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Propolis-enhanced Calcium Hydroxide Combination for Direct Pulp Capping: Impact on Oxidative Stress Markers in Wistar Rat Teeth

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Introduction: This study explored the effects of propolis extract calcium hydroxide ($Ca(OH)_2$) combination on malondialdehyde and superoxide dismutase expression in dental pulp, aiming to assess its potential as a direct pulp capping material. **Materials and Methods**: Thirty male Wistar rats were randomly assigned to three groups. Mandibular molar teeth were prepared using a low-speed round bur. In Group I, no material was applied; in Group II, teeth were treated with $Ca(OH)_2$; and in Group III, teeth were treated with Propolis extract-$Ca(OH)_2$, followed by Cention N filling. Immunohistochemistry was conducted on pulp tissue samples obtained on the third and seventh days post-treatment to assess malondialdehyde and superoxide dismutase expression. Statistical analyses included the Shapiro-Wilk test, Levene test, ANOVA, and Tukey's HSD. **Results**: The samples treated with propolis extract-$Ca(OH)_2$ combination exhibited significantly lower malondialdehyde expression on both days compared to samples treated with $Ca(OH)_2$ ($P<0.05$), indicating reduced oxidative stress. Superoxide dismutase expression in the propolis extract-$Ca(OH)_2$ group was higher ($P<0.05$), suggesting an enhanced antioxidant activity. The control group showed intermediate results. Statistical analyses confirmed significant differences between groups for both malondialdehyde and superoxide dismutase expressions ($P<0.05$). **Conclusion**: The study suggests that the propolis extract-$Ca(OH)_2$ combination holds promise for direct pulp capping applications by minimizing oxidative stress and promoting antioxidant defense mechanisms in dental pulp.

Keywords: Calcium Hydroxide; Direct Pulp Capping; Malondialdehyde; Propolis; Superoxide Dismutase

Introduction

The selection of direct pulp capping material is one of the important factors affecting the outcome of the treatment. Pulp capping material must have biocompatible properties and good physio-chemical content [1]. Calcium hydroxide ($Ca(OH)_2$) is the gold standard for direct pulp capping; it is widely used and has antibacterial properties [2, 3]. The formation of reparative dentin takes place when the protective role of the pulp tissue is disturbed in response to an elevation in the levels of oxygen free radicals due to the application of $Ca(OH)_2$. A certain concentration of $Ca(OH)_2$ damages the pulp tissue by eliminating fibroblast cells and forms tunnel defects in the pulp tissue as a result of irregular reparative dentin [2, 4].

The mediators identified during the inflammatory process are platelet aggregation factors, arachidonic acid metabolites, tumor necrosis factor-α, interleukins and free radicals [5]. A free radical created by physiological activities is known as reactive oxygen species (ROS). Cell damage can be the consequence of an imbalance between oxidants and antioxidants, and an excessive level of oxidative stress in a process. Lipid peroxidation of dental pulp occurring in polyunsaturated fatty acids in the phospholipid membrane of fibroblast and odontoblast cells is triggered by the oxidative stress of the pulp tissue [6].

Malondialdehyde (MDA) is a biomarker often used to measure the extent of cell or tissue breakdown. Superoxide dismutase (SOD) is an endogenous antioxidant that controls the amounts of ROS in

the body. The physiological levels of SOD and MDA must be in a dynamic balance to control ROS and maintain cell function [7].

Propolis is a strong antioxidant containing important compounds, such as flavonoids and caffeic acid phenethyl ester that have anti-inflammatory properties. Propolis has been shown to have significantly better antimicrobial properties than $Ca(OH)_2$ due to containing flavonoids and having approximately 10 times less toxic effects on periodontal ligament fibroblasts and dental pulp [8]. In vital pulp therapy, propolis can induce the formation of TGF-1, which is imperative for odontoblast differentiation, inhibits osteoclastic activity or resorption, induces the formation of high-quality tubular dentin, and promotes synthesis of collagen *via* pulp cells [8, 9]. The antioxidant effect of propolis can cause the synthesis of antioxidant enzymes like SOD. In some studies, the addition of propolis can increase SOD and decrease MDA [10].

The hydroxyl ions nearby the cell may interfere with the biological function and cause cell necrosis due to the higher pH of $Ca(OH)_2$ compared to that of propolis [11]. Calcium hydroxide application exhibited lower cell survival compared to propolis application. In some studies, it was found that propolis extract-$Ca(OH)_2$ combination was biocompatible with rat connective tissue, had no unfavorable side effects, and significantly reduced inflammation [12]. In a previous study, propolis extract-$Ca(OH)_2$ combination could repair pulp tissue damage through the formation of reparative dentine. Several anti-inflammatory markers such as IL-10, VEGF and TGF-β increased after the application of propolis extract-$Ca(OH)_2$ combination [4].

The benefits of propolis have motivated researchers to explore the potential of propolis extract-Ca(OH)2 combination as a direct pulp capping material. This approach aims to address the limitations of existing materials and improve outcomes for patients.

Materials and Methods

The outline of the present *in vivo* study was approved by Ethics Committee of Faculty of Dental Medicine Airlangga University, Surabaya, Indonesia (Ethical code: 277/HRECC.FODM/X/2018

Sample preparation

Thirty male Wistar rats (Rattus novergicus) weighing around 200-300 grams were used. The samples were randomly divided into 3 groups, each group consisting of 10 rats (n=10). The procedure was performed on the mandibular molars, and the pulp chamber was accessed after Class I cavity preparation utilizing a low-speed handpiece and a round diamond bur (0.84 mm in diameter). Group I did not receive any pulp canal treatment and was filled with Cention. Group II was treated with $Ca(OH)_2$ mixed with distilled water in a 1:1 ratio and then filled with Cention. Group III was treated with propolis extract-$Ca(OH)_2$ combination with 40% Propylene Glycol made with a 1:1.5:1 ratio. Wistar rats were then terminated on third and seventh day. The tissue sections were stored in a 10% formalin buffer fixation solution for 24 h and then prepared as tissue block paraffin.

Immunohistochemistry

Teeth were decalcified using 10% Ethylenediaminetetraacetic acid for 30-60 days until the tissues were soft. Graded alcohol (70%, 80%, 90%, 96%, and 100%) was used to dehydrate, each for 2 h at a time. The samples were cleaned twice for an hour each, using a 1:1 xylol alcohol solution and were placed in the oven after being dipped in liquid paraffin for 60 min at 48°C. The embedding process (block making) was carried out on the specimens and they were labeled. Hematocillin-Eosin staining and immunohistochemical examination of the expression of MDA and SOD (NP 000445.1; StressMarq Biosciences, Victoria, British Columbia, Canada) were carried out. After blocking with 3% hydrogen peroxide for 20 min, the pulp tissue slide preparations were treate with 3% hydrogen peroxide for 20 minutes followed by rinsing with sodium perborate having a pH of 7.4. The rinsing was performed once every minute. Diammonium benzine was then dripped on, and the incubation process continued for 10 min. The counterstaining process took an extra ten min using Mayer Hematoxylin. Rinsing with sodium perborate with a 7.4 pH was done three times for five min at each incubation change. The pulp tissue preparations were observed using a light microscope starting at 400× magnification, and 1000× for 20 fields of view. Analysis was carried out on the immunohistochemical results of the pulp tissue of Wistar rat teeth for MDA and SOD examination. The obtained data are the results of histological observations of each tail in each group.

Data analysis

The Shapiro-Wilk test was used to examine whether the data in each sample group was normally distributed. To ascertain whether the sample groups had the same variation, the Levene Test was applied. To determine whether there were any differences among the full group of animals, the ANOVA test was utilized. If there were differences, Tukey's HSD would continue to find out the differences between each group. In this study, statistical tests were carried out at the 95% confidence level (P<0.05).

Results

The histopathological examination of odontoblast cells in the pulp chamber was used to determine their characteristics. Figure 1 shows odontoblast cells were found to be located at the periphery of the chamber.

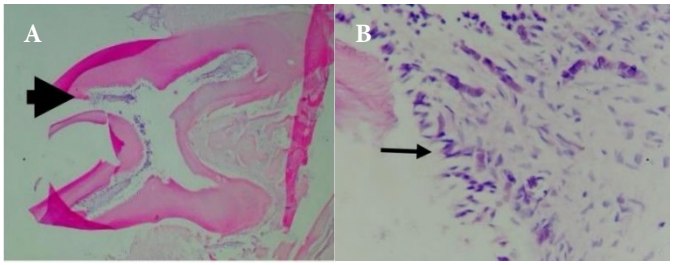

Figure 1. Histopatology depiction of HE-stained rat pulp tissue odontoblast cells with; *A)* Black arrow indicate the perforation area (40× magnification); *B)* Black arrow indicate odontoblast-like cell morphology with a rounded shape along the dentin margin (100× magnification)

The primary step of this research is analyzing the normality of data distribution using Shapiro-Wilk normality test. The following results were obtained: in the control group significance value was 0.853; in $Ca(OH)_2$ group significance value was 0.96; in propolis extract-$Ca(OH)_2$ combination group significance value was 0.814. In all three groups p-value was higher than 0.05; therefore the MDA data on the third day was normally distributed. To analyze whether the data had a homogeneous variant or not, the data was tested using the Levene test. Malondialdehyde levels on the third day had a 0.223 value so it has homogeneous variance (P>0.05). The ANOVA test findings show a 0.000 significance value (P<0.05), which implies that there is a significant difference in MDA levels on the third day between the treatment groups. A post hoc test using the Tukey's HSD was performed to determine whether groups were significantly different and the results are presented in Table 1.

Based on the results of the Shapiro-Wilk normality test, MDA levels on the seventh day was normally distributed (P>0.05) with 0.670 significance value in the control group, 0.777 in the $Ca(OH)_2$ group, and 0.314 in the $Ca(OH)_2$-propolis group. The MDA levels on the seventh day was acquired with a significance value of 0.140, therefore it was decided to have a homogenous variant (P>0.05) when the homogeneity test was conducted using the Levene Test. There was a significant difference in MDA levels on the seventh day between the treatment groups, according to the One-way ANOVA test, which was conducted because the data was normally distributed. Its significance value was 0.000 (P<0.05). The analysis continues with a post hoc test to find out the details of the differences in each group, the test used was Tukey's HSD. The results of the analysis are presented in Table 2.

Table 1. Mean (SD) of Superoxide dismutase (SOD) and Malondialdehyde (MDA) of all groups [**significant difference value (P<0.05)*]

	Group	Mean (SD)
3rd day of MDA expression	Control	18.2 (2.9)
	$Ca(OH)_2$	16.0 (1.5)
	$Ca(OH)_2$+Propolis	8.4 (1.14)
7th day of MDA expression	Control	17.8 (3.11)
	$Ca(OH)_2$	11.8 (1.4)
	$Ca(OH)_2$+Propolis	3.8 (0.83)
3rd day of SOD expression	Control	6.4 (2.07)
	$Ca(OH)_2$	9.6 (1.5)
	$Ca(OH)_2$+Propolis	13.2 (2.38)
7th day of SOD expression	Control	10.6 (1.5)
	$Ca(OH)_2$	14.8 (2.4)
	$Ca(OH)_2$+Propolis	18.6 (1.14)

Table 2. Significant analysis based on Two-way ANOVA Tukey's HSD of Malondialdehyde (MDA) and Superoxide dismutase (SOD) expression on the third and seventh days

	Group	Control	CaOH	$Ca(OH)_2$+Propolis
3rd day of MDA expression	Control		0.000*	0.000*
	$Ca(OH)_2$	0.000*		0.000*
	$Ca(OH)_2$+Propolis	0.000*	0.000*	
7th day of MDA expression	Control		0.000*	0.000*
	$Ca(OH)_2$	0.000*		0.000*
	$Ca(OH)_2$+Propolis	0.000*	0.000*	
3rd day of SOD expression	Control		0.001*	0.000*
	$Ca(OH)_2$	0.001*		0.001*
	$Ca(OH)_2$+Propolis	0.000*	0.001*	
7th day of SOD expression	Control		0.001*	0.000*
	$Ca(OH)_2$	0.000*		0.000*
	$Ca(OH)_2$+Propolis	0.001*	0.000*	

**significance value (P-value<0.05)*

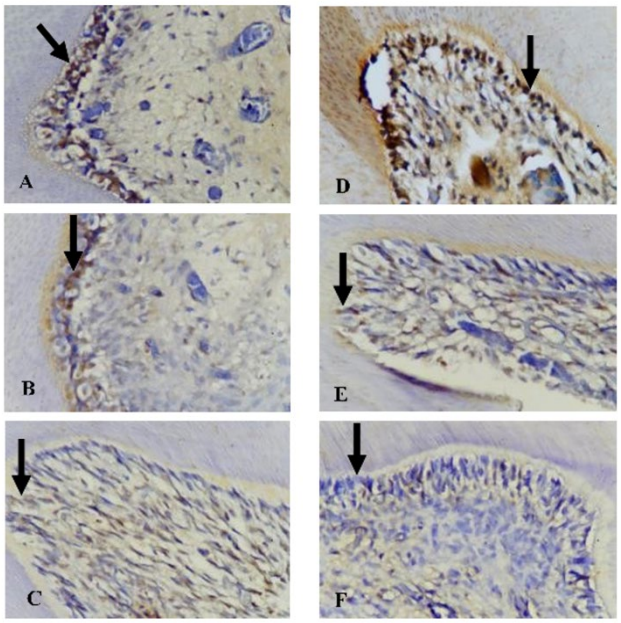

Figure 2. Odontoblastic layer expressing Malondialdehyde (MDA) on the third day and seventh day; Black arrows indicate MDA expression; *A)* Odontoblast-like cells expressing MDA in the dental pulp after application of Cention (Control) on the third day of observation; *B)* Odontoblast-like cells expressing MDA in the dental pulp after application of $Ca(OH)_2$ on the third day of observation; *C)* Odontoblast-like cells expressing MDA in the dental pulp after application of $Ca(OH)_2$-propolis on the third day; *D)* Odontoblast-like cells expressing MDA in the dental pulp after application of Cention (Control) on the seventh day of observation; *E)* Odontoblast-like cells expressing MDA in the dental pulp after application of $Ca(OH)_2$ on the 7th day of observation; *F)* Odontoblast-like cells expressing MDA in the dental pulp after application of $Ca(OH)_2$-propolis on the seventh day (400× magnification)

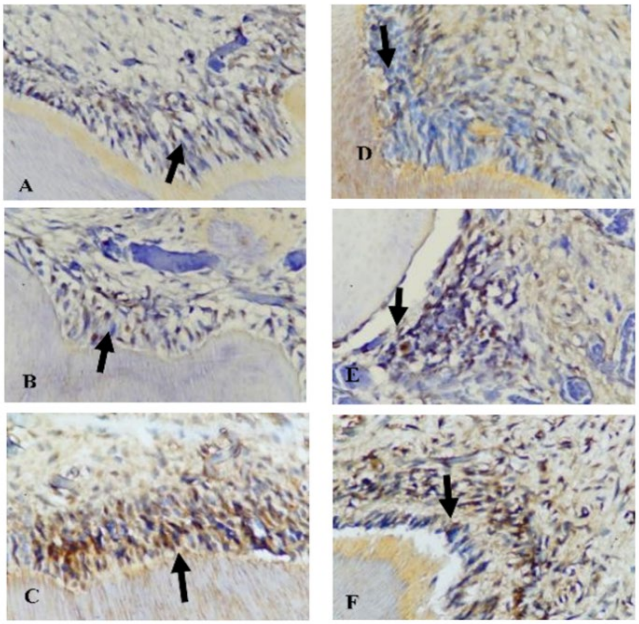

Figure 3. The black arrow indicates the Superoxide dismutase (SOD) expression on the third and seventh day (brown color); *A)* Odontoblast-like cells expressing SOD in dental pulp after Cention application (Control) on the third day; *B)* Odontoblast-like cells expressing SOD in dental pulp after $Ca(OH)_2$ application on the third day; *C)* Odontoblast-like cells expressing SOD in the dental pulp after $Ca(OH)_2$-propolis application on the third day; *D)* Odontoblast-like cells expressing SOD in the dental pulp after Cention application (control) on the seventh day; *E)* Odontoblast-like cells expressing SOD in the dental pulp after $Ca(OH)_2$ application on the seventh day; *F)* Odontoblast-like cells expressing SOD in the dental pulp after $Ca(OH)_2$-propolis application on the seventh day (400× magnification)

Immunohistochemical examination of the dental pulp of rats expressing MDA was carried out by examining immunohistochemistry using anti-MDA, which has a brown color. Figure 2 shows the immunohistochemistry of MDA expression on the third and seventh day for the three groups.

The SOD data obtained were the results of histological observations of each rat in each group. By using the Shapiro-Wilk sample normality test, it was determined that the outcomes were normally distributed (P>0.05), with the control group having 0.754 P-value, the $Ca(OH)_2$ group having 0.492 P-value, and the propolis extract-$Ca(OH)_2$ group having 0.899 P-value. Levene test was done on the third day, SOD data was obtained with a significance value of 0.506 (P>0.05) so the data had homogeneity of variance.

Comparative analysis was carried out using Two-way ANOVA to determine the differences in MDA and SOD levels of each different treatment observed at two different times, day 3 and day 7. The results of Two-way ANOVA analysis can be seen in the Table 2. The P-value obtained from the Two-way ANOVA test shows that there is a significant difference in MDA and SOD levels with observation time and treatment group (P<0.05). However, the interaction between the variables observation time along with treatment did not present significant differences for MDA and SOD levels (P>0.05).

Tukey's HSD was used for determine the significance of the differences between groups. According to the findings, data were substantially different if P-value was less than 0.05. The results of the Tukey's HSD analysis show that all treatment groups have significant data. Regarding the Shapiro-Wilk test's results for the normality test in the table presenting the seventh day, SOD levels showed 0.086 significance value in the control group, 0.0384 significance value in the $Ca(OH)_2$ group, and 0.814 significance value in the propolis extract-$Ca(OH)_2$ group, indicating that the sample is normally distributed. Levene test was done to determine the homogeneity and the result was 0.292 significance level (P>0.05) on the seventh day, so data had homogeneity of variance.

Immunohistochemical examination of the dental pulp of Wistar rats expressing SOD was carried out by examining immunohistochemistry using anti-SOD which has a brown color. Figure 3 describes the appearance of immunohistochemical in SOD expression on the third day for the three groups.

Discussion

There are many cell mediators that can trigger pulp inflammation as a reaction after tooth preparation, such as interleukins and several enzymes. When inflammation occurs, cell metabolism and ROS increases. Administration of substances containing antioxidants can reduce the severity of the inflammatory response to injury [13]. Reactive oxygen species have a significant role in the fundamental biochemical functions of the human body. However, reactive stress can be a result of these molecules' increased activity which can harm the body's functional-morphological systems and adversely affect the complex of dentine and pulp [14]. This research used direct pulp capping with $Ca(OH)_2$ and propolis extract-$Ca(OH)_2$ combination to examine the variations in MDA and SOD expression in odontoblast-like cells.

The results of the research showed that on the third day, the mean MDA expression was highest in the control group while there were no changes in the $Ca(OH)_2$ group. This was because the control group was covered with a resin-based restorative material consisting of monomer. Monomers in synthetic materials may cause accumulation of ROS which can increase inflammation by activating NF-κB and replicating TNF-secreting genes [15]. The tissue damage induced by excessive production of ROS which leads to oxidative stress [16] will result in an inflammatory phase lasting for 0-3 days. Increased MDA levels signify damage to the membrane caused by the oxidation of unsaturated fatty acids in cell membranes. MDA is produced as a result of the cell membranes' altered fluidity and permeability [14].

Propolis extract-$Ca(OH)_2$ combination caused the lowest mean expression of MDA on the third and seventh day, with significant difference compared to the control group and the $Ca(OH)_2$ group. Propolis can inhibit peroxidation and lipid hemolysis, and therefore, reduces MDA. The chemical composition of propolis affects the quality of antioxidants. Quercetin, caffeic acid phenethyl ester, p-vanillin, p-coumaric acid, apigenin, and cinnamic acid are phenolic compounds and flavonoids that can neutralize ROS produced by macrophages and neutrophils during the inflammatory phase, resulting in a reduction in MDA levels [17]. Several studies also stated that caffeic acid phenethyl ester can suppress lipid peroxidation [18]. Hydrogen ions in the flavonoids of the propolis are released and bind the hydroxyl ions (OH^-) to decrease the oxidation reactions in free radical cells [19]. Propolis exhibits its anti-inflammatory properties by inhibiting NF-κB activation, decreasing the number of inflammatory cells that produce of cytokines, and lowering free radicals [14]. Widjiastuti *et al.* [20] stated that propolis can inhibit the expression of NF-κB caused by various inflammatory agents such as inflammatory cytokines, bacterial products and oxidative stress. This leads to the significant decrease in formation of MDA in the propolis extract-$Ca(OH)_2$ group. According to acquired data, there is no apparent distinction between the control group and the $Ca(OH)_2$ group regarding the expression of SOD on the third day. The majority of body's physiological and pathological metabolic processes are tightly correlated with ROS. Reactive oxygen species will be generated in excessive amounts when the tissue is damaged, causing oxidative stress.

In propolis extract-$Ca(OH)_2$ combination group, propolis enhanced the endogenous antioxidant SOD by activating the NrF2 factor, which is a protein that regulates antioxidant protection, resulting in SOD cellular antioxidant enzymes enhancement [14]. On the third and seventh day, the propolis extract-$Ca(OH)_2$ combination group considerably outperformed the $Ca(OH)_2$ and control groups in terms of SOD expression. Several studies also stated SOD increases during cell differentiation. This occurs as a defensive strategy to counteract the elevated oxygen demand and free radical production caused by active mast cells when the cells differentiate [21]. This could explain why the mean SOD level showed an increase on the seventh day when compared to the third day in all groups. The pulp tissue began to experience chronic inflammation involving fibroblasts on the seventh day which marked the change from acute inflammation. There was a stage consisting of fibroblast cell expansion and differentiation into cells that resembled odontoblasts along with collagen synthesis [16, 22].

Conclusions

The findings of the study indicate that treatment with propolis extract and $Ca(OH)_2$ combination results in lower MDA expression on the third and seventh day, than treatment with $Ca(OH)_2$. After applying the propolis extract and $Ca(OH)_2$ combination, SOD expression was higher on the third and seventh day when compared to only applying $Ca(OH)_2$.

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Conflict of interest

None.

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Authors' contributions

Supervision and First Author: Ira Widjiastuti, Formal Analysis: Sri Kunarti, Evri Kusumah Ningtyas, Ayu Rafania Atikah, Writing-review & editing Khadijah Fauzi Basalamah, Amelia Evita Puspita, Novelia Ayu Widianti, Tengku Natasha Eleena binti Tengku Ahmad Noor.

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