The Effect of Addition Cacao Pod Shell and Green Tea for Pulp Capping Materials on Reducing Malondialdehyde and Apoptosis Expressions

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Abstract

Calcium Hydroxide Ca(OH)2 has been used since years ago as pulp capping material in inducing reparative dentin for pulp complex preservation. Calcium Hydroxide has effect by *stimulating* mineralization and inhibiting bacterial growth. However, Ca(OH)2 also has shortcomings which theoretically could be boosted by high polyphenol level in extract of green tea and extract of cacao pod shell which is effective as antibacterial, anti-inflammation and antioxidant. The addition of green tea extract and cacao pod shell extract with Ca(OH)2 are supposed to improve physical properties of Ca(OH)2.

This study expected to compare the reduction of malondialdehyde and odontoblast apoptosis expressions as the effects of addition green tea extract and cacao pod shell extract with Ca(OH)2 on exposed dental pulp.

Thirty six rats were divided to 3 groups: The positive control group was given Ca(OH)2 and aquadest, group II was given cacao pod shell with Ca(OH)2, groups III was given extract of green tea with Ca(OH)2. On the third and seventh day an incision was made and examined to check the malondialdehyde and odontoblast cells apoptosis expressions. Data analysis result using Tukey HSD test , the Data showed there were significant differences between each groups.

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Introduction

The inflammatory response begins when microorganisms and their byproducts irritate the pulp. This inflammation response to injury through the expressions of several cytokines, including TNF-? (Tumor Necrosis Alpha), interferon-? (IFN-?), Interleukin 6 (IL-6), Interleukin 1 (IL-1). These inflammatory conditions can also increase the production of reactive oxygen species (ROS), which induces oxidative stress in cells and lead to worse condition which lead to cell apoptosis.[1]

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ROS will initiate lipid peroxidation reactions in cell membranes which are composed of polyunsaturated fatty acids (PUFA) protein structures where these structures are very sensitive to free radicals reaction.[2] Free radicals have been measured by various methode. One of these method by measured Malondialdehyde (MDA) levels which reflect free radical production.3The chemically stable nature of MDA makes this compound more often used as a biomarker of oxidative stress than other compounds.[3]

Increased ROS affected the integrity of the mitochondrial membrane which can induced apoptosis. Apoptosis is induced when the Bcl2 family proapoptotic proteins cause the opening of mitochondrial permeability transition pore and proapoptotic proteins into cytoplasm by interacting with apoptotic protease-activating factor 1 (Apaf-1) to CARD (forms Caspase Recruitment Domain). Several CARDs merge to

form an apoptotic cell and activate caspase 9, and then activate caspase 3 which is an effector caspase that induced apoptosis.[4]

Utilize natural resources that have antioxidant properties such as cacao pod shell and green tea. According to Yuwono et al. (2019) cacao pod shell contains flavonoid compounds, natural antioxidant compounds such as tannins (proanthocyanidins) 58%, catechins 37%, anthocyanins 4%, and quercetin.5 Likewise, green tea is known for its benefits as a source of natural antioxidants. Green tea contains of catechins (flavan-3-ol) belonging to the group of flavonoids such as: epigallocatechin 3 gallate (EGCG), epigallatocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC). 6 Flavonoid compounds can reduce the formation of ROS levels by capturing ROS and inhibiting the formation of cytokines proinflammatory, thereby preventing inflammatory mediator reactions, reducers of accumulated of nitric oxide (NO) or reactive oxygen species (ROS).7

Studied by Fitriani et al., (2019) regarding the cytotoxicity test of cacao pod shell extract against BHK-21 fibroblast cells found that concentrations of 6.25%, 12.5%, 25%, 50%, and 100% were toxic because the percentage of dead cells was more than 50 %, while at a concentration of 3.12% it was non-toxic because the percentage of dead cells was less than 50%.8.Based on previous studies, cacao pod shell extract with a concentration of 3.12% and green tea extract with a concentration of 0.8% (8mg/ml is equivalent to 8000ppm, then converted in percentage to 8000/10000%) proved to be non-toxic to fibroblast cells and have anti inflamatory ability.9

Based on the description above, the presence of flavonoid compounds in cacao pod shell and green tea combined with Ca(OH)2 as a pulp capping material is expected to reduce ROS levels and suppress the number of odontoblast cell apoptosis. According to Duggal and Singh (2015) stated that from a number of studies on the analysis of apoptosis, it appears that the initial phase of apoptosis varies between 0-3 days while the peak of apoptosis varies between 4-7 days.10 Another study by Dou et al. (2020) regarding the test of Ca(OH)2 as a pulp capping material on the viability, proliferation, and apoptosis of human dental pulp cells, it was found that the peak apoptotic rate occurred on day-3.11 Thus, the authors wanted to conduct a study on the differences in the decrease in MDA expression and apoptosis of odontoblast cells after the application of Ca(OH)2 combined with 3.12% cacao shell extract compared to Ca(OH)2 combined with 0.8% green tea as a pulp capping materials.

Materials and methods

Research Sample

Thirty six male Rattus norvegicus (wistar rats) with average body weight 200-300 grams aged 2-3 months.

Research Methods

The samples distributed into three treatment groups in random; The first group (groupI) is control group which given $Ca(OH)_2$ and aquadest, the second (groupII) was given cacao pod shell with $Ca(OH)_2$, the third group (groupIII) was given extract of green tea with $Ca(OH)_2$ and filled all cavities with RMGIC after application pulp capping materials. On the third and seventh day rats from each groups terminated by peritoneal injection and examined to check the malondialdehyde and odontoblast cells apoptosis expressions.

Results

The results of IHC observations of malondialdehyde expression in odontoblast cells in each group were as follows:

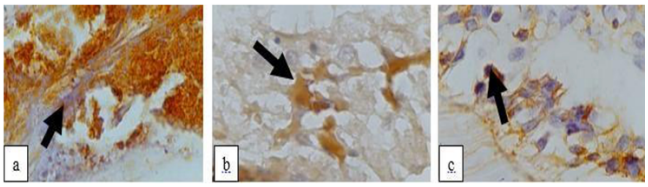

Figure 1. IHC observations of malondialdehyde expressions on day-3 (1000x magnification) shown with brownish colored cells; (a) $Ca(OH)_2$ + aquadest, (b) $Ca(OH)_2$ with extract of cacao pod shell, (c) $Ca(OH)_2$ with green tea extract.

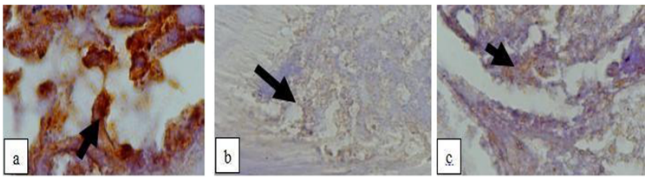

Figure 2. IHC observations of malondialdehyde expression on day-7 (1000x magnification) shown with brownish colored cells; (a) $Ca(OH)_2$ + aquadest, (b) $Ca(OH)_2$ with extract of cacao pod

shell, (c) $Ca(OH)_2$ with green tea extract.

Table 1 shows that the number of sample from each group is 6. The smallest mean of MDA expression is group III, $Ca(OH)_2$ with green tea extract, on day-7.

No	Group	Observation day	n	Mean	Std Deviation
1	$Ca(OH)_2$ with aquadest	3	6	10,67	1,63
		7	6	6,50	1,51
2	$Ca(OH)_2$ with cacao pod shell	3	6	6,83	2,48
		7	6	5,83	2,13
3	$Ca(OH)_2$ with green tea	3	6	6,16	1,47
		7	6	5,50	1,87

Table 1. Sample, Mean, dan Standard deviation of MDA Expression.

To determine the difference in MDA expression between groups, the ANOVA test was performed. The results of the ANOVA test for MDA expression on day-3 obtained p = 0,002 (p< 0,05). This indicates that there is a significant difference in MDA expression between groups on day-3. While the results of the ANOVA test for MDA expression on day-7 obtained p = 0,646 (p > 0,05). This indicates that there is no significant difference in MDA expression between the groups on the day-7. To identify differences between groups I,II and III, Tukey HSD tests were carried out. The result of the Tukey HSD test can be seen in table 2 and table 3 below.

	$Ca(OH)_2$ with aquadest	$Ca(OH)_2$ with cacao pod shell	$OH)_2$ with green tea
$Ca(OH)_2$ with aquadest		p = 0,009*	p = 0,003*
$Ca(OH)_2$ with cacao pod shell			p = 0,821
$Ca(OH)_2$ with green tea			

Table 2. Tukey HSD test of MDA expression on day-3.

From Table 2 above showed that the MDA expression on day-3 between group 1 with group II and group I with group III had p value < 0,05. This data means that there is a significant difference between group I with group II and group I with group III. Meanwhile, the MDA expression on day-3 between the group II and group III had p value > 0,05. This means that there is no significant difference between the group II and group II.

	$(OH)_2$ with aquadest	$Ca(OH)_2$ with cacao pod shell	$(OH)_2$ with green tea
$Ca(OH)_2$ with aquadest		p = 0,811	p = 0,629
$Ca(OH)_2$ with cacao pod shell			p = 0,948
$Ca(OH)_2$ with green tea			

Table 3. Tukey HSD test of MDA expression on day-7.

From table 3 above, it can be seen that on the day-7 MDA expression in all between groups has p value > 0,05. This data showed that there is insignificant difference in all between each groups.

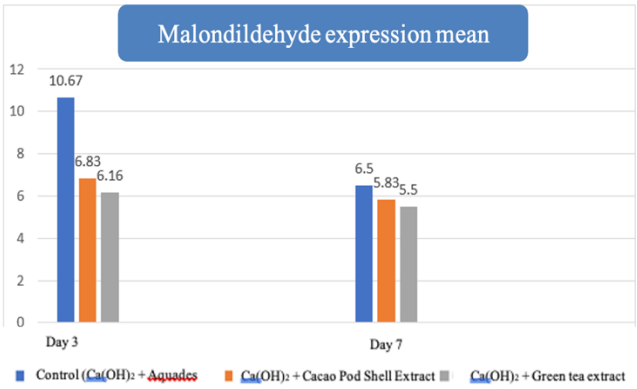

Figure 3. Mean of MDA expression all groups on the day-3 and day-7

Figure 3 significant difference was shown in the decrease of malondialdehyde expression in the control group, but group II and group III have lower mean malondialdehyde expression compared to the group I or control group on day 3 and the results of the TUNEL Assay observations of odontoblast cell apoptosis expression in each group are as follows:

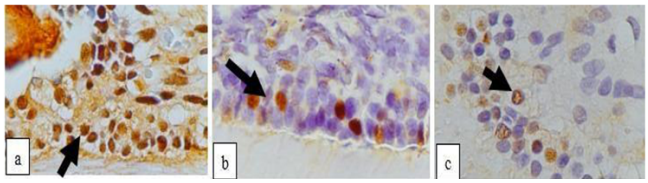

Figure 4. TUNEL Assay observations of apoptosis expression on day-3 (1000x magnification) shown with brownish colored cells; (a) $Ca(OH)_2$ + aquadest, (b) $Ca(OH)_2$ with extract of cacao pod shell, (c) $Ca(OH)_2$ with green tea extract.

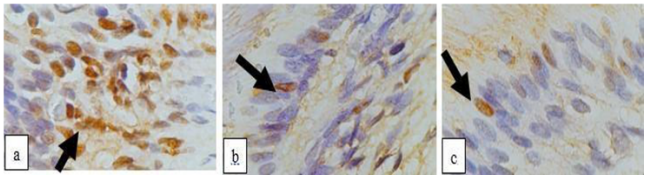

Figure 5. TUNEL Assay observations of apoptosis expression on day-7 (1000x magnification) shown with brownish colored cells; (a) $Ca(OH)_2$ with aquadest, (b) $Ca(OH)_2$ with extract of cacao pod shell, (c) $Ca(OH)_2$ with green tea extract.

Table 4 bellow shows that the number of sample from each group is 6. The smallest mean of apoptosis expression is $Ca(OH)_2$ with green tea extract group on day-7.

No	Group	Observation day	n	Mean	Std deviation
I	$Ca(OH)_2$ with aquadest	3	6	9,50	1,87
		7	6	8,67	1,36
II	$Ca(OH)_2$ with cacao pod shell	3	6	6,50	1,37
		7	6	5,16	1,16
III	$Ca(OH)_2$ with green tea	3	6	5,66	1,21
		7	6	2,16	0,75

Table 4. Sample, Mean, dan Standard deviation of Apoptosis Expression.

To determine the differences apoptosis expression between each groups, the ANOVA test was performed and obtained p value = 0,001(p <0,05). This indicates that there is a significant difference in the expression of apoptosis between groups on day-3 and day-7. To identify the differences between groups, Tukey HSD tests were carried out. The results of the Tukey HSD in table 5 below.

	$Ca(OH)_2$ with aquadest	$Ca(OH)_2$ with cacao pod shell	$Ca(OH)_2$ with green tea
$Ca(OH)_2$ with aquadest		p = 0,006*	p = 0,001*
$Ca(OH)_2$ with cacao pod shell			p = 0,884
$Ca(OH)_2$ with green tea			

Table 5. Tukey HSD test of apoptosis expression on day-3

* = significant difference

Table 5 shows the expression of apoptosis on day-3 between group I with group II and between group I with group III had p < 0,05. This shows that there is a significant difference between the group I with group II and group I with group III. Meanwhile, the expression of apoptosis on day-3 between group III and group III had p value > 0,05. This shows that there is no significant difference between the group II and group III.

	$Ca(OH)_2$ with aquadest	$Ca(OH)_2$ with cacao pod shell	$Ca(OH)_2$ with green tea
$Ca(OH)_2$ with aquadest		p = 0,001*	p = 0,001*
$Ca(OH)_2$ with cacao pod shell			p = 0,006*
$Ca(OH)_2$ with green tea			

Table 6. Tukey HSD test of apoptosis expression on day-7.

* = significant difference.

Table 6 shows that on day-7 of apoptosis expression in all groups has p value < 0,05.

This data shows that there is significant differences among all groups.

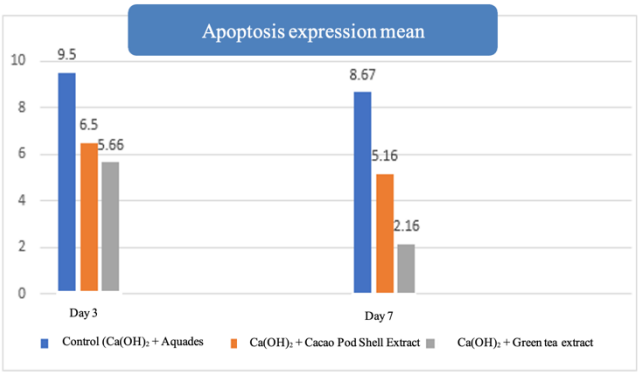

Figure 6. Mean of apoptosis expression all groups on the day-3 and day-7.

Figure 6 shows that there is a significant difference in the decrease in apoptosis expression in the $Ca(OH)_2$ with cacao pod shell extract and $Ca(OH)_2$ with green tea extract to control group on both day-3 and day-7.

Discussion

Combination of calcium hydroxide with proanthocyanidin compounds will lead a reaction. Ca^{2+} ions and OH^- ions from calcium hydroxide bind to phenol groups to form ionic bonds between Ca^{2+}, OH^-, and C. Meanwhile, the OH^- groups on the phenol groups will form H_2O in hydrogenic bonds.[11]

Whereas in the combination of calcium hydroxide with EGCG compounds, Ca^{2+} ions and OH^- ions from calcium hydroxide will bind to the hydroxyl group on ring B which then forms ionic bonds between Ca^{2+}, OH^-, and C. Meanwhile, the OH^- group occupies the position of ring B

(3'4'5'- OH) will form H2O in hydrogenic bonds.[12] The main polyphenol content in cacao pod shell is tannin. Tannins in cacao pod shell are condensed tannins (proanthocyanidins), which are polymers of catechins and epicatechins. The function of Tannins not only as primary antioxidants (donates hydrogen atoms or electron), but also as secondary antioxidants.[13]

Green tea contains the largest polyphenols, especially catechins, which are derivatives of flavans, a flavonoid group. Green tea polyphenols can scavenge free radicals by providing stable phenol radicals.[6] Catechins can quickly act as free radical scavengers because they are able to transfer electrons quickly. Catechins can transfer electrons rapidly to free radical sites induced by ROS in DNA. The largest catechin in green tea is EGCG.[14] Yongtao et al., (2021) conducted a study on how EGCG inhibits apoptosis of human pulp cells. when administered with nitroprusside (SNP) as an induced nitric oxide synthase (stimulus of the oxidation reaction). The results showed that EGCG has a defense effect against apoptosis caused by INOS in human pulp cells by scavenging ROS and modulating the Bcl-2 family. EGCG ameliorated caspase 9 and caspase 3 activity and increased cytochrome c due to SNP. SNPs cause increased expression of Bax (pro-apoptotic Bcl-2 family), and decreased expression of Bcl-2 (anti-apoptotic Bcl-2 family). SNPs increase the release of cytochrome c from the mitochondria to the cytosol and increase the activity of caspase9 and caspase3, which are markers of apoptosis.[10,15]

The addition cacao pod shell extract in $Ca(OH)_2$ containing proanthocyanidin and the combination of $Ca(OH)_2$ with green tea extract containing the EGCG will bind to ROS so that they become more stable compounds. Flavonoid compounds contained in cacao pod shell extract and green tea extract such as proanthocyanidin and EGCG are compounds that contain many groups OH^- which are multifunctional can act with free radicals as reducing agents, free radical scavengers, metal chelators, and reducers of singlet oxygen formation[7]. Flavonoids have antioxidant activity because they have Intramolecular hydrogen-bonds and can reduce the antioxidant activity of hydroxyl groups, acting as hydrogen-bond donors (5−OH, 3−OH and 3'−OH) so that ROS become stable and new free radicals are formed which are less reactive.[16]

Flavonoid compounds can protect the lipid bilayer membrane by donating one of the H^+ ions to lipid peroxyl (LOO^-). LOO^- is the result of lipid peroxidation due to ROS binding to the lipid bilayer. Giving H^+ ions can stop further radical reactions and reduce malondialdehyde levels because lipid peroxidation does not occur.[17]

The largest group of polyphenols in green tea is EGCG (Epigallocatechin gallate) which has high antioxidant properties. EGCG suppressed signal transduction pathway in the cell nucleus such as IL-1β, IKKβ, TLR, IRAK-1 and ERK1/2, associated with limitation in the number of inflammation[18]. In addition to the mechanism of antioxidant activity, EGCG compounds also have other mechanisms to reduce the level of oxidation. EGCG may have utility as an anti-inflammatory material in pulpitis that activate transcription factors for oxidation reactions.[6,10] Thus it will suppress ROS activity in producing malondialdehyde and suppress ROS activation to activate the apoptotic pathway so that the amount of malondialdehyde and apoptosis of odontoblast cells will decrease.

Based on the results of the phytochemical test of the extract used in this study, it was found that flavonoid compounds were greater in green tea extract (34.59%) than in cacao pod shell extract (27.23%), so green tea extract had higher antioxidant power based on its flavonoid content. Research conducted by Yuanita (2022) regarding the difference in antioxidant properties of green tea extract compared to cacao pod shell extract, it was found that the antioxidant qualities of green tea extract was higher than cacao pod shell extract, but there was no significant difference.[19] This is in line with the outcome of this study where there was insignificant difference between decreased expression of malondialdehyde and decreased expression of apoptosis in the $Ca(OH)_2$ with green tea extract group (groupIII) compared to the $Ca(OH)_2$ with cacao pod shell extract group (groupII) although based on the average number of decreased expression of malondialdehyde and expression of apoptosis in the groupII was higher than group III.

Conclusion

The addition of cacao pod shell extract and green tea extract to calcium hydroxide on

exposed dental pulp can reduce the malondialdehyde expression and odontoblast apoptosis.

Acknwoledgements

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Declaration of Interest

We require that authors disclose any personal and colflicts of interest.

Ethical policy and institutional review board statement

Ethical clearance had been obtained from the Ethics Commission of the Faculty of Dentistry, Universitas Airlangga, Surabaya (No.397/HRECC.FODM/IV/2020), 2020.

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