

## ORIGINAL ARTICLE

# The Effect of the Combination of Calcium Hydroxide With Green Tea Extract and Calcium Hydroxide With Cacao Peel Extract on the Number of Odontoblast-like Cells and Collagen Type I

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## ABSTRACT

**Introduction:** Pulp capping is an endodontic treatment that maintains the vitality of the pulp tissue. Green tea and cacao have antioxidant properties that may inhibit inflammation, thus improve pulp capping result when mixed with calcium hydroxide (Ca(OH)<sub>2</sub>), which has been the gold standard for pulp capping treatment. The objective of this study was to determine the effect of green tea and cacao extracts combined with calcium hydroxide on the number of odontoblast like-cells and type I collagen expression, which marks the repair process. **Methods:** The occlusal surface of the right maxillary first molar of rats was prepared until pulp was exposed. The combined materials were directly applied to the exposed pulp. All cavities were restored with GIC. The rats were sacrificed on day 7 and 28. Histological and immunohistochemical examinations were performed to determine the number of odontoblast-like cells and type I collagen expression. **Results:** The highest average number of odontoblast-like cells on day 28 in the calcium hydroxide-cacao, calcium hydroxide-green tea, and the control group were 14.4, 13.2 and 7.8 cells/region, respectively, while the type I collagen expression were observed at 14.0, 13.0 and 6.8 cells/region, respectively. **Conclusion:** Cacao peel and green tea extract can be added to the current endodontic treatments to improve results.

**Keywords:** Calcium hydroxide, Green tea, Odontoblasts like-cell, Type I collagen expression, Health and wellbeing.

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## INTRODUCTION

Dentin and pulp have the same embryological origin and are closely related in terms of anatomy and physiology, so that they are described in terms of the dentin-pulp complex. This dentin-pulp complex is usually separated from the oral environment by a protective layer of enamel on the tooth crown and cementum at the roots. When this protective layer is lost the dentin-pulp complex is exposed to the irritant and responds in various ways (1). Tertiary dentin is formed in response to pathological external stimuli (eg. caries, micro-leakage, abrasion). Tertiary dentin formation is part of the defensive reaction of the dentin-pulp complex which aims to protect the pulp and can further be divided into reactionary and reparative dentin. When destruction to odontoblast occurs because of deep caries or trauma, progenitor cells or stem cells located in dental pulp proliferated and differentiated into odontoblast-like cells, wherein forms reparative dentin eventually. The proliferation and differentiation activity influenced by morphogen secreted by dentin matrix (2).

The most common type of collagen found in dentin is collagen type I. Collagen type 1 plays a crucial role in the biomineralization process and accounts for 90% of the total protein in the organic matrix of bone and dentin (1). It induces the formation of hydroxyapatite crystals, which is the initial formation of reparative dentine, as part of pulp and dentine regeneration. Fibroblasts will differentiate to form odontoblast-like cells and increase the expression of collagen type 1 to replace damaged odontoblasts (3).

Pulp capping prevents pulp from necrosis after being exposed or nearly exposed (4). Some factors that influence this treatment are cytotoxicity, biological characteristics of the material and ability to control infection. Pulp capping is divided into 2 categories, indirect pulp capping and direct pulp capping (5). When the dental pulp is exposed, the technique used is direct pulp capping. The indirect pulp capping technique is used in conditions where there is still a thin layer of dentin (6). Calcium hydroxide (Ca (OH)<sub>2</sub>) is gold standard material for pulp capping. It has alkali properties with pH increased to 12.5 at 37° C for antibacterial (7) and ability to stimulate reparative dentin formation. Calcium hydroxide has the potential to degrade into calcium and hydroxyl ions, stimulating odontoblasts and other pulp cells to create reparative dentin in a variety of ways (4) However, it

does not provide long-term good adaptation to dentin, does not support consistent odontoblast differentiation and can cause pulp irritation (5). Furthermore, when calcium hydroxide comes into touch with dentinal fluid, it dissolves the substance and reduces its usefulness, leaving the cavity open and the restoration ineffective (7). The deficiency of calcium hydroxide as a pulp capping material includes possibility of pulp inflammation for more than 3 months and formation of a tunnel defect. But with all its shortcomings, calcium hydroxide is still worth maintaining because it has high antibacterial abilities (9). Considering the weaknesses, alternative materials from natural sources may be introduced to improve the quality of calcium hydroxide. In this study, green tea extract and cacao peel extract were selected as additions to calcium hydroxide.

Green tea (*Camellia sinensis*) is a high source of polyphenols, of which polyphenols are components of antioxidants. Polyphenols contained in green tea consist of several chemical compounds such as: flavonols, flavandiol, flavonoids and phenolic acids which total up to 30% by weight of dry tea (10). Among the polyphenol content, 16 of them are catechins. There are 4 main catechin compounds found in green tea, they are Epigallocatechin gallate (EGCG), Epigallocatechin (EGC), Epicatechin gallate (ECG) and epicatechin (EC). Among these, EGCG is the dominant catechin in green tea (11). Cacao (*Theobroma cacao* L.) processing produces cacao peel waste as much as 74% of the total pods. As a cacao processing waste, the peels are rich in flavonoids which can suppress the inflammatory process, so they are better utilized. According to phytochemical analysis research, there are groups of many active compounds, such as alkaloid, tannin, saponin, terpenoid, and flavonoid that have antibacterial activity in cacao peel (12). Extract of cacao peel contains polyphenols which have antimicrobial power against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, and *Bacillus cereus* bacteria.

Epicatechin (EC), epigallocatechin (EGC), epigallocatechingallate (EGCG), and quercetin are flavonoids found in green tea and cocoa peel. Antioxidant properties have long been known to exist in this substance. EGCG is a strong antioxidant that helps in the process of osteogenesis. TNF- (tumor necrosis factor) and interleukin-6 (IL-6) production are suppressed by EGCG, which enhances osteoblast lifespan (13). The benefits of calcium hydroxide, cacao peel and green tea are the basis for combining calcium hydroxide with cacao peel extracts or with green tea extracts as pulp capping material. This study aims to compare the effect of green tea extract and cocoa peel extract combined with calcium hydroxide on the expression of odontoblast-like cells and type I collagen which marks the repair process.

## MATERIALS AND METHODS

### Animal Preparation

Prior to conducting the research, approval was obtained from the ethical feasibility team of the Faculty of Dental Medicine, Universitas Airlangga with the certificate number 197/HRECC.FODM/IX/2017. The sample used the first molars of 30 rats with the following criteria: 12 to 16 weeks of maturity, body weight of 200 to 300 g, giving standard feed and drinking water ad libitum, perfect growth of first molar teeth, and giving feed consisting of BR 1 type chicken food mixed with one-part flour made into 15 to 40 g of pellets, daily.

### Tooth Preparation

All rats were anesthetized with ketamine (Ketalar, Warner- Lambert, Ireland). Class I preparation (Black's classification) was made on the right maxillary molar using a handpiece (NSK Ltd., Japan) with a round bur, 0.8 mm in diameter (SS White Diamond) at a low speed, until the pulp chamber was reached. After perforation, the cavity was cleaned with a drop of sterile aquadest solution and dried with cotton pellets.

### Green tea extract preparation

The green tea extract was made using maceration techniques. Crushed dry tea leaves until it becomes a powder weighing 250 g. Green tea leaf powder was added to the macerator, 70% ethanol was added as a solvent in a ratio of 1:10 times the simplicia, namely 2000 ml, then stirred until homogeneous. The mixture was allowed to macerate for 48 hours in a closed macerator with daily stirring. Maserat filtered from the pulp using filter paper. Then evaporated using a rotary evaporator at a temperature of 70°C and a pressure of 80 mBar until a thick extract was obtained (100% concentration).

### Cacao peel extract preparation

Ripe cacao with yellow marks is used in this study. Before processing, the fruit is left for approximately 5 days to facilitate the release of the seeds from the fruit peel. The extraction process of the cacao peel is carried out by maceration. The cacao peel used in this study was fresh peel weighing 6 kg, then cut and aerated. When the peel is half dry then put in the oven with a temperature of 50°C. After drying, 1 kg of milled cacao peel is then macerated with 70% ethanol solvent for 24 hours and then filtered. After filtering the peel, the filtrate and dregs are obtained. The waste obtained is then soaked again after it is filtered again. The maceration and filtering processes occur repeatedly until a clear filtrate is obtained. After obtaining the clear filtrate, ethanol is evaporated using a Rotary Vacuum Evaporator at a temperature of 40°C to obtain a thick brown peel extract. The pure extract was sterilized in an autoclave at a temperature of 121°C in 15 minutes.

### Combination of calcium hydroxide with green tea and cacao peel extract

Calcium hydroxide was obtained by mixing pure calcium hydroxide powder with sterile aquadest at a ratio of 1:1 (0.1 g calcium hydroxide powder and 0.1 mL sterile aquadest). It was then stirred using a cement spatula on a glass slab until it formed a dough-like consistency. The combination of calcium hydroxide and cacao peel extract was used by mixing calcium hydroxide powder and cacao peel extract with a ratio of 1:2 (0.1 g Ca(OH)<sub>2</sub> powder and 0.2 mL cacao peel extract. The combination of calcium hydroxide and green tea extract was used by mixing calcium hydroxide powder and green tea extract with a ratio of 1:2 (0.1 g Ca(OH)<sub>2</sub> powder and 0.2 mL green tea extract (12).

### Application of combination of calcium hydroxide with green tea and cacao peel extract

The 30 rats were divided randomly into 5 groups. All groups were treated with pulp perforation preparation on first molar teeth, given capping materials and filled with dual cured glass ionomer cement (GIC) (Cention N, Ivoclar Vivadent, India) as the final restoration.

Group I : control group. The tooth was given calcium hydroxide combination with sterile aquadest.

Group II : P1 group. The tooth was given cacao peel extract.

Group III : P2 group. The tooth was given the application of calcium hydroxide in combination with cacao peel extract in a ratio of 1 : 1.

Group IV : P3 group. The tooth was given the green tea extract.

Group V : P4 group. The tooth was given the application of calcium hydroxide combined with green tea extract in a ratio of 1: 1.

Of the 5 treatment groups, each was further divided into 2 groups to be observed on the 7th and 28th days. Preparation and staining of Haematoxylin Eosin (HE) to see the number of odontoblast cells and

immunohistochemical examination to observe the expression of collagen type 1, each slide in the field of view with 400x magnification. The results of each calculation are written on a worksheet and the average value per field of view is taken.

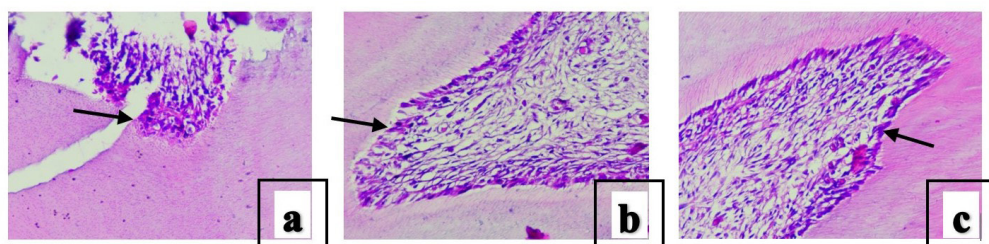
### Statistical Analysis

This is a study that is being conducted as an experiment. Because initial measurement is not practicable, a randomized post-test only control group design was adopted as the research design. The mean and standard deviation of the study's findings were determined and then analyzed using SPSS for Windows. To see if there were any differences between the groups, one-way analysis of variance test was performed and followed with the Tukey's honestly significant difference test with a significance level of 0.05.

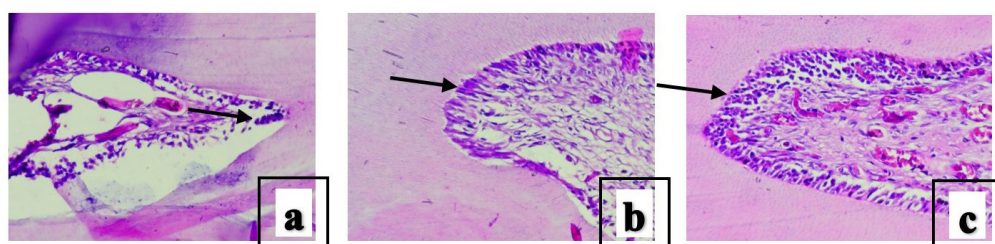
### RESULTS

#### Odontoblast-like Cells (OLC) Expression

A histopathological examination of anatomy in rat dental pulp odontoblast cells showed the morphology of odontoblast-like cells located in the peripheral part of the pulp chamber (Figure 1 and 2). Treatment with a combination of calcium hydroxide and green tea extract (P4 group) showed the highest OLC expression compared with calcium hydroxide and sterile aquadest (control group) ( $p = 0.000$ ), cacao peel extract (P1 group) ( $p = 0.140$ ), calcium hydroxide and cacao peel extract (P2 group) ( $p = 0.649$ ), green tea extract (P3 group) ( $p = 1.000$ ) on day 7 (Figure 3). On day 28, treatment with combination of calcium hydroxide and cacao peel extract (P2 group) showed the highest OLC expression compared with calcium hydroxide and sterile aquadest (control group) ( $p = 0.000$ ), cacao peel extract (P1 group) ( $p = 0.979$ ), green tea extract (P3 group) ( $p = 0.749$ ), and combination calcium hydroxide with green tea extract (P4 group) ( $p = 0.993$ ) (Figure 3).



**Figure 1: Histopathological description of (a) calcium hydroxide control group (b) cacao peel extract group (c) green tea extract group on day 7. Description: black arrows indicate OLC. Magnification 400x.**



**Figure 2: Histopathological description of (a) calcium hydroxide control group (b) cacao peel extract group (c) green tea extract group on day 28. Description: black arrows indicate OLC. Magnification 400x.**

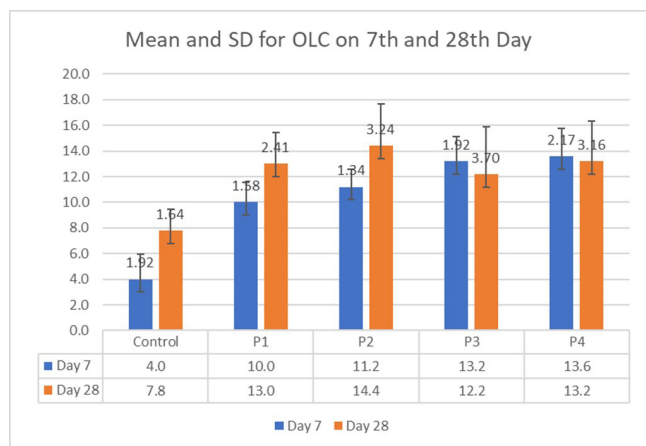


Figure 3: The mean and standard deviation (SD) of OLC expression after being treated with a combination of calcium hydroxide and sterile aquadest (control), cacao peel extract (P1), calcium hydroxide and cacao peel extract (P2), green tea extract (P3), calcium hydroxide and green tea extract (P4).

### Collagen type 1 (COL 1) expression

The collagen type 1 expressed by the pulp odontoblast cells with DAB staining appear as brown discoloration (Figure 4 and 5). Treatment with a combination of calcium hydroxide and green tea extract (P4 group) showed the highest COL 1 expression compared with calcium hydroxide and sterile aquadest (control group) ( $p = 0.000$ ), cacao peel extract (P1 group) ( $p = 0.030$ ), calcium hydroxide and cacao peel extract (P2 group) ( $p = 0.060$ ), green tea extract (P3 group) ( $p = 1.000$ ) on day 7 (Figure 6). On day 28, treatment with combination of green tea extract (P3 group) showed the highest OLC expression compared with calcium hydroxide and sterile aquadest (control group) ( $p = 0.000$ ), cacao peel extract (P1 group) ( $p = 0.998$ ), combination calcium hydroxide and cacao peel extract (P2 group) ( $p = 1.000$ ), and combination calcium hydroxide with green tea extract (P4 group) ( $p = 0.963$ ) (Figure 6).

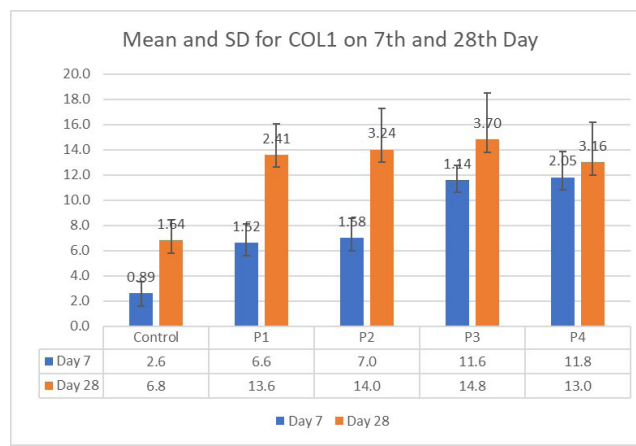


Figure 6: The mean and standard deviation (SD) of COL 1 expression after being treated with a combination of calcium hydroxide and sterile aquadest (control), cacao peel extract (P1), calcium hydroxide and cacao peel extract (P2), green tea extract (P3), calcium hydroxide and green tea extract (P4).

### DISCUSSION

Pulp inflammation is a response to tooth tissue damage caused by caries or trauma. During inflammation, the release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was upregulated, producing excessive intracellular reactive oxygen species (ROS) (14). Excessive oxidative stress will reduce the number of osteoblasts through Nuclear Factor Kappa-light-enhancer of Activated B Cell (NF- $\kappa$ B) and reduce the rate of bone formation through the Wnt/ $\beta$ -catenin transmission line (15). ROS produced by various extracellular inflammatory stimuli activates NF- $\kappa$ B, a transcriptional factor that results in gene expression of proinflammatory-related proteins (16). Flavanol groups such as EGCG function to protect cells from oxidative stress by binding ROS, preventing caspase-3 activation, and increasing antioxidant enzymes such as glutathione peroxidase, glutathione

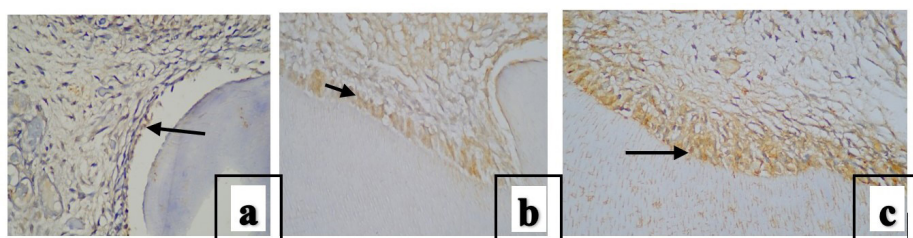


Figure 4: Immunohistochemical description of (a) calcium hydroxide control group (b) group of cacao peel extract (c) green tea extract group on day 7. Description: black arrow indicates COL 1. Magnification 400x.

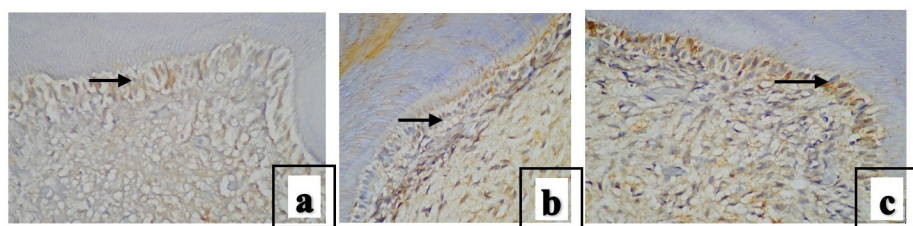


Figure 5: Immunohistochemical description of (a) calcium hydroxide control group (b) group of cacao peel extract (c) green tea extract group on day 28. Description: black arrow indicates COL 1. Magnification 400x.

reductase, and glutathione-S-transferase and can inhibit cyclooxygenase and phospholipase A2 chains, so that they can suppress inflammatory response (17). When the activation of NF- $\kappa$ B is suppressed by antioxidants, it will indirectly produce an anti-inflammatory effect, suppressing the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-alpha) and production of interleukin-6 (IL-6), so that the inflammatory reaction will be better controlled (16). EGCG has a non-polar or hydrophilic characteristics which helps to infiltrate cytoplasm and the cell nucleus by diffusing through protein pore channel in the cell membrane and interacting with intracellular molecules (14). EGCG can also enhance osteogenic differentiation in a dose-dependent manner. The effects of EGCG are similar to those on murine BMSC: increased expression of associated osteogenic genes, including Runx2, BMP2, ALP, COL1 (type 1 collagen), osteonectin and osteocalcin; increased ALP activity; increased fibroblast proliferation, increased collagen and, finally, increased mineralization (13).

The results of this study showed that the highest mean amount of odontoblast count showed that the highest average number of odontoblasts on the 7th day of observation was the treatment group with a combination of calcium hydroxide and green tea extract (P4). Meanwhile, the highest mean odontoblast count was observed on the 28th day in the combination treatment group of calcium hydroxide and cacao peel extract (P2). OLC is the result of differentiation from Human Dental Pulp Stem Cell (hDPSC). Differentiation from hDPSC to OLC is influenced by stimulation of the cytokine TGF  $\beta$  which regulates the expression of DSPP and DMP 1, which play a role in the odontogenic differentiation signaling pathway (18). The highest amount of collagen type 1 (COL I) on the 7th day of observation was the treatment group with combination of calcium hydroxide and green tea extract (P4). Meanwhile, the highest average number of COL I on the 28th day of observation was the green tea extract group (P3). The amount of COL I is influenced by Runx2 which is a transcription factor that can increase the expression of specific osteoblast genes such as COL I, ALP and DSP (18). Runx2 which is responsible for expressing the collagen gene to produce COL I begins to proliferate after 24 hours of injury and the number will increase on the third day. Based on research by Majumdar (18), the number of COL I will increase from the 3rd day and reach its peak on the 7th day (19).

## CONCLUSION

Since combined with calcium hydroxide, the antioxidant qualities of green tea and chocolate may help to reduce inflammation and so improve pulp capping results. When calcium hydroxide was combined with cocoa peel extract, the number of odontoblast-like cells and type I collagen expression were both higher than when

calcium hydroxide was combined with green tea extract. To improve endodontic treatment outcomes, green tea and chocolate can be added.

## ACKNOWLEDGEMENT

The authors would like to thank the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia and staff of Endodontic Division Faculty of Dental Medicine, Universitas Airlangga Surabaya, Indonesia for funding this study.

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