ORIGINAL RESEARCH

Effect of Calcium Hydroxide Combinations with Green Tea Extract and Cocoa Pod Husk Extract on p38 MAPK and Reparative Dentine

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Abstract

Aim and objective: The aim of this research is to analyze the effect of calcium hydroxide combinations with green tea extract and the combination of calcium hydroxide with cocoa pod husk extract on the activation of p38 MAPK and wide area of reparative dentin in mice dental.

Materials and methods: This study used 36 rats that were randomly divided into three treatment groups: positive control group was applied calcium hydroxide and aquades (group I), the test group was applied calcium hydroxide combined with cocoa pod husk extract (group II), and the next test group was applied using calcium hydroxide combined with green tea extract (group III); all the cavities were restored with RMGIC. On day 7 and 28, experimental animals from each treatment group were killed by peritoneal injection to see the activation of p38 MAPK, while reparative dentin was only seen on day 28.

Results: The result of data analysis using Multiple Comparison Tukey HSD test showed significant difference between the positive control group and the test groups for the average p38 MAPK activation value on day 7 and 28. But there was no significant difference between two test groups. The same thing was obtained in the calculation of the average area of reparative dentin, where group I had the lowest value compared to groups II and III on day 28 with a significant difference. There was no significant difference between groups II and III.

Conclusion: The use of combination calcium hydroxide with green tea extract and combination calcium hydroxide with cocoa pod husk extract have significant effect on p38 MAPK activation and wide area of reparative dentin in mice dental.

Clinical significance: The use of combination calcium hydroxide with green tea extract and combination calcium hydroxide with cocoa pod husk extract have been proven to activate more p38 and form a wider reparative dentin.

Keywords: Calcium hydroxide, Cocoa pod husk extract, Green tea extract, p38 MAPK, Pulp capping direct, Reparative dentin.

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Introduction

Dental pulp can be exposed to stimuli from the outside, such as caries or trauma. The response to the higher intensity of tissue damage such as deep carious lesions can cause odontoblast cell death and loss of dentin tissue beneath the lesion, resulting in exposure to pulp tissue.[1]

The formation of hard tissue by odontoblasts, fibroblasts, and pulp cells is an important protective response to external stimuli.[2] Cellular response due to extracellular stimulation is mediated through marker pathways such as mitogen-activated protein kinase (MAPK). p38 from mammals show a similar role, and activation has shown its emergence in response to extracellular stimuli.[3]

Direct pulp capping is a treatment in dental conservation involving the placement of biocompatible materials on the pulp tissue that is accidentally exposed due to trauma or iatrogenic factors.[4–6]

Calcium hydroxide [$Ca(OH)_2$] has become the standard of therapy as an ingredient of pulp capping for a long time. The advantages of calcium hydroxide is that it has excellent antimicrobial properties.[5] But calcium hydroxide has also some disadvantages, including the absence of adaptation to dentin, did not increase odontoblast differentiation consistently, and has shown cytotoxic reactions in cell culture, which results in the presence of tunnel defect on the formed reparative dentin matrix.[5,7,8]

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Cocoa pods have potential as natural, anti-inflammatory, antioxidant, and antimicrobial properties because they contain polyphenols in the form of flavonoids or condensed tannins.[9,10] Proanthocyanidin is the largest polyphenol group in cocoa.[11,12] The total phenolic content of cocoa pods was significantly higher than other parts of the cocoa.[13] The role of polyphenols in inflammatory response is done through inhibition of the nuclear factor kappa B (NF-kB) and the MAPK signaling pathway.[14] Some previous studies have proven that Cocoa polyphenols significantly prevented tumor necrosis factor α (TNF-α)-induced NF-kB translocation. The induction of NF-kB by TNF-α is partially mediated by members of the MAPK family. The cocoa polyphenols induced the activation and

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increased phosphorylation of ERK and p38 but strongly inhibited the activation of JNK, MEK-1, and P13K.[15–17]

Green tea is considered a food source rich in antioxidants because it is rich in polyphenols. The largest polyphenol group in green tea that provides a powerful antioxidant effect is epigallocatechin gallate (EGCG). EGCG is able to reduce the expression of proinflammatory cytokines and reduce TLR4 so that there is a decrease in the expression of TNF-α and free radicals that can oxidize tissue so as to prevent damage to cell membranes.[14]

The aim of this research is to analyze the effect of calcium hydroxide combinations with green tea extract and the combination of calcium hydroxide with cocoa pod husk extract on the activation of p38 MAPK and wide area of reparative dentin in mice dental.

Materials and Methods

The total sample size of the mice used in this study was 36 white male Wistar strain rats (*Rattus novergicus*) aged 12–15 weeks and weighing 200–250 g. Then, samples were selected and divided into three groups randomly. The sample size in each group was 12 white male Wistar strain rats. All procedures performed in this study was obtained ethical eligibility from the Faculty of Dentistry Ethics of Airlangga University No. 052/HRECC.FODM/II/2019.

Green tea extract preparation was performed at the faculty of Pharmacy, Widya Mandala Chatolic University, Surabaya. The extract was derived from dry green tea leaves (Rollaas green tea, Yobukita tea, Indonesia) by maceration technique using 70% ethanol (Medis, Indonesia).

Next, 200 g dry green tea leaves (Rollaas green tea, Yobukita tea, Indonesia) was grinded to become powder. Ethanol of 70% was added until the powder was submerged for 3 days, shaken, and stirred. The solution was then filtered using filter paper (Whatmann filter paper 1; GE Healthcare Life Science, USA), and then the extract was evaporated using a rotary vacuum evaporator (Shreeji, India) until a thick green tea extract with a weight of 33 g was obtained. The extract was stored in a closed container (Fig. 1).

Cocoa pod husk extract was prepared at the faculty of Pharmacy, Widya Mandala Chatolic University, Surabaya. The extract was derived from 1 kg of Forastero type cocoa pod husk by maceration teqhnique using 70% ethanol (Medis, Indonesia).

First, cocoa fruit was dried for 5 days and then separated from the seeds. Cocoa pod was cleaned and then sliced with a thickness of about 1–2 mm, then put into an oven (WTC Bindder Oven FD 53; Tuttlingen, Germany) at the temperature of 50°C for 24 hours. Cocoa pod that has been dried was grinded to become powder. Ethanol of 70% was added until the powder was submerged for 3 days, shaken, and stirred. The solution was then filtered using filter paper (Whatmann filter paper 1; GE Healthcare Life Science, USA), and then the extract was evaporated using a rotary vacuum evaporator (Shreeji, India) until a thick chocolate extract with a weight of 101 g was obtained. The extract was stored in a closed container (Fig. 2).

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). Mice were placed on a container.

The occlusal surface of the tooth to be prepared was disinfected and cleaned with cotton pellet previously dipped in 95% alcohol solution. Class I cavity preparation (Black classification) was made on the occlusal surface of the maxillary right first molars using low-speed handpiece with round diamond bur (SS White Dental, Lakewood, NJ) to reach the pulp roof. The depth of preparation was estimated to be as large as a bur head. The perforation action on the pulp chamber was done using a k-file no.8. After perforation, the cavity was rinsed with saline solution and dried with cotton pellet.

In group I, $Ca(OH)_2$ was combined with aquades in a ratio of 1:1; in group II, $Ca(OH)_2$ was combined with cocoa pod husk extract in a ratio of 1:2; and in group III, $Ca(OH)_2$ was combined with green tea extract in a ratio of 1:2. The material was applied on the surface of the pulp and then the cavity was restored with Cention N (Ivoclar Vivadent, Schaan, Liechtenstein). Rats were placed in cages and provided with standard food and water.

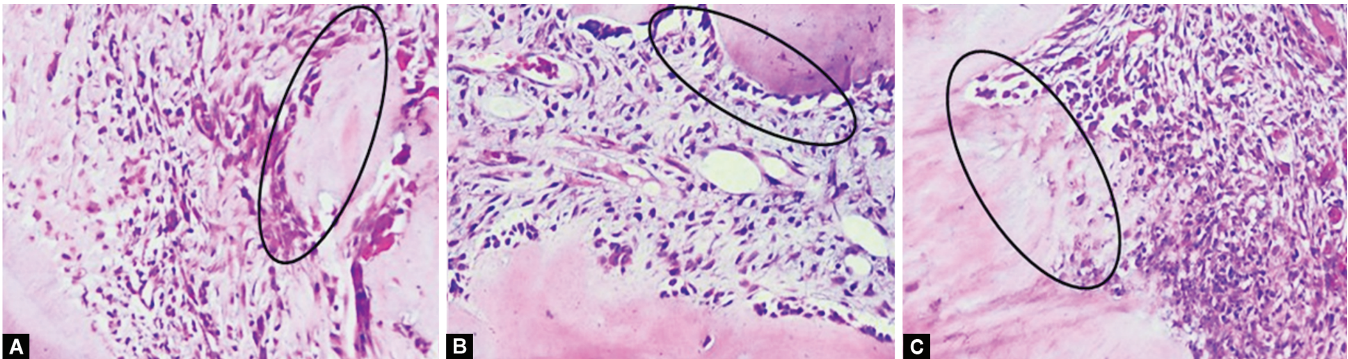
Experimental animals from each treatment group were killed by peritoneal injection according to the time determined after treatment. Twelve mice were killed on day 7 to see activation of p38 with 4 mice in each group. On day 28, 12 mice were killed to see activation of p38 with 4 mice in each group, and 12 other mice were killed on day 28 to see reparative dentin also with 4 mice in each group.

After removal or decapitation, the jawbone in the interdental area of the maxillary right first molar was obtained. Histological preparation of ±6 μm thickness parallel to the long axis of the tooth was obtained. In this study, two slides obtained from each specimen.

Histopathological preparations of the pulp tissue were observed using a 400× magnification Olympus BX51 light microscope with a 10× division of the visual field to see the area of reparative dentin formed. Reparative dentin area measurement (dentin bridge) used Image Raster 4.1 software (OptiLab).

Figs 1A to C: (A) Dry green tea leaves; (B) Filtered process of green tea extract; (c) Green tea extract

Figs 2A to D: (A) Cocoa pod husk was separated from the beans; (B) Cocoa pods washed; (C) Thinly sliced cocoa pod husk and then baked in to the oven; (D) Cocoa pod husk extract



Figs 3A to C: (A) Histologic features demonstrating dentin bridge formed on day 28th in positive control group; (B) $Ca(OH)_2$ combined with cocoa pod husk extract group; (C) $Ca(OH)_2$ combined with green tea extract group

The preparations were stained using the hematoxylin–eosin technique, and photos were taken at 200× magnification of the microscope, 10× visual field. Dentin bridge measurement used the "multiline" mode according to the flow from dentin bridge surrounding until two points meet. The measurement results obtained was then tabulated. The measurement results were then analyzed using a different test (average) with the analysis of variance (ANOVA) on the SPSS software ver. 21. (Pizem, J., Cor, A., 2003).

Immunohistochemical (IHC) observation on p38 MAPK activation calculations were carried out using antibodies rabbit anti p-p38 MAPK monoclonal antibody (Cell Signaling Technology; Isotype IgG) so that the binding of proteins and antibodies can be seen. The enzyme was then reacted with a chromogen substrate that can be observed with a light microscope. For calculation purposes, the code slide was closed and randomly given a new number. So the examiner was blinded to the group sample slides. Inspections were carried out separately by two examiners.

This was an experimental research. Randomized posttest only control group design was used as research design, since the initial measurements were not possible. The results of this study were calculated as a mean and standard deviation. Shapiro-Wilk test was used to find out the distribution of data less than 50 and proceed with homogeneity test. To test the difference, ANOVA was used with a significance level of α of 0.05 which was then followed by the Tukey test.

Results

Histologic features that showed the formation of reparative dentine on the day 28 in each group are presented in the Figure 3. The average area of reparative dentin area in each group was 14.66667 μm^2 for group I, 23.66667 μm^2 for group II, and 25.00000 μm^2 for group III (Fig. 4).

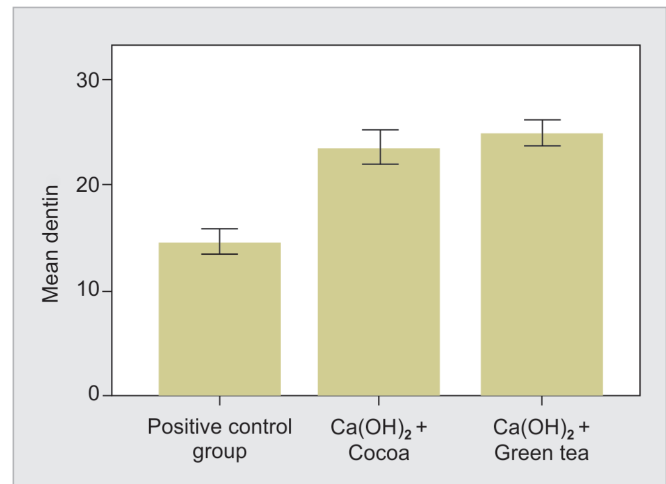


Fig. 4: Graph of mean value of dentin bridge calculation on day 28th

The result of the ANOVA on the reparative dentin formation (Table 1) revealed the significant difference among the sample group with significance value 0.000 (p value < 0.05).

Based on reparative dentin formation, the sample of group III have significant difference compared to group I with significance value 0.000 (p value < 0.05). The sample of group II also showed significant difference when compared to group I with significance

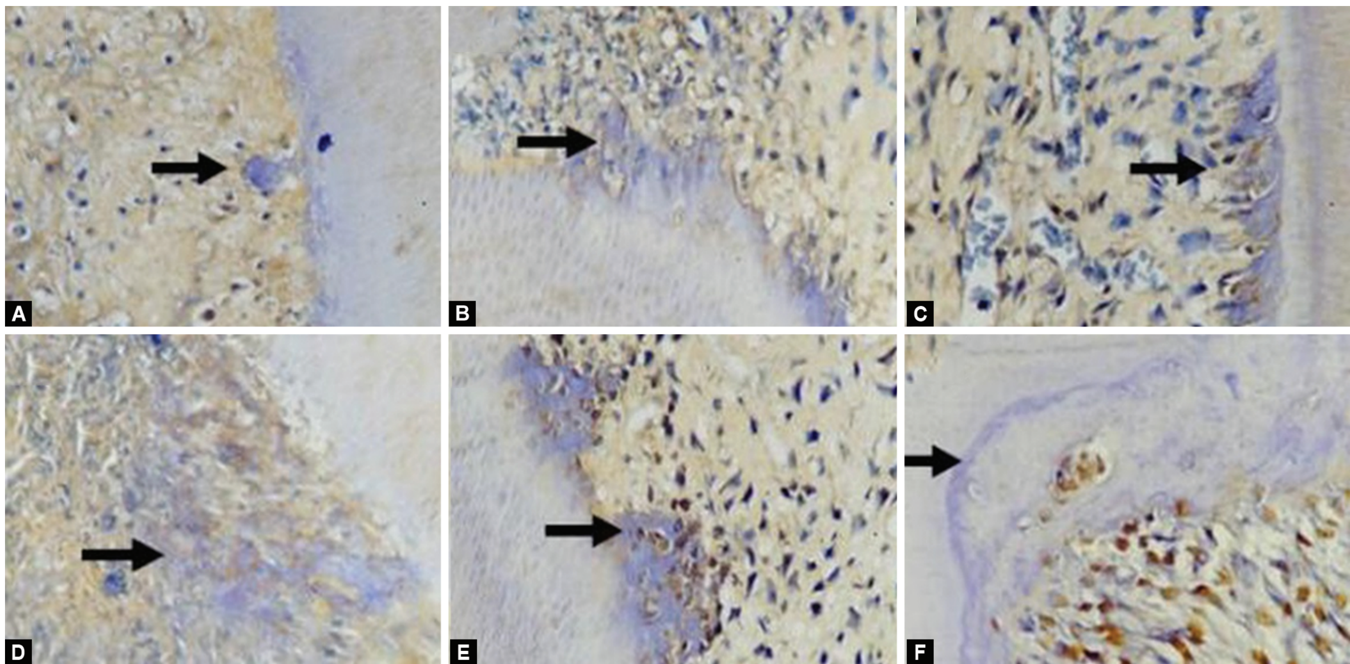
Table 1: Result of ANOVA on the reparative dentin formation

	Sum of squares	*df*	*Mean square*	*F*	*Sig.*
Between groups	4390.222	5	878.044	158.048	0.000
Within groups	166.667	30	5.556		
Total	4556.889	35			

Table 2: Result of Tukey HSD test on calculations of dentin bridge on day 28th

Treatment groups	*Positive control*	*$Ca(OH)_2$ + cocoa pod husk extract*	*$Ca(OH)_2$ + green tea extract*
Positive control	–	–	–
$Ca(OH)_2$ + cocoa pod husk extract	0.000*	–	–
$Ca(OH)_2$ − green tea extract	0.000*	0.921	–

*There are significant differences (*p* value < 0.05)



Figs 5A to F: IHC detection of p38 MAPK. Brown dots indicate the activated of p38, while the blue line shows candidate dentin bridge on day 7th (arrow): (A) Positive control group; (B) Cocoa group; (C) Tea group. Dentin bridge formed on day 28th (arrow): (D) Positive control group; (E) Cocoa group; (F) Tea group

value 0.000 (*p* value < 0.05). But there was no significant difference between group II and group III, with significance value of 0.921 (Table 2).

IHC detection of p38 MAPK on day 7 and day 28 can be seen in Figure 5. Brown dots indicate p38 activated that enters into the nucleus, while the blue line shows candidate dentin bridge on day 7 (arrow) and dentin bridge formed on day 28 (arrow). The average of p38 activation on day 7 in each group was 3.50 for the group I; 10.17 for the group II; and 12.50 for the group III. On day 28, the average of p38 activation in each group was 6.33 for the group I; 11.67 for group II; and 12.83 for group III (Fig. 6).

The result of the ANOVA on p38 activation (Table 3) revealed the significant difference among the sample group with significance value 0.000 (*p* value < 0.05).

Based on p38 activation on day 7, the sample of group III has significant difference compared to group I with significance value 0.001 (*p* value < 0.05). The sample of group II also showed significant difference when compared to group I with significance value 0.011 (*p* value < 0.05). But there was no significant difference between group III and group II, with significance value 0.964 (Table 4).

Based on p38 activation on day 28, the sample of group III have significant difference compared to group I with significance value 0.000 (*p* value < 0.05). The sample of group II also showed significant difference when compared to group I with significance value 0.000 (*p* value < 0.05). But there was no significant difference between group III compared to group II. The significance value for this pair was 0.118 (Table 5).

The inference of this study reported the combination of calcium hydroxide with green tea extract, and the combination of calcium hydroxide with cocoa pod husk extract have the significant effect on the activation of p38 MAPK and wide area of reparative dentin in mice dental.

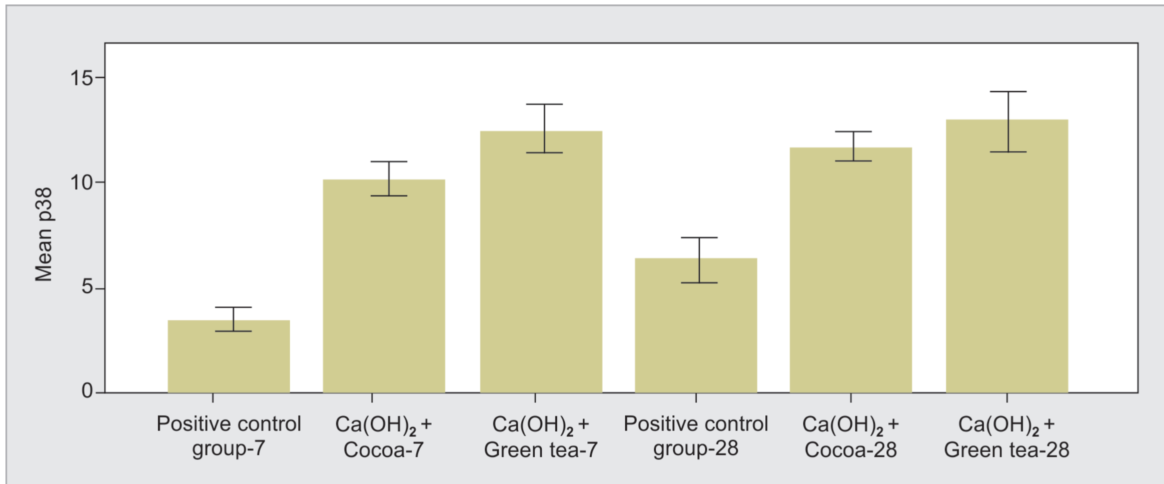


Fig. 6: Graph of mean value of p38 calculation on day 7th and 28th

Table 3: Result of ANOVA on p38 MAPK activation

	Sum of squares	*df*	*Mean square*	*F*	*Sig.*
Between groups	427.667	5	85.533	13.553	0.000
Within groups	189.333	30	6.311		
Total	617.000	35			

Table 4: Result of Tukey HSD test on activation p38 on day 7th

Treatment groups	*Positive control*	*$Ca(OH)_2$ + cocoa pod husk extract*	*$Ca(OH)_2$ + green tea extract*
Positive control	–	–	–
$Ca(OH)_2$ + cocoa pod husk extract	0.011*	–	–
$Ca(OH)_2$ + green tea extract	0.001*	0.964	–

*There are significant differences (*p* value < 0.05)

Table 5: Result of Tukey HSD test on activation p38 on day 28th

Treatment groups	*Positive control*	*$Ca(OH)_2$ + cocoa pod husk extract*	*$Ca(OH)_2$ + green tea extract*
Positive control	–	–	–
$Ca(OH)_2$ + cocoa pod husk extract	0.000*	–	–
$Ca(OH)_2$ + green tea extract	0.000*	0.118	–

*There are significant differences (*p* value < 0.05)

Discussion

Referring to the mean of reparative dentin area on day 28, the control group shows the lowest mean value compared to the treatment groups, given a combination of $Ca(OH)_2$ with cocoa pod husk extract and combination of $Ca(OH)_2$ with green tea extract. This happens because calcium hydroxide has a deficiency as a pulp-capping agent. Calcium hydroxide directly applied to the open pulp will cause superficial necrosis coagulation, slight irritation to the underlying tissue, tunnel defect, and inadequate quality of the dentin formed.

Referring to the difference test on day 28, there was a significant difference between the mean values of the reparative dentin area in the positive control group and the two treatment groups with p value < 0.05; however, there were no significant differences between the combination of $Ca(OH)_2$ with cocoa cocoa pod husk extract and combination of $Ca(OH)_2$ with green tea extract with p value = 0.921.

The combination of $Ca(OH)_2$ with cocoa pod husk extract and combination of $Ca(OH)_2$ with green tea extract used as an pulp capping material can show better reparative dentin formation than $Ca(OH)_2$ combined with aquades.

This occurred because proanthocyanidin contained in cocoa pod husk extract and EGCG contained in green tea extract as the largest polyphenol component has anti-inflammatory and antioxidant ability that can bind to free radicals, namely, hydroxyl ions (OH-) derived from $Ca(OH)_2$, so as to prevent lipid peroxidation and cell death. Proanthocyanidin and EGCG will support the dissociation of the Nrf2/Keap1 complex which will produce inhibitory effects on NF-kB activity and promote activation of various antioxidant enzymes/proteins that will reduce oxidative stress. This finding also proves that green tea extract and cocoa pod husk extract can be applied in the treatment of pulp capping direct.

Procyanidins BI and B3 and (+) - Catechins found in cocoa pods showed that these polyphenols were able to trap hydrophilic peroxyl radicals *in vitro*, and radical cleaning activity increased with

polyphenol concentrations. In addition, the antioxidant activity of polyphenols has been demonstrated *in vitro* to prevent lipid peroxidation, a type of cell injury induced by ROS and oxidation of low-density lipoproteins (LDL).[18] Phenolic compounds are reported to be able to react with reactive oxygen compounds; this is due to one or two hydroxyl groups in the aromatic ring that can act as hydrogen donors.[19]

On the activation of p38 MAPK, there were significant differences between the positive control group with both treatment groups on the day 7 and 28 with p value < 0.05; however, there was no significant difference between the two treatment groups with p value $= 0.964$ (day 7th) and p value $= 0.118$ (day 28th).

This occurred because the cocoa pods husk extract and green tea extracts have the main content of polyphenols that play a role through three pathways, namely, through MAPK regulation, inhibition of the NF-kB pathway, and inhibition through cyclooxygenase and lipoxygenase. EGCG and proanthocyanidin have been shown to have anti-inflammatory and antioxidant effects. EGCG is able to reduce the expression of proinflammatory cytokines and decrease TLR4 so that there is a decrease in the expression of TNF-α and free radicals that can oxidize tissues so as to prevent damage to cell membranes.[14]

The increased phosphorylation and activation of p38 MAPK by polyphenols have been reported in several previous studies. Previous study reported the anti-inflammatory effect of cocoa polyphenol in the colon of rats shown by reduced both the phosphorylation of JNK and the nuclear translocation of NF-kB induced by TNF-α, and induced the activation of ERK and p38.[16] The other previous study reported that cocoa polyphenol suppressed TNF-α induced phosphorylation of Akt, p70S6K, ERK, p90 kDa ribosomal S6 kinase (p90RSK), mitogen-activated protein kinase kinase 4 (MKK4), and c-Jun N-terminal kinase (JNK) and increased the phosphorylation of p38 in mouse epidermal cell.[17] An *in vitro* study reported that EGCG inhibits human villous trophoblast growth through increase in activities of ERK MAPK, p38 MAPK, and MAPK proteins.[20]

The p38 MAPK signaling pathway is involved in many fundamental cellular processes, including proliferation, differentiation, motility, apoptosis, and survival. Some previous studies have reported that the p38 MAPK signaling pathway is instrumental in human dental pulp cell migration and dentinogenesis.[21–23]

The insignificant difference between the green tea extract group and the cocoa extract group might be due to the fact that both of them have a large enough polyphenol content and both have anti-inflammatory, antioxidant, and immunomodulatory effects. Therefore, both groups can reduce the effects of inflammation while scavenging free radicals and reduce oxidative stress that occurs.

Based on the duration of p38 MAPK application in each treatment group, it was found that there was no significant difference in the observations of each group both control and treatment on day 7 compared to day 28. This can occur because the p38 signaling pathway plays an important role in the repair response rather than dentin-like complexes during the tertiary dentinogenetic process (in this case reparative dentin).[24]

The limitation in this study is the lack of a treatment group to know the extent of the effects cocoa pod husk extract and green tea extract without addition of calcium hydroxide on dentin reparative and p38 activation.

However, so far the researchers have not gotten any similar studies conducted by other researchers, so it is difficult to compare the results obtained in this study with other studies.

Conclusion

The use of combination calcium hydroxide with green tea extract and combination calcium hydroxide with cocoa pod husk extract have significant effect on p38 MAPK activation and wide area of reparative dentin in mice dental. Further research about effectiveness of green tea extract and cocoa pod husk extract on p38 MAPK activation and reparative dentin formation without the combination of calcium hydroxide is required.

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