie, Netherlands) (7 mg kg^{-1} body weight), and then Class I cavities (Black Classification) were prepared on the occlusal surface of right maxillary first molar using a low-speed tapered round diamond bur (Intensiv, Switzerland) (0.84 mm in diameter). The pulp was then exposed at the cavity floor using a dental explorer (Martin, Germany) (0.35 mm in tip diameter) and allowed in the oral environment for 1 h, after that, the pulp directly capped with Ethanolic Extract Propolis (EEP) (0.5 mg) (Group II), Extract Flavonoid-Propolis (EFP) (0.5 mg) (Group III), Extract Non-Flavonoid Propolis (ENFP) (0.5 mg) (Group IV), or Calcium Hydroxide ($Ca(OH)_2$) (0.5 mg) (Group V). Each cavity was then air-dried and filled with Glass Ionomer Cement (HS Posterior Extra, GC, Tokyo, Japan) as permanent filling. The experimental protocol was approved by the ethical committee of Faculty of Medicine, Hasanuddin University.

Four rats were sacrificed at 6 h, 2 days, 4 days and 7 days respectively. The teeth and surrounding bone were resected, fixed in Bouin's fixative for 24 h, decalcified with acetic acid/formal saline for 7 days, embedded in paraffin and sectioned serially at 6 μm thickness. The sections were stained with IL-6 monoclonal antibody (Neuromics, USA) using immunohistochemistry method and viewed by light microscopy. Immunohistochemistry evaluation was carried out as described previously (Faleiro-Rodrigues et al., 2004). Data were statistically analyzed by Freidman and Kruskal Wallis non-parametric tests. Data were analyzed using the SPSS 20.0.1 software (SPSS Inc, Chicago, IL, USA). Significance was established at $P < 0.05$ level.

3. Results

Except group I as negative control (no treatment), all treatment group showed IL-6 expression on inflamed rats dental pulp tissue after 6 h, 2 days, 4 days and 7 days application, but the expression was decreased with the longer of observation time periods. However, EEP was looks more stronger than other material test in inhibit IL-6 expression on inflamed rat dental pulp tissue (Fig. 1). No evidences of necrotic pulp tissues in all groups of animals were found throughout the study. For the sake of clarity and brevity, the photomicrograph of IL-6 expression is presented here in only by the section from all groups at 6 h and 7 days (Fig. 2).

The results of Freidman test revealed that there was no significant difference of IL-6 expression among time periods for each group (Table 1). Meanwhile, Kruskal Wallis test showed that there was significant difference ($P < 0.05$) of IL-6 expression between group I and other groups in 6 h and 2 days but not in 4 days and 7 days time periods (Table 2).

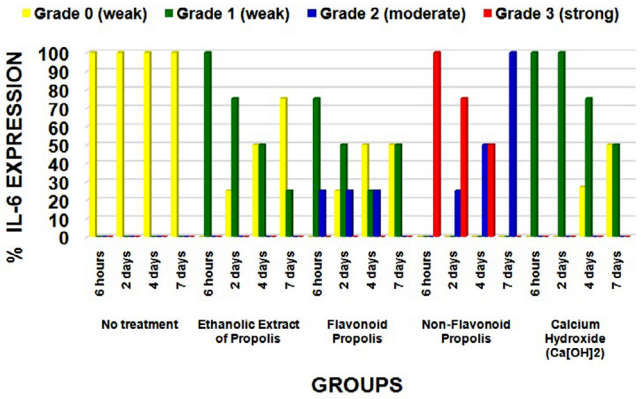

Figure 1. Histogram of percentage of IL-6 expression at group I (normal dental pulp) and inflamed rats dental pulp tissue group II, III, IV, V was capped with Ethanolic Extract Propolis (EEP), Extract Flavonoid-Propolis (EFP), Extract Non-Flavonoid Propolis (ENFP), and Calcium Hydroxide ($Ca(OH)_2$), respectively.

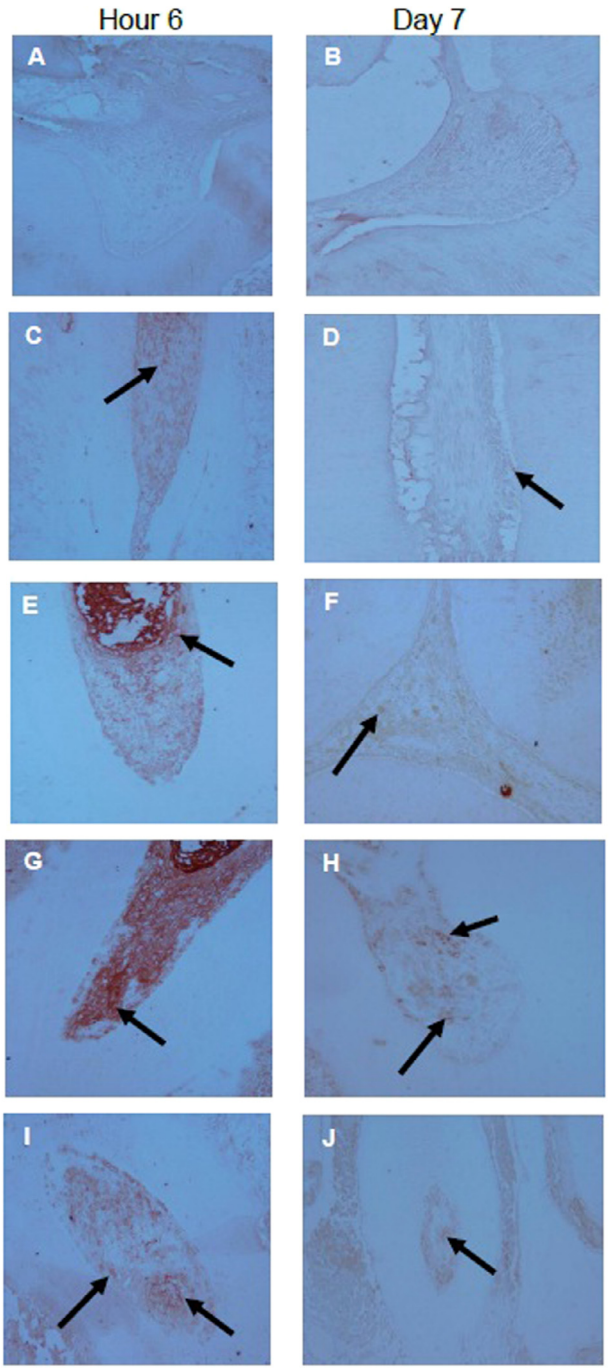

Figure 2. Interleukin-6 expression on rats dental pulp tissue. Normal dental pulp with no treatment (negative control) in groups I (A–B) and inflamed dental pulp in groups II (C–D), III (E–F), IV (G–H) and V (I–J), capped with Ethanolic Extract Propolis (EEP), Extract Flavonoid-Propolis (EFP), Extract Non-Flavonoid Propolis (ENFP), and Calcium Hydroxide ($Ca(OH)_2$), respectively. Arrows show IL-6 expression. Immunohistochemistry method, DAB chromogen, original magnification 200×.

4. Discussion

Interleukin-6 displays multiple biological effects and acts as a major mediator of the host response following tissue injury and infection as well as inflammation. Study by El Salhy et al. (2013) showed that levels of IL-8 and the ratios of IL-6/IL-10 and IL-8/IL-10 have the potential to be indicators of dental pulp inflammation in caries exposure cases. The level of IL-6 was significantly higher in tooth with caries exposure and irreversible pulpitis as compared

Table 1
The difference grade of IL-6 expression among time periods for each group.

Groups	*Mean rank*				Freidman test	*P*
	6 h	2 days	4 days	7 days		
No treatment	2.50	2.50	2.50	2.50	0.00	1.00
EEP	3.13	2.13	2.50	2.25	2.111	0.550
Flavonoid	2.50	2.50	2.50	2.50	0.00	1.00
Non-flavonoid	2.13	2.38	2.38	3.13	2.25	0.522
$Ca(OH)_2$	2.00	2.88	2.50	2.63	1.345	0.719

Table 2
The difference grade of IL-6 expression among groups for each time period.

Time periods	*Mean rank*					Kruskal Wallis test	*P*
	No treatment	EEP	Flavonoid	Non-flavonoid	Ca $(OH)_2$		
6 jam	5.50	10.25	11.66	13,50	11.00	0.000	0.038*
2 hari	5.00	9.75	10.83	11.13	10.01	2.235	0.047*
4 hari	5.75	6.63	7.25	8.75	6.95	4.156	0.385
7 hari	5.50	5.75	6.88	7.25	5.98	3.341	0.503

Note: *Significant at $P < 0.05$.

to healthy tooth, so he suggest that cytokine estimation in pulpal blood may help in the diagnosis of pulpal inflammation. Previous study also detected the presence of IL-6 in dental granulomas (De Sa et al., 2003) and high levels of IL-6 in inflamed human dental pulp and periapical lesions compared with healthy pulp, suggesting that IL-6 is released locally in endodontic lesions (Barkhordar et al., 1999). Furthermore, IL-6 levels were found to be elevated in symptomatic human periapical lesions compared with asymptomatic lesions and uninflamed periradicular tissues, suggesting that excessive IL-6 release may be linked with worsening of inflammation and therefore of the clinical symptoms (Prso et al., 2007).

Anti-inflammatory and immunomodulatory properties of propolis and its constituents have been study by a number of researchers. The results of this study demonstrated that 6 h after EEP and $Ca(OH)_2$ application, only weak expression of IL-6 occured on inflamed rat dental pulp. In contrast, propolis-derived flavonoids as well as non-flavonoids stimulated moderate and strong IL-6 expression at the same time period, but this expression was decreased with the longer of observation time periods. In addition, EEP was looks more stronger than other material test in inhibit IL-6 expression on inflamed rat dental pulp tissue. Our previous study also found the suppression of dental pulp inflammation by propolis (Sabir, 2005).

The present results are not surprising, since propolis was known to have anti-inflammatory properties, perhaps via suppression of immune cell activation, macrophage-derived nitric oxide, neutrophil activation and cytokines production (Sforcin, 2007). However, in this present study, the exact mechanism of propolis to suppress IL-6 production still unclear, perhaps via suppression of activation and differentiation of mononuclear macrophages (Fuliang et al., 2005). The anti-inflammatory activity of propolis not only depends on the presence of flavonoids and caffeic acid phenethyl ester (CAPE), but also by additional active compounds, such as ferulic acid, (hydroxyl) cinnamic acid and diterpene derivatives (Borelli et al., 2002; Ramos and Miranda, 2007). Study by Bachiega et al. (2012) on peritoneal macrophages from BALB/c mice found that IL-6 production was significantly inhibit by propolis and phenolic compound in propolis such as cinnamic and coumaric acids. However, no single ingredient is predominantly active rather than all work together (Sforcin and Bankova, 2011).

Presently, $Ca(OH)_2$ is the most commonly used medication for direct pulp capping treatment (Lacević et al., 2003). The weak

expression of IL-6 on inflamed rat dental pulp 6 h after $Ca(OH)_2$ application and less marked on day 7 caused of its alkalinity. This alkalinity actually has a beneficial effect on the injured dental pulp tissue, insofar as it causes mild irritation and stimulates the conjunctive tissue to defend and repair itself, initiating an mild inflammatory reaction to control and eliminate the irritating agent (Fransson, 2012). Previous studies have reported that $Ca(OH)_2$ induces a lesser degree of inflammatory infiltrate in the initial hours, and inducing subsequent tissue repairing (Nelson-Filho et al., 1999). An recent *in vitro* study using ELISA found that $Ca(OH)_2$ inhibited IL-6 which produced by mouse fibroblasts (Gomes-Filho et al., 2009), but the exact mechanisms still unclear.

5. Conclusions

The present result obtained in rats suggest that *Trigona* sp. propolis from Luwu regency, south Sulawesi province, Indonesia could suppressed the expression of IL-6 on inflamed rats dental pulp tissue.

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