

Propolis extract as pulp capping material enhances odontoblast-like cell thickness and type 1 collagen expression (in vivo)

Ira Widjiastuti, Ari Subiyanto, Evri Kusumah Ningtyas, Rendy Popyandra, Michael Golden Kurniawan and Fauziah Diajeng Retnaningsih
Department of Conservative Dentistry,
Faculty of Dental Medicine, Universitas Airlangga,
Surabaya – Indonesia

ABSTRACT

Background: Propolis is a natural biocompatible material that has been widely studied in dentistry because of its inflammatory, anti-microbial and immunomodulatory properties. One of the active components is caffeic acid phenethyl ester (CAPE). CAPE is effective in stimulating collagen as well as inhibiting the inflammation and degeneration of dental pulp. **Purpose:** To investigate the post-administration of propolis extract as pulp capping material enhances odontoblast-like cell thickness and type 1 collagen expression in Wistar rats (*Rattus Norvegicus*) **Methods:** This research was a true experimental design with a posttest-only control group design. Sixty-three Wistar rats were randomly divided into three groups, with each group consisting of 21 rats: Group I: Positive control; no capping material was administered; Group II: CAPE was administered; Group III: 11% of the propolis extract was administered. All samples were filled with glass ionomer cement. Seven rats from each group were sacrificed after days 7, 14 and 28 of post-pulp capping administration, and their afflicted teeth were subsequently extracted for histologic analysis. **Results:** No significant difference was seen in odontoblast-like cell thickness after the application of CAPE and propolis on days 7 and 14 ($p > 0.05$). However, a significant difference was noticed on day 28 ($p < 0.05$), with the thickness of odontoblast-like cell in CAPE being thinner than that in propolis. A significant difference in the expression of type 1 collagen was observed on days 7, 14 and 28 after the application of the propolis extract compared with CAPE ($p < 0.05$). **Conclusion:** The post-administration of propolis extract as a pulp capping material could enhance odontoblast-like cell thickness and type 1 collagen expression in Wistar rats.

Keywords: CAPE; odontoblast-like cell; propolis extract; pulp capping; type 1 collagen

Correspondence: Ira Widjiastuti, Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Jl. Mayjen Prof. Dr Moestopo 47, Surabaya – Indonesia. Email: ira-w@fkg.unair.ac.id

INTRODUCTION

Dental caries can lead to degenerative changes in teeth structures. The products of bacterial metabolism destroy enamel and dentine, which can subsequently lead to pulp disease. Basic measures to protect the pulp against caries include decreasing dentine permeability, reducing immune and inflammatory reactions and inducing tertiary dentine formation.¹ Pulp tissues consist of blood vessels, innervation, connective tissue fibres and cells, such as fibroblasts and odontoblasts. Odontoblasts are unique cells that produce collagen and non-collagen proteins for the formation of dentine extracellular matrix; they are also the first response to exogenous stimuli or dental materials.²

Dental pulp is similar to other connective tissues with the capability of restoration. The characteristics of pulp healing include the restoration of damaged soft tissues, differentiation of subodontoblasts into odontoblast-like cells and the construction of dentine bridges in perforated pulp tissues.³

Cavity preparation often causes pulp perforation when removing the infected tissue in deep cavity lesion. This mechanical traumatic perforation results from the use of dental burs or other dental instruments. When the pulp is exposed, direct pulp capping can be performed to reduce the need for complicated treatment, such as root canal treatment or extraction.^{4,5} The regenerative pulp tissue treatment aims to regenerate normal tissues between pulp and dentine.

This tissue is responsible for regulating the formation of tertiary dentine. The pulp tissue consists of progenitor cells that proliferate and differentiate into odontoblasts. Damaged odontoblasts can be replaced by odontoblast-like cells, originating from pulp fibroblast cells.⁶ The differentiation of odontoblasts involves several growth factors and extracellular matrices. At this stage, types 1 and 3 collagens play an important role. Type 1 collagen can induce the differentiation and formation of the dentine component. Type 1 collagen is a specific extracellular dense matrix produced at the beginning of the reparative dentine formation. Type 1 collagen in odontoblast cells is considered the initial marker of the reparative dentine formation process.⁷

The most recognised and popular pulp capping material is calcium hydroxide. Nevertheless, direct pulp capping with calcium hydroxide has some disadvantages. It can cause pulp inflammation for three months, have an unpredictable tissue response and have irregular reparative dentine formed below calcium hydroxide, probably forming the tunnel defect. These conditions can increase the dentinal bridge's permeability, which can cause a bacterial invasion.⁸ Nowadays, in dentistry, propolis is studied as a natural pulp capping material. Propolis is a popular medicine in the world with therapeutic effects, such as anti-carcinogenic, anti-oxidant, anti-inflammatory, antibiotic, antifungal and antihepatotoxic.^{9–11} Caffeic acid phenethyl ester (CAPE) is one of the active components of propolis, which has been investigated *in vitro* and *in vivo*. The function of CAPE has been shown to be effective in preventing cancer, inflammation and immunomodulation.^{12,13} CAPE can induce the formation of collagen in the dental pulp and reduce inflammation and degeneration of the pulp.⁹ Thus, this study aims to investigate the effect of propolis extract as a pulp capping material on odontoblast-like cell thickness and type 1 collagen expression in Wistar rats (*Rattus norvegicus*).

MATERIALS AND METHODS

This research was a true experimental design and approved by the ethics committee (No. 10/KKEPK.FKG/II/2015) of the Faculty of Dental Medicine, Universitas Airlangga. Sixty-three healthy, 8–16-month-old male Wistar rats (*Rattus norvegicus*), weighing 200–250 g and being fully erupted molars, were used in this study. The sample was fed a standard feed of 20 g/day per rat plus *ad libitum* drinking water. The Wistar rats were anaesthetised using 100 mg of ketamine (Ketalar®, Warner–Lambert, Irlandia) and 10 mg/kg of xylazine HCl (Rompun®, Bayer, Leverkusen, Jerman) in sterile phosphate-buffered saline (PBS) placed on fixation board. The occlusal surface of the right maxillary first molar was perforated using a low-speed handpiece with a round diamond bur (diameter 0.46) until it reached the pulp chamber.

The sample was divided into three groups, with each consisting of 21 rats ($n = 7$): on day 7, Group I: Positive control; no pulp capping material was administered, with glass ionomer cement (GIC) restoration only; Group II: CAPE was administered; Group III: 11% of the propolis extract was administered. On day 14, Group I: Positive control; Group II: CAPE was administered; Group III: 11% of the propolis extract was administered. On day 28, Group I: Positive control; Group II: CAPE was administered; Group III: 11% of the propolis extract was administered. Propolis was obtained from extract *Apis mellifera* of bees and dissolved with 96% of ethanol. CAPE was obtained from the propolis extract and then analysed using visible spectrophotometer UV. For all groups, a micro applicator was used to put the pulp capping materials on dentine surface. Then, all the samples were filled with restoration material (GIC, Fuji IX®, GC Tokyo, Japan). In addition, the samples were sacrificed after days 7, 14 and 28 post-pulp capping administration. The afflicted tooth and mandible were extracted and fixed in 10% of buffered formalin; they were then decalcified with 10% of ethylenediaminetetraacetic acid (EDTA) (Onemed Dental, PT. Jaya Mas Mandiri, Medika Industri, Sidoarjo, Indonesia) for 28–30 days, embedded in paraffin and cut with microtome in a buccolingual plane parallel to the tooth vertical axis into sections of 6-micro thickness. The samples were then stained with hematoxylin–eosin (HE) to analyse odontoblast-like cell thickness; they were viewed using a light microscope (Nikon E100, Tokyo, Japan) in 400x magnification and counted in 1000x magnification on five different fields of view by two observers. To analyse type 1 collagen, the samples were stained with immunohistochemical (IHC) and monoclonal antibody (COL1A1 Antibody #sc-293182, Santa Cruz Biotechnology, USA) and viewed using a light microscope (Nikon E100, Tokyo, Japan) in 1000x magnification.

SPSS software was used to analyse the data. One-way analysis of variance (ANOVA) ($p < 0.05$) was used to compare the groups. Subsequent pairwise comparisons were made among means by means of Tukey's honestly significant difference (HSD) ($p < 0.05$).

RESULTS

The thickness of odontoblast-like cells and type I collagen expression was higher in the propolis group than the other groups. The highest expression of type I collagen and thickness of odontoblast-like cells was found in group propolis (Tables 1 and 2). The data on odontoblast-like cell thickness and type 1 collagen were analysed. ANOVA test revealed significant differences in the thickness of odontoblast-like cells and type 1 collagen after days 7, 14 and 28 between the groups (p -value < 0.05). Tukey's HSD test was subsequently used to find out the differences between the groups. Table 3 shows the significant

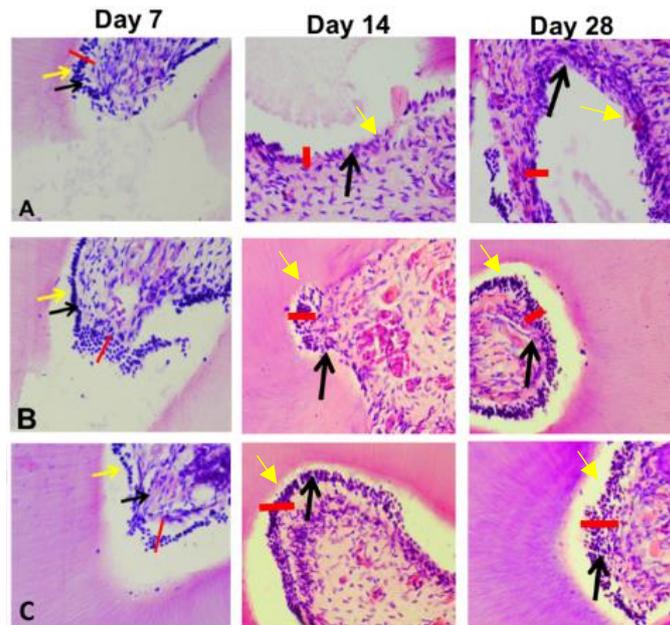


Figure 1. Odontoblast-like cell in (A) positive control group, (B) CAPE group and (C) propolis group at 400x magnification. The black arrow shows the morphological of the odontoblast-like cell; the red line shows the thickness of the odontoblast-like cell; the yellow arrow shows the continuity of the odontoblast-like cell.

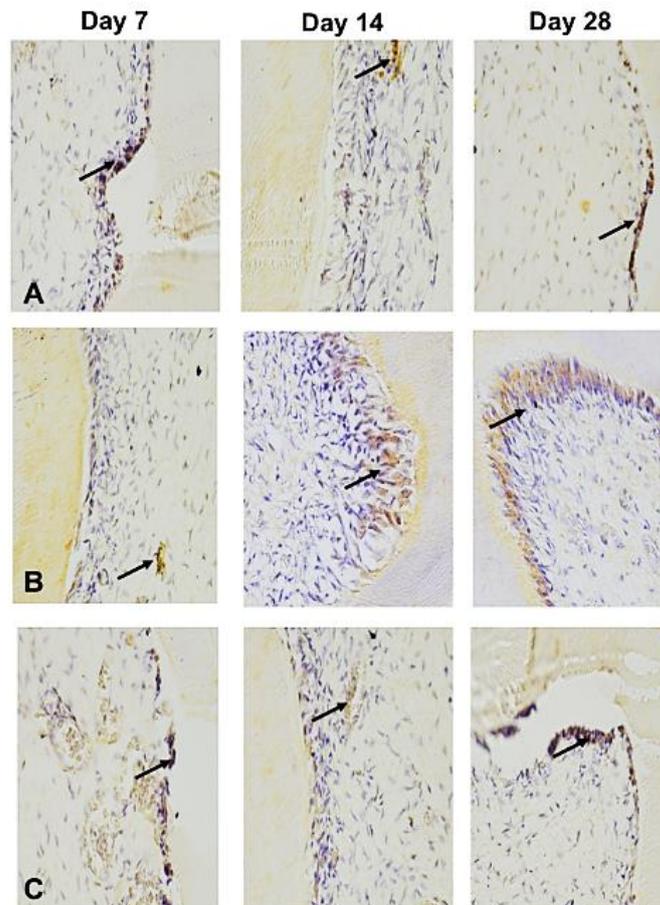


Figure 2. Type I collagen expression in (A) positive control group, (B) CAPE group and (C) propolis group at 1000x magnification. The black arrow shows type I collagen expression.

differences in the thickness of odontoblast-like cells between the CAPE and positive control groups on days 7 and 14, whereas no significant differences were seen on day 28. The odontoblast-like cells in the propolis group were significantly thicker than those in the positive control group on days 7, 14 and 28. Meanwhile, between the propolis and CAPE groups, significant differences in the thickness of odontoblast-like cells were noticed on day 28 (Figure 1). According to Table 4, the propolis group showed more significant differences in the expression of type I collagen than the CAPE and positive control groups on days 7, 14 and 28 (Figure 2). Significant differences in type I collagen between the CAPE and positive control groups were recorded after days 7, 14 and 28 (p -value<0.05).

Table 1. Mean and standard deviation of odontoblast-like cell thickness on days 7, 14 and 28

Groups	N	Mean \pm SD		
		Day 7	Day 14	Day 28
Positive control	7	0.01 \pm 0.00	0.02 \pm 0.00	0.04 \pm 0.01
CAPE	7	0.07 \pm 0.02	0.05 \pm 0.01	0.06 \pm 0.03
Propolis	7	0.07 \pm 0.01	0.07 \pm 0.03	0.12 \pm 0.01

Table 2. Mean and standard deviation of type I collagen expression on days 7, 14 and 28

Groups	N	Mean \pm SD		
		Day 7	Day 14	Day 28
Positive control	7	2.57 \pm 1.13	2.71 \pm 1.38	2.43 \pm 0.78
CAPE	7	8.42 \pm 2.07	7.43 \pm 1.51	8.43 \pm 1.51
Propolis	7	13.14 \pm 3.97	13.71 \pm 3.72	17.57 \pm 2.57

Table 3. Tukey HSD test results of odontoblast-like cell thickness on days 7, 14 and 28

Days	Groups	Positive control	CAPE	Propolis
7	Positive control	-	0.000*	0.000*
	CAPE		-	0.657
	Propolis			-
14	Positive control	-	0.008*	0.005*
	CAPE		-	0.107
	Propolis			-
28	Positive control	-	0.032	0.000*
	CAPE		-	0.002*
	Propolis			-

*information: significant at $p < 0.05$

Table 4. Tukey HSD test results of type I collagen expression on days 7, 14 and 28

Days	Groups	Positive control	CAPE	Propolis
7	Positive control	-	0.000*	0.000*
	CAPE		-	0.010*
	Propolis			-
14	Positive control	-	0.006*	0.000*
	CAPE		-	0.010*
	Propolis			-
28	Positive control	-	0.000*	0.000*
	CAPE		-	0.000*
	Propolis			-

*information: significant at $p < 0.05$

DISCUSSION

Damage to the pulp tissue will set off an inflammatory reaction. Inflammatory reactions are the initial stages of a series of healing processes. When the tissue is affected, fibroblasts migrate immediately to the wound, proliferate and produce collagen matrices; these later become hard tissue barriers against the remaining pulp tissues from irritants and set to repair the damaged tissues.¹⁴ The healing characteristics of the perforated pulp tissues include the restoration of the destroyed soft tissues, the differentiation of sub-odontoblasts into odontoblast-like cells and formation of reparative dentine bridge.³

In this research, the thickness of odontoblast-like cells on days 7, 14 and 28 showed significant differences across all groups. The odontoblast-like cells in the positive control group were thinner than those in the CAPE and propolis extract groups, perhaps because the positive control group was not treated with any pulp capping materials and that the cavity was only filled with restoration materials. Karube *et al.*'s research suggests that sodium fluoride (NaF) can induce apoptosis in odontoblast¹⁴ and that GIC contains fluoride that is probably cytotoxic to odontoblast. The odontoblast-like cells on days 7, 14 and 28 in the propolis group were significantly thicker than those in the control group. These results may have been caused by the properties of propolis, which are known to have antibacterial, anti-inflammatory, antioxidant and immunomodulatory characteristics. These properties can cause the healing process in the dental pulp, which begins with the formation of collagen fibres, to occur more easily because propolis can prevent infection and accelerate cell regeneration.¹⁵

The active components of flavonoids and caffeic acid in the propolis extract play a crucial role in inhibiting

the arachidonic acid lipoxygenase pathway, which can slow down the inflammatory response and increase the phagocytic activity and the stimulating cellular immunity. The propolis extract also functions as an antibacterial by destroying bacterial cell walls and preventing bacterial cell reproduction. The propolis extract offers better results arising from the dentine formation.¹⁶

The results of this study suggest that the propolis extract can accelerate the formation of odontoblast-like cells. The propolis extract is known to be able to stimulate TGFβ1 production.¹³ TGFβ1 stimulates the proliferation of fibroblasts and accelerates the formation of collagen content, which may participate in the healing process of the dental pulp.¹⁷ The potential of propolis to stimulate TGFβ1 production, differentiation of fibroblasts and inhibition of inflammation, was significantly thicker than that of the CAPE group. The use of CAPE as the pulp capping material did not inhibit the inflammatory response of the pulp tissue in the experimental animals, nor did it accelerate the healing and reparative processes of the dentin–pulp complex.¹⁸

The main compositions of propolis extracts comprise phenolic acids and esters, flavonoids (flavones, flavonones, flavonols, dihydroflavonols, chalcones), sesquiterpenes, terpenes, β steroids, naphthalene, aromatic aldehydes, alcohols, stilbene derivatives of benzopyran, caffeic acid, benzophenone, cinnamic acid derivatives and benzoic acid.¹⁹ CAPE is an active component in propolis, which inhibits the production of cytokines and chemokines, with T-cell proliferation and lymphokine production slowing down the inflammatory process. The anti-inflammatory activity of propolis concerns flavonoids, especially galangin and quercetin. The mechanism of CAPE involves inhibiting NF-κB; flavonoids, in turn, inhibit NF-κB and the activities of cyclooxygenase and lipoxygenase.²⁰ Flavonoids can also stimulate TGFβ1 induction and collagen synthesis by fibroblasts.¹⁸ In this study, the expression of type 1 collagen on days 7, 14 and 28 is higher in the propolis extract group than that in the control and CAPE groups. This explains why the odontoblast-like cells and the expression of type 1 collagen in the propolis extract group in this study were thicker than those in the CAPE group, which could be attributed to the propolis containing both CAPE and flavonoids, with some other substances containing quercetin, naringenin, salicylic acid, apigenin, ferulic acid, vestitol and galangin. These substances work together to accelerate the healing process by stimulating the expression of collagen type 1 during the initial stage of wound healing.²⁰ In conclusion, the propolis extract produced thicker odontoblast-like cell layers and showed more expression of type 1 collagen than that in CAPE on days 7, 14 and 28. Further study is required for the pulp capping material containing propolis to qualify as a medicine for human teeth.

REFERENCES

- Berman LH, Hargreaves KM, Cohen S. Cohen's pathways of the pulp expert consult. 10th ed. St. Louis: Mosby Elsevier; 2010. p. 8–9.
- Baldión PA, Velandia-Romero ML, Castellanos JE. Odontoblast-like cells differentiated from dental pulp stem cells retain their phenotype after subcultivation. *Int J Cell Biol.* 2018; 2018: 1–12.
- Parolia A, Kundabala M, Rao NN, Acharya SR, Agrawal P, Mohan M, Thomas M. A comparative histological analysis of human pulp following direct pulp capping with Propolis, mineral trioxide aggregate and Dycal. *Aust Dent J.* 2010; 55(1): 59–64.
- Kunarti S. Pulp tissue inflammation and angiogenesis after pulp capping with transforming growth factor β1. *Dent J (Majalah Kedokt Gigi).* 2008; 41(2): 88–90.
- Zakaria MN. Save the pulp is the essential issues on pulp capping treatment. *J Dentomaxillofacial Sci.* 2016; 1(2): 73–6.
- Bergenholtz G, Hørsted-Bindslev P, Reit C. Textbook of Endodontology. 2nd ed. UK: Wiley-Blackwell; 2010. p. 3–7.
- He G, George A. Dentin matrix protein 1 immobilized on type I collagen fibrils facilitates apatite deposition in vitro. *J Biol Chem.* 2004; 279(12): 11649–56.
- Dwiandhono I, Effendy R, Kunarti S. The thickness of odontoblast-like cell layer after induced by propolis extract and calcium hydroxide. *Dent J (Majalah Kedokt Gigi).* 2016; 49(1): 17–21.
- Djurica G, Vesna D, Elena K. The effect of caffeic acid phenethyl ester on healing capacity and repair of the dentin–pulp complex: in vivo study. *Acta Vet Brno.* 2008; 58: 99–108.
- Abbasi AJ, Mohammadi F, Bayat M, Gema SM, Ghadirian H, Seifi H, Bayat H, Bahrami N. Applications of propolis in dentistry: a review. *Ethiopian J Health Sci.* 2018; 28(4): 505–12.
- Sabir A, Tabbu CR, Agustiono P, Sosroseno W. Histological analysis of rat dental pulp tissue capped with propolis. *J Oral Sci.* 2005; 47(3): 135–8.
- Elkhenany H, El-Badri N, Dhar M. Green propolis extract promotes in vitro proliferation, differentiation, and migration of bone marrow stromal cells. *Biomed Pharmacother.* 2019; 115: 108861.
- Al-Shaher A, Wallace J, Agarwal S, Bretz W, Baugh D. Effect of propolis on human fibroblasts from the pulp and periodontal ligament. *J Endod.* 2004; 30(5): 359–61.
- Karube H, Nishitai G, Inageda K, Kurosu H, Matsuoka M. NaF activates MAPKs and induces apoptosis in odontoblast-like cells. *J Dent Res.* 2009; 88(5): 461–5.
- Yu C, Abbott P V. An overview of the dental pulp: its functions and responses to injury. *Aust Dent J.* 2007; 52: S4–6.
- Sabir A. Respons inflamasi pada pulpa gigi tikus setelah aplikasi ekstrak etanol propolis (EEP) (The inflammatory response on rat dental pulp following ethanolic extract of propolis (EEP) application). *Dent J (Majalah Kedokt Gigi).* 2005; 38(2): 77–83.
- Lin PS, Chang HH, Yeh CY, Chang MC, Chan CP, Kuo HY, Liu HC, Liao WC, Jeng PY, Yeung SY, Jeng JH. Transforming growth factor beta 1 increases collagen content, and stimulates procollagen I and tissue inhibitor of metalloproteinase-1 production of dental pulp cells: Role of MEK/ERK and activin receptor-like kinase-5/Smad signaling. *J Formos Med Assoc.* 2017; 116(5): 351–8.
- Wang LC, Lin YL, Liang YC, Yang YH, Lee JH, Yu HH, Wu WM, Chiang BL. The effect of caffeic acid phenethyl ester on the functions of human monocyte-derived dendritic cells. *BMC Immunol.* 2009; 10: 39.
- Widjiastuti I, Suardita K, Saraswati W. The expressions of NF-κB and TGFβ-1 on odontoblast-like cells of human dental pulp injected with propolis extracts. *Dent J (Majalah Kedokt Gigi).* 2014; 47: 13–8.
- Oryan A, Alemzadeh E, Moshiri A. Potential role of propolis in wound healing: Biological properties and therapeutic activities. *Biomed Pharmacother.* 2018; 98: 469–83.