Comparison of the Effect of Calcium Hydroxide Combination with Cocoa Pod Husk Extract and Green Tea Extract on Fibroblast and Alp Activation

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Abstract

To analyze the effect of the combination of calcium hydroxide with cocoa pod husk extract and calcium hydroxide with a combination of green tea extract on fibroblast and Alkaline Phospatase (ALP) activation in mice perforation dental pulp and Immunology.

Sixty upper molars in Wistar rats were perforated mechanically and applied the combination material of pulp capping then divided into five groups. The first group was treated with calcium hydroxide and aqudes, the second group was treated with cocoa pod husk extract, the third group was treated with green tea extrac, the Fourth group was treated with calcium hydroxide and cocoa pod husk extract, the fifth group was treated with calcium hydroxide and green tea extract, then the cavity was restored. Rats from each group were killed after being treated according to a predetermined time by peritoneal injection to see the number of fibroblast cells and ALP activation.

The average value of the number of fibroblasts in group I was lower compared to the other test groups. There were statistically significant differences between groups. between groups I with groups IV and V on day 7 and day 28 with $p < 0.05$. In ALP activation, the average value of ALP activation in group I was lower compared to the other test groups and there were statistically significant differences between groups group 1 and 4 other groups on day 7 and day 28. The expression of ALP in the wistar (rattus norvegicus) rat pulp after administration of calcium hydroxide mixed with green tea extract was higher than administration of calcium hydroxide mixed with cocoa pod extract.

The use of combination calcium hydroxide with green tea extract has been proven to activate more ALP than the combination of calcium hydroxide with cocoa pod husk extract.

Experimental article (J Int Dent Med Res 2021; 14(4): 1465-1471)

Keywords: ALP, Calcium hydroxide, Cocoa pod husk extract-Green tea extract, Fibroblast, Immunology.

Received date: 01 July 2021 **Accept date:** 26 October 2021

Introduction

Dental pulp is a connective tissue that is rich in blood vessels and nerves, located in the cavities of teeth.[1] Pulpal lesions due to mechanical stimulation, trauma and caries can cause pulp tissue to be exposed.[2] Pulp capping is a protective treatment on exposed pulp tissue to maintain the vitality of the pulp tissue, eliminate irritation in the pulp tissue contaminated by bacteria, and stimulate the formation of dentinal bridges.[1]

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Fibroblasts are the most numerous cells in the pulp appearing as specific tissues or cells capable of generating cells that will differentiate into odontoblasts when given the right signal. Fibroblasts make a major contribution in the formation of connective tissue extracellular matrix components, i.e. the synthesis of collagen, elastin, glycosaminoglycans, proteoglycans, and multiadhesive glycoproteins.[3-5] In the healing phase of the pulp, there is an increase in fibroblast activity marked by fibroblast cell proliferation and fibroblasts differentiation into *odontoblast like*-cell. This new odontoblast cell will synthesize the Alkaline Phosphatase (ALP) enzyme to start mineralization. Fibroblast cells will continue to proliferate until reparative dentin is formed. ALP is involved in the initiation stage of tissue mineralization. ALP is an important component of the repair and healing mechanism

of pulp tissue.[6-9]

The most often used material for *pulp capping* maintenance on caries with open pulp is calcium hydroxide Ca $(OH)_2$. Calcium hydroxide is proven to form *dentin bridge* when placed on the pulp tissue. Recent studies have reported that calcium hydroxide shows several disadvantages including liquid necrosis on the pulp surface, the formation of tunnel defects in dentinal bridges that fail to provide hermetic sealing to protect the pulp from repeated infections due to microleakage, high solubility in oral fluid, and degraded.[6] Based on some of the limitations of calcium hydroxide, other alternative materials which can be combined with Ca $(OH)_2$ is needed, so that the antiinflammatory and antimicrobial properties of Ca $(OH)_2$ increase.

Many natural products have been proven beneficial in the healing process. One of the plants found in Indonesia that has the potential as an anti-inflammatory, antioxidant and natural antimicrobial is cacao (*Theobroma Cacao L.*). Cocoa contains polyphenols which are potential natural antioxidants that can modulate the immune system and provide a chemopreventive effect and ward off free radicals. The total phenolic content reaches 611 mg GAE per serving, while the flavonoids reach 564 mg ECE per serving.[7,8,10] 12 Proanthocyanidin is the largest polyphenol group in cocoa, i.e. 58% - 65% of the total cocoa polyphenols.[11,12] Research on the effectiveness of cocoa pod extract with an optimal concentration of 15% in periodontal dressing has been carried out. This research was conducted in vivo on rabbit gingiva to see an increase in the number of fibroblast cells. The active compounds of catechins, tannins, and anthocyanins, the contents of cocoa pod extract, can suppress the number of inflammatory cells and free radicals produced during the inflammatory phase, so that the migration activity and proliferation of fibroblasts occur more quickly.[7]

Besides, tea is also a well-known plant and one of the most consumed beverages in Indonesia. Green tea (*Camellia sinensis*) contains high polyphenols since it has a minimal amount of oxidation processes. The polyphenols contained in green tea are various, especially flavonoids. The main flavonoid in green tea which has an important role is catechins. Catechins have antioxidant activity for their phenol groups. This compound has benefits for the wound healing process, especially in increasing the number of fibroblasts.[13]

Objectives to combine natural medicine with modern medicine by combining calcium hydroxide with cocoa pod extract and calcium hydroxide with green tea extract as pulp capping material. This study examined the effect of administering a combination of green tea extract with calcium hydroxide and a combination of cocoa pod extract with calcium hydroxide on the number of fibroblast cells and the expression of Alkaline Phosphatase (ALP) in the exposed pulp.

Materials and methods

Research Samples

The total sample size of this study was 60 males Rattus novergicus aged 12-16 weeks with a body weight of 200-250 grams. The samples were collapse and divided into 5 groups randomly.

Research Methods

Both Green tea and cocoa pod extracts were prepared at the faculty of Pharmacy, Widya Mandala Catholic University, Surabaya. The green tea extract was made using maceration technique from dry green tea leaves (Rollaas green tea, Yobukita tea, Indonesia). Dry tea leaves were mashed to powder. Green tea leaf powder was put in a macerator, 70% ethanol was added as a solvent in the ratio of 1:10 times (40 grams of simplicia to 400 mL of liquid), then was stirred until homogeneous for 3 days. The mixture was left macerated for 48 hours in a closed macerator for stirring process each day. Macerated mixture was filtered from its pulp using filter paper. Afterwards, it was evaporated using *rotary* evaporator at a temperature of 70°C and pressure of 80 mBar until thick extract was obtained.

Cocoa pod extract was obtained from one kilogram of Forastero type cocoa pod husk from PT. Perkebunan Nusantara XII-Kibuns Kalkaska Banyuwangi. The cleaned cocoa pods sliced to a thickness of about 1-2 mm then dried in the open air for five days. Dried Cocoa pods were grinded to powder. A total of 40 grams of cocoa powder was macerated using a solvent by immersing 400 ml of 70% ethanol in a shaker (Shreeji, India) continuously for 24 hours. The solution was then filtered using Whatmann filter paper no.41, (GE Healthcare Life Science, USA) so that it was macerated. The solvent (ethanol) in the macerate

was evaporated using a rotary vacuum evaporator (Shreeji, India) until an extract with constant weight was obtained.

The tools used were first disinfected with 95% alcohol. All rats were anesthetized with 100 mg ketamine (Ketalar, Warner Lambert, Ireland) (65 mg / kg body weight) and xylazine HCl (Rompun, Bayer, Leverkusen, Germany) dissolved in sterile phosphate buffered saline (PBS), and then all rats were placed on a container. Cleaning and disinfection were carried out on the occlusal tooth surface to be prepared using a cotton pellet which was previously immersed in a 95% alcohol. Preparation of Class I-like cavity was made on the occlusal surface of the right maxillary first molar until it reached the pulp roof using a low speed handpiece with a round diamond bur

(SS White Dental, Lakewood, NJ. Pulp exposure was performed using k-file no. 10. The perforated teeth were rinsed with a saline solution and then dried with a cotton pellet. In this study, the concentration of cocoa pod husk extract was used 3.125% while the concentration of green tea extract was used 0.8%. The extract concentration was determined based on the toxicity test of previous studies.

In group I, calcium hydroxide and aquadets were applied to the pulp surface using sonde. In group II, 0.25 ml cocoa pod extract was applied to the pulp surface using 1 cc syringe. In group III, calcium hydroxide was mixed with cocoa pod extract with a ratio of 1:2 on the surface of the pulp using sonde. In group IV, green tea extract

(0.25ml) was applied to the surface of the pulp using a 1 cc syringe. In group V, calcium hydroxide was mixed with green tea extract in a ratio of 1:2 on the surface of the pulp using a pulp surface using sonde. Then, the cavity was restored with Cention restoration material (Ivoclar Vivadent, Schaan, Liechtenstein). Rats were returned to the cages after being tagged. Rats from each group were eutenized after being treated depend to a predetermined time by peritoneal injection on day 7 and 28.

Histopathological preparations of pulp tissue were observed using a 400x magnification Olympus BX51 light microscope in 10 visual fields on each glass object with a 0.0154 mm diameter field of view, and then counted the number of fibroblast cells by 2 examiners.

Immunohistochemistry (IHC) ALP expression and ALP expression calculations were performed using an Olympus BX51 light microscope. Examination and calculation of ALP expression were observed by using mouse Anti-ALP Antibody monoclonal IgG2a (Santa Cruz Biotechnology) so that the binding of proteins and antibodies can be seen. The examinations were conducted blinded and separately by two examiners.

The Shapiro-Wilk and Levene test were used to find out the distribution the homogeneity of data. Anova was used to test the difference with a significance level of α 0.05. If there is a difference, it was then proceeded with the Tukey HSD test to determine the differences in each group.

Results

Histopathology Observation Results of Pulp Fibroblast Cells

To determine the characteristics of pulp fibroblast cells histopathological examination using a microscope with 400x magnification. Pulpal fibroblast cells are elliptical with a large nucleus. The results seen in five groups of perforated mice were the control group with the application of calcium hydroxide mixed with distilled water; the treatment group with the application of cocoa pod extract; the treatment group with the application of a combination of calcium hydroxide mixed with cocoa pod extract, the treatment group with the application of green tea extract, and the treatment group with the combination application of calcium hydroxide mixed with green tea extract observed on days 7 and 28. Fibroblast cell on day 7 and 28 in each group was presented in the Figure 1 A-E and Figure 2 A-E.

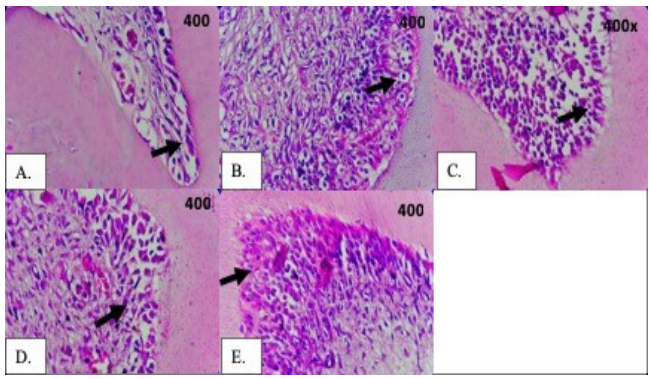

Figure 1. HPA depiction of fibroblast cells in *wistar (Rattus norvegicus)* rat pulp tissue preparation day 7 with a magnification of 400x

(Black arrows indicate fibroblasts). A) in the control group with the application of $Ca(OH)_2$; (B) in groups with the application of cocoa pod extract; (C) in groups with the application of a combination of $Ca(OH)_2$ with cocoa pod extract, (D) in the group with the application of green tea extracts, (E) in the group with the application of a combination of Ca(OH)2 with green tea extracts.

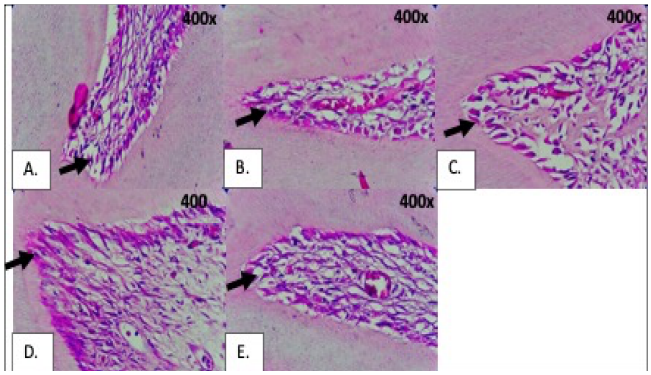

Figure 2. HPA depiction of fibroblast cells in *wistar (Rattus norvegicus)* rat pulp tissue preparation day 28 with a magnification of 400x (Black arrows indicate fibroblasts). A) in the control group with the application of Ca(OH)2; (B) in groups with the application of cocoa pod extract; (C) in groups with the application of a combination of Ca(OH)2 with cocoa pod extract, (D) in the group with the application of green tea extracts, (E) in the group with the application of a combination of Ca(OH)2 with green tea extracts.

The Results of Immunohistochemical Examination

The results of immunohistochemical (IHC) observations to determine the distribution of odontoblast cells expressing ALP show in the Figure 3 A-E and Figure 4 A-E.

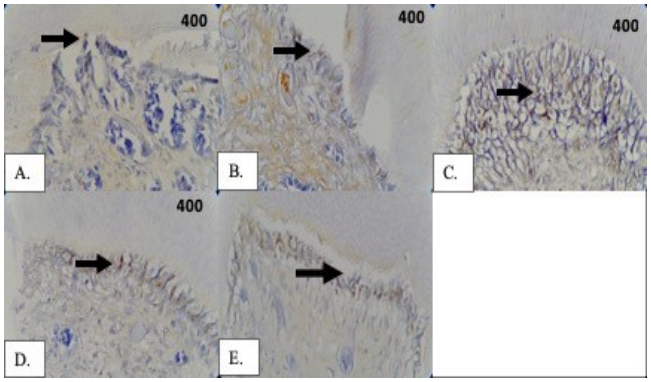

Figure 3. Black arrows indicate Odontoblast cells expressing ALP on day 7 with 400x (A) magnification in the control group with the application of Ca(OH)2; (B) in groups with the application of cocoa pod extract; (C) in groups with the application of a combination of Ca(OH)2 with cocoa pod extract, (D) in the group with the application of green tea extracts, (E) in the group with the application of a combination of Ca (OH) 2 with green tea extracts.

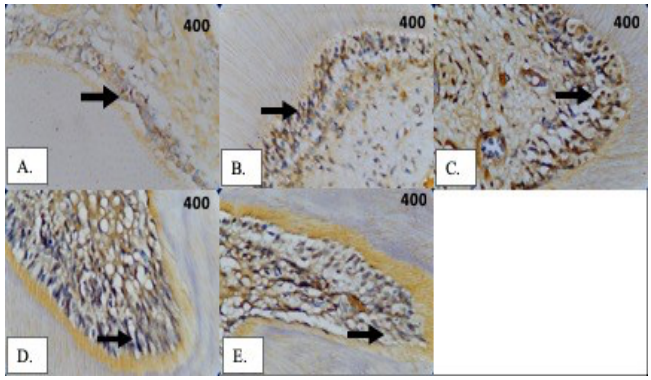

Figure 4. Black arrows indicate Odontoblast cells expressing ALP on day 28 with 400x (A) magnification in the control group with the application of Ca(OH)2; (B) in groups with the application of cocoa pod extract; (C) in groups with the application of a combination of Ca(OH)2 with cocoa pod extract, (D) in the group with the application of green tea extracts, (E) in the group with the application of a combination of Ca (OH) 2 with green tea extracts.

	Day 7		Day 28	
Treatment Group	**N**	**Mean ± SD**	**N**	**Mean ± SD**
I	6	3.50 ± 1.64	6	8.0 ± 3.10
II	6	8.0 ± 4.15	6	11.83 ± 1.94
III	6	6.5 ± 2.88	6	11.83 ± 1.94
IV	6	11.17 ± 2.48	6	13.17 ± 4.02
V	6	9.83 ± 2.04	6	13.50 ± 3.27

Table 1. The results of calculation of the mean value and standard deviation of the number of Fibroblast Cells on the day 7 and 28.

Statistical Analysis of Fibroblast Cell Counts and Alkaline Phosphatase (ALP) Expression

The results of data analysis of differences in the number of fibroblast cells and the expression of Alkaline Phosphatase (ALP) on day 7 and 28 of the 5 treatment groups with 6 N

each can be seen in the table 1-6. The average value of the number of fibroblasts in group I was lower compared to the other test groups and there were significant differences between groups I with groups IV and V on day 7 and day 28 with $p < 0.05$, but there were no significant differences between the other groups with $p > 0.05$. In ALP activation, the average value of ALP activation in group I was lower compared to the other test groups and there was a significant difference between group 1 and 4 other groups on day 7 and day 14.

Treatment Group	I	II	III	IV	V
I					
II	0.066				
III	0.358	0.880			
IV	0.001*	0.306	0.053		
V	0.005*	0.782	0.260	0.918	

Table 2. Difference test among treatment groups using *Tukey Multiple Comparison* on the number of fibroblast cells on day 7.
*there are significant differences (*p-value*<0.05)

Treatment Group	I	II	III	IV	V
I					
II	0.199				
III	0.199	1.000			
IV	0.042*	0.934	0.934		
V	0.027*	0.865	0.865	1.000	

Table 3. Difference test among treatment groups using *Tukey Multiple Comparison* on the number of fibroblast cells on day 28.
*there are significant differences (*p-value*<0.05)

	Day 7		Day 28	
Treatment Group	N	Mean ± SD	N	Mean ± SD
I	6	3.00 ± 1.09	6	4.83 ± 2.48
II	6	9.00 ± 1.41	6	10.83 ± 2.32
III	6	8.83 ± 1.47	6	11.67 ± 2.16
IV	6	13.67 ± 2.64	6	13.67 ± 3.56
V	6	13.67 ± 3.08	6	14.17 ± 3.56

Table 4. The results of the mean and standard deviation of Alkaline Phosphatase (ALP) Expressions on days 7 and 28.

Treatment Group	I	II	III	IV	V
I					
II	0.00*				
III	0.00*	1.00			
IV	0.00*	0.002*	0.001*		
V	0.00*	0.006*	0.004*	0.993	

Table 5. Difference test between groups using *Multiple Comparison* Tukey HSD to Alkaline Phosphatase (ALP) Expression on day 7.
*there are significant differences (*p-value*<0.05)

Treatment Group	I	II	III	IV	V
I					
II	0.011*				
III	0.003*	0.987			
IV	0.00*	0.450	0.750		
V	0.00*	0.450	0.750	1.000	

Table 6. Difference test between groups using *Multiple Comparison* Tukey HSD to Alkaline Phosphatase (ALP) Expression on day 28.
*there are significant differences (*p-value*<0.05).

Discussion

The effectiveness of $Ca(OH)_2$ calcium hydroxide depends on the mixing agent used to be prepared as a paste. This mixing agent plays an important role in the rate of decomposition of ions, so that the rate of penetration of hydroxyl ions into the dentinal tubules is more effective to produce an adequate pH.[14] These hydroxyl ions make the environment alkaline, which helps activate the enzyme alkaline phosphatase (ALP) inducing mineralization tissue, thereby helping the tissue repair process. This calcium hydroxide mixing agent can increase the antibacterial activity of calcium hydroxide.[15-17]

In the calculation of the number of fibroblast cells, the highest average was found in the treatment group of the combination of green tea extract and $Ca(OH)_2$ on day 28 which was equal to 13.5. This is because the high content of EGCG in green tea extract can inhibit the translation of NF-KB into the cell nucleus. A study stated that EGCG inhibits the IKK/NF-KB signal transduction pathway because it inhibits IKK phosphorylation. Thus, IKB inhibition occurs and consequently reduces NF-KB activity and inhibits TNF-B expression resulting in limitation of the number of inflammatory cells that migrate to the injured area.[17] This causes a shorter and more active inflammatory reaction than TGFB. TGFB is the main factor to stimulate proliferation of fibroblasts so that the number of fibroblast cells increases.[18] The inflammatory phase ended on day 3 and then entered the proliferation phase.[19] Fibroblast cells appeared in the injured area after 3 days after injury and reached a peak after day 7. This statement explains the results of research that until day 28, the proliferation of fibroblasts continued. In the inter-group test with *multiple comparisons* shows significant differences between $Ca(OH)_2$ of control group on day 7 of the green tea extract treatment group and a group of combination of green tea extract and Calcium Hydroxide on day 7. There was also a significant difference between the $Ca(OH)_2$ of control group on day 28 towards green tea extract group on day 28 and green tea extract combination group and $Ca(OH)_2$ on day 28. This shows that the green tea extract treatment group whether with or without a combination of Ca $(OH)_2$ had increased fibroblast proliferation on day 7 and 28 compared to the control group. However, there were no significant differences between the groups of cocoa pod extract and green tea extract. This is because green tea extracts and cocoa pod extracts have comparable polyphenol contents so that they have the effect of increasing the number of fibroblast cells that are relatively similar.[20]

The highest average in the ALP expression study was found in the combined treatment group of green tea extract and $Ca(OH)_2$ on day 28, this was because the high EGCG content in green tea extract could increase ALP expression.[21-23] Alkaline phosphatase (ALP) is an enzyme that plays a role in the mineralization process by forming calcium phosphatase sediments in the organic matrix through the release of phosphate ions which react with calcium ions in the bloodstream. Calcium phosphatase sediments are molecular units of hydroxyapatite that play a role in the formation of dentin bridges.[24,25] ALP activation in endodontic cases is usually influenced by the presence of $Ca(OH)_2$ which causes an alkaline atmosphere.[23] This study used observations of the number of ALP on day 7 and 28,because based on research by Williams et al. (2019), ALP activity is very low on the first 7 days and starts to increase after the second week.[9,26]

The results of difference test between treatment groups using multiple comparisons Tukey HSD on ALP expression on days 7 and 28 day show that there were significant differences between the control group $Ca(OH)_2$ towards the treatment group of cocoa pod extract, combination of cocoa pod extract mixed with calcium hydroxide, green tea extract, and a combination of green tea extract mixed with Calcium Hydroxe with varying values of 0.00, 0.011, and 0.003 ($p < 0.05$). Significant differences were also found in the treatment group of cocoa pod extract towards the green tea extract group and the combination group of green tea extract mixed with calcium Hydroxide on day 7. In addition, there were significant differences in the combination group of cocoa pod extract mixed with calcium hydroxide towards the green tea extract group and the combination of green tea extract mixed with calcium hydroxide groups on the same day. This shows that the treatment group of green tea extract and cocoa pod extract whether with or without a combination of Calcium Hydroxide increased ALP expression on days 7 and 28 compared to the control group and the cocoa pod extract group which indicated the healing process of the pulp.[27,28]

However, similar studies conducted by other researchers have not been found, so it is difficult to compare the results of this study with other studies.

Conclusions

There were no statistically significant differences in the number of fibroblast cells in *Wistar (rattus norvegicus)* rat pulp between the administration of calcium hydroxide mixed with green tea extract compared to calcium hydroxide mixed with cocoa pod extract. The expression of ALP in the *wistar (Rattus norvegicus)* rat pulp after administration of a combination of calcium hydroxide with green tea extract was higher than administration of a combination of calcium hydroxide with cocoa pod extract.

Declaration of Interest

The authors declare that there are no conflicts of interest.

Ethical policy and institutional review

All procedures performed in this study was obtained ethical eligibility from the Faculty of Dentistry Ethics of Airlangga University No. 063/HRECC.FODM/II/2019.

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