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ORIGINAL ARTICLE

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The effect of calcium hydroxide in combination with propolis extract on decreasing nerve growth factor and substance P expression in rat dental pulp neuron

O efeito do hidróxido de cálcio em combinação com o extrato de própolis na diminuição da expressão do fator de crescimento nervoso e da substância P em neurônio de polpas de ratos

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ABSTRACT

Objective: Pain sensitivity was reflected in the expression of nerve growth factor (NGF) and substance P (SP), which we used in a rat tooth perforation model to assess the efficacy of $Ca(OH)_2$ combined with propolis extract direct pulp capping agent. The model was used to investigate the synergistic effects of $Ca(OH)_2$ and propolis extract. **Material and Methods:** This experimental study used 36 Wistar rat mandibular incisors randomly divided into three groups. The rats were euthanized after 12 and 24 h. Specimens underwent histological evaluation under a light microscope to identify the presence of neuron cells; an immunohistochemistry method using anti-NGF and anti-SP1 was used to evaluate the expression of NGF and SP. The results were then statistically evaluated by one-way analysis of variance and Tukey's honestly significant difference tests. **Results:** Significant differences were found in NGF expression between the $Ca(OH)_2$ + propolis and control group (12h group $p<0.001$; 24h group $p<0.001$) and between $Ca(OH)_2$ and the control group (12h group $p=0.001$; 24h group $p<0.001$). Significance differences in SP expression were also found between the $Ca(OH)_2$ + propolis and $Ca(OH)_2$ groups and between the $Ca(OH)_2$ + propolis and control groups (12h group $p<0.001$; 24h group $p<0.001$). **Conclusion:** $Ca(OH)_2$ combined with propolis extract as a direct pulp capping agent can inhibit dental pain response because the main active ingredients of propolis are caffeic acid phenethyl ester and flavonoids which can inhibit the release of prostaglandin, thereby inhibiting SP secretion, but not NGF expression.

KEYWORDS

Calcium hydroxide; Dental pulp capping; Nerve growth factor; Propolis; Substance P.

RESUMO

Objetivo: Avaliar a eficácia do $Ca(OH)_2$ associado com extrato de própolis como agente de capeamento pulpar direto e como a sensibilidade à dor pode refletir na expressão do fator de crescimento nervoso (NGF) e substância P, em ratos que tiveram dentes inferiores perfurados. O estudo foi usado para investigar os efeitos sinérgicos do $Ca(OH)_2$ e extrato de própolis. **Material e Métodos:** Esse estudo experimental usou 36 incisivos inferiores de ratos Wistar divididos aleatoriamente em três grupos. Os ratos foram sacrificados depois de 12 e 24 horas. Os espécimes foram submetidos à avaliação histológica em um microscópio de luz para identificar a presença de células neuronais; um método de imuno-histoquímica usando anti-NGF e anti-SP1 foi usado para avaliar a expressão de NGF e SP. Os resultados então foram analisados estatisticamente análise de variância one-way e teste de Tukey. **Resultados:** Diferenças significativas foram encontradas na expressão de NGF entre $Ca(OH)_2$ + própolis e grupo controle (grupo 12h $p<0.001$; grupo 24h $p<0.001$), e entre $Ca(OH)_2$ e grupo controle (grupo 12h $p=0.001$; grupo 24h $p<0.001$). Diferenças significativas na expressão de SP também foram encontradas entre $Ca(OH)_2$ + própolis e grupo $Ca(OH)_2$, e entre $Ca(OH)_2$ + própolis e grupo controle (grupo 12h $p<0.001$; grupo 24h $p<0.001$). **Conclusão:** $Ca(OH)_2$ associado com extrato de própolis como agente de capeamento pulpar direto pode inibir a resposta à dor dentária porque os principais componentes ativos do própolis são o éster fenetílico do ácido cafeico e os flavonoides, que podem inibir a liberação de prostaglandina, inibindo assim a secreção de SP, mas não a expressão de NGF.

PALAVRAS-CHAVE

Hidróxido de cálcio; Capeamento da polpa dentária; Fator de crescimento nervoso; Própolis; Substância P.

INTRODUCTION

The inflammation of the pulp and the activation of the dental pulp nerve fibers are the root causes of dental pain [1]. The pain in the trigeminal nociceptor is characteristic of the pulp illness known as pulp inflammation or pulpitis [2]. The dental pulp is innervated by sensory nerve fibers containing neuropeptides, which are released by afferent nerve fibers and are associated with neurogenic inflammation and wound healing. There are only two types of nerve fibers, Aδ and C, which mainly innervate the pulp tissue and act as polymodal receptors [3]. Approximately 25-50% of dental nerve fibers are Aδ-myelinated nerve fibers containing the neuropeptides calcitonin gene-related peptide (CGRP) and nerve growth factor (NGF) [2]. NGF is a neuropeptide neurotrophic factor, a well-known mediator for persistent pain. Pain occurs because a reaction originates from NGF, a neuropeptide produced when tissue damage occurs and an important component of inflammation [4]. NGF acts directly on peptidergic C-fiber nociceptors, which express the NGF thyroid kinase receptor, TrkA, as well as the low-affinity neurotrophic receptor p75 [5]. When stimulated, the terminal part of the C nerve fiber will produce NGF receptors and release several neuropeptides, namely substance P (SP), CGRP, and neurokinin A (NKA) [6]. SP is considered a major mediator of neurogenic inflammation and hyperalgesia. This mechanism not only involves nerve fibers in the area of tissue damage but also extends to the surrounding damaged tissue, where it causes secondary hyperalgesia [7].

Direct pulp capping is a procedure that is used for maintaining the vitality of the dentin-pulp complex when the dental pulp is exposed and is still vital, caused by caries, trauma or iatrogenic causes, such as accidental opening of the pulp due to tooth preparation and removal of carious tissue [8]. The pulp requires protection against bacterial invasion, thermoelectric conduction, and chemical protection of the overlying restorative materials. Calcium hydroxide $Ca(OH)_2$ is the gold standard for pulp capping treatment. The biocompatibility of $Ca(OH)_2$ and other pulp capping materials can be used to maximally reduce the pain and inflammation response [9]. One of the alternative pulp capping materials that can be developed is propolis. Some authors recommend the use of propolis as a carrier in a mixture of $Ca(OH)_2$ paste is recommended because propolis contributes to the antimicrobial effects of $Ca(OH)_{2,}$ which other carriers, such as saline and propylene glycol, do not [10-12].

The pharmacologically active chemical components of propolis that are the most widely known are flavonoids, isoflavonoids, phenolics, caffeic acid and aromatic acid [13]. The content of flavonoids and caffeic acid derivatives in propolis has an anti-neuroinflammatory effect and can provide a degree of healing of pulp inflammation and reduce pain [14,15]. Because nerve fibers that innervate the pulp produce NGF and SP molecules and their receptors, this study aimed to investigate the effects of expression in nerve cells.

MATERIALS AND METHODS

Materials

Raw propolis extract was combined with $Ca(OH)_2$ paste at a 1:1.25 ratio (125 mg $Ca(OH)_2$ and 0.1875 propolis extract). This combination was mixed with a cement spatula until it was homogeneous. The materials used in this study were propolis extract, $Ca(OH)_2$ powder (Merck, Darmstadt, Germany), cotton pellets, paper point, 10% formalin solution, 70% alcohol, 95% ketamine HCl (Ketamine®, Kepro BV, Deventer, Netherland), povidone-iodine (Betadine®, PT Mahakam Beta Farma, Jakarta, Indonesia), xylazine HCl (Xyla®, De Adelaar BV, Venray, Netherland), phosphate-buffered saline (PBS), Cention filling (Ivoclar Vivadent, Schaan, Liechtenstein), paraffin wax, and hematoxylin and eosin stain.

Propolis preparation

Propolis extract was created by treating dried Apis mellifera honeycomb (100% propolis) and processed at Balai Penelitian dan Konsultasi Industri, Surabaya, East Java, Indonesia. One kg of propolis was macerated and cut to a thickness of 0.5 to 1 cm. The mixture was then homogenized for 24 h by shaking 1,000 mL of 96% ethanol into the closed container at 80 rpm. The mixture of propolis and ethanol that has been shaken is then filtered with a vacuum filter. The filtrate liquid is transferred into an evaporator cup, then evaporated in a vacuum evaporator until all the solvent is separated at a temperature of 50-60 °C for 3-5 h. The resulting extract residue (thick, brownish liquid) is transferred into a brown glass bottle and stored at a temperature of 20-25 °C [16].

Tools

The tools used consisted of a cage for containing the subjects, 3 cc and 1 cc syringes, a micromotor, a low-speed handpiece (NSK, Tokyo, Japan), a 0.8 mm round diamond bur, a 0.46 mm diameter probe tip, a half-moon explorer, tweezers, glass plates, cement spatulas, cement stoppers, a plastic filling instrument, a headlamp, pot urine, surgical masks, surgical gowns, surgical nurse caps, gloves, cutting tools and a digital camera.

Subjects

The procedures used in this study were in accordance with the ethical standards instituted by the Health Research Ethical Clearance Commission of the Universitas Airlangga Faculty of Dental Medicine No: 259/HRECC. FODM/V/2020. The subjects were male Wistar rats *(Rattus novergicus)* between 12-18 weeks old with a body weight of around 200-300 grams each. The sample number was calculated using the Federer formula of (t-1) (n-1) ≥15 [17]. Six groups were created, with six rats for each group and 36 samples in total were obtained from that estimation. The groups were divided into $Ca(OH)_2$ and propolis extract groups, $Ca(OH)_2$ groups and control groups. The tools used in this research were sterilized in an autoclave at 121 °C for 30 minutes. During the procedure in the treatment group, the tools used were first disinfected with 70% alcohol for aerobic bacterial decontamination [18]. All rats were prepared with povidone-iodine antiseptic at the injection site, then anesthetized by peritoneal injection with ketamine HCl and xylazine HCl. The incisal surface of the mandibular central incisor was cut 4 mm, and the cavity was prepared on the incisal surface using a low-speed handpiece with a round tapered ring bur (diameter 0.8 mm) until the pulp chamber perforated. The depth of preparation was estimated to be 1.5 mm. Perforation of the pulp chamber was accomplished using a probe (0.46 mm tip diameter). Then the cavity was dripped with sterile saline solution and dried with a cotton pellet and paper point. In groups 3 and 4, $Ca(OH)_2$ mixed with distilled water was applied until its thickness reached 0.8 mm. In groups 5 and 6, $Ca(OH)_2$ combined with propolis extract was applied until its thickness was 0.8 mm. A carrier was used to apply the material on the pulp surface, and the material was compacted with an ultrafine micro brush. After applying the pulp capping material, the cavity was restored with a Cention filling material. The rats were observed in the cage after being labeled. At the end of the experimental period of 12 and 24 h after procedure, the animals were euthanized by guillotine decapitation. Sections of the teeth were obtained. The specimens were the alveolar bone and the teeth; they were taken, and decalcification continued. The specimens were fixed for four days using a 10% formaldehyde solution. Decalcification was accomplished using a 10% EDTA solution 10% for 30 days. The EDTA solution was changed every day. The specimen was cut at the pulp site and fixed again for 24 h using 2% sodium sulfate. The specimens were placed in paraffin wax, labeled, then sliced using a 6μm width microtome and put on a glass plate.

Application of the combination of calcium hydroxide and propolis

After pulp chamber perforation in the mandibular central incisor, the 36 rats were divided into six groups:

Group 1: Control group. The pulp chamber is applied with Cention. Decapitation after 12 h;

Group 2: Control group. The pulp chamber is applied with Cention. Decapitation after 24 h;

Group 3: Treatment group. The pulp chamber is applied with calcium hydroxide. Decapitation after 12 h;

Group 4: Treatment group. The pulp chamber is applied with calcium hydroxide. Decapitation after 24 h;

Group 5: Treatment group. The pulp chamber is applied with a combination of calcium hydroxide and propolis. Decapitation after 12 h;

Group 6: Treatment group. The pulp chamber is applied with a combination of calcium hydroxide and propolis. Decapitation after 24 h.

Histopathology and immunohistochemistry

A light microscope (Olympus BX51, Olympus®, Tokyo, Japan) was used to perform histopathology and immunohistochemistry. The calculation was done by counting brown dots as visualized at the site. The brown color formed because of the reaction of DAB (diaminobenzidine) and HRP (enzyme peroxidase). Two different people did the calculation, and then the average was obtained.

Statistical analysis

All data are expressed as means and SD. This research was experimental. Levene's test was used to test homogeneity. Saphiro-Wilk was chosen for the normality test because the sample was less than 50. One-way ANOVA testing was implemented to determine whether there was an effect on the treatment group. Tukey's honestly significant difference (HSD) test measured the differences between each group post hoc. Results were considered statistically different at $p < 0.05$. Data were analyzed using SPSS version 25.0 for Windows.

RESULTS

Histopathology (HPA) was done to make sure the sliced specimens were exemplary. Figure 1 shows the histopathology results.

After confirmation with histopathology, immunohistochemistry (IHC) was done to determine the differences between NGF and SP expression (Figure 2 and Figure 3). The mean and standard deviation results of NGF IHC can be seen in Table I and SP in Table II.

Normality and homogeneity tests were carried out prior to data analysis. The results of the Saphiro-Wilk test for normality and Levene's test for homogeneity obtained $p > 0.05$ for all treatment groups, establishing that the data were normally distributed and homogeneous. Then the ANOVA test was conducted to see if the treatment group affected NGF and SP expression. In this test, $p = 0.000$ ($p < 0.05$) at 12 h and 24 h indicated a significant difference between the treatment groups regarding NGF and SP expression. The analysis was continued post hoc using Tukey's HSD test to determine the differences in each group. NGF results are reported in Tables III and IV, and Tables V and VI show SP results.

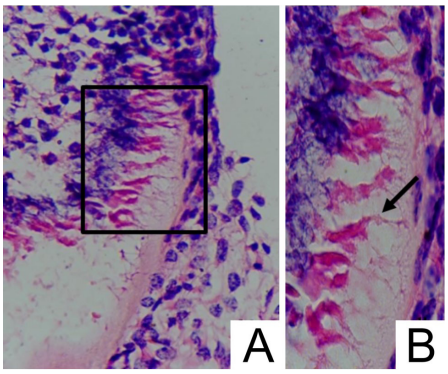

Figure 1 - Histopathology morphology pulp nerve cells of rat lower incisor (A) 100x magnification, (B) 400x magnification.

Table I - Sample size, mean, and standard deviation of NGF expression at 12 and 24 h

Group	*N*	12 h		24 h	
		Mean	SD	Mean	SD
$Ca(OH)_2$ + Propolis Extract	6	3.83	2.137	3.17	1.169
$Ca(OH)_2$	6	6.00	1.789	4.83	2.229
Control	6	11.67	2.503	12.33	2.582

Table II - Sample size, mean, and standard deviation of NGF expression at 12 and 24 h

Group	*N*	12 h		24 h	
		Mean	SD	Mean	SD
$Ca(OH)_2$ + Propolis Extract	6	4.00	1.673	2.83	1.472
$Ca(OH)_2$	6	10.83	2.317	12.17	2.317
Control	6	11.17	2.994	12.67	2.066

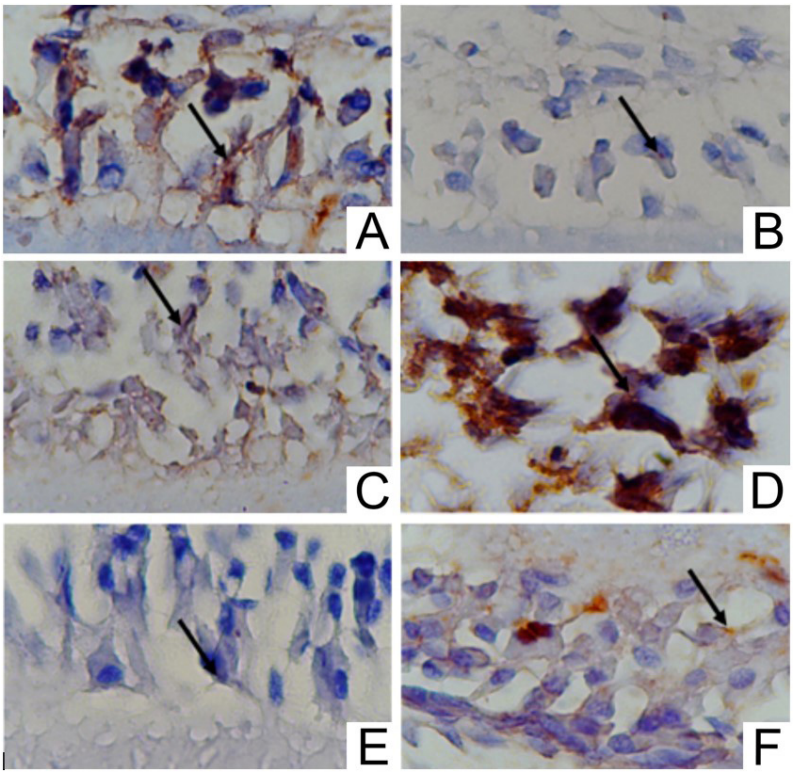

Figure 2 - Immunohistochemistry pulp nerve cells of rat lower incisor that express NGF at 1000x magnification. Black arrows indicate NGF expression at 12 h and 24 h: (A) NGF expression after perforation at 12 h; (B) NGF expression after $Ca(OH)_2$ application at 12 h; (C) NGF expression after application of the combination of $Ca(OH)_2$ and propolis at 12 h; (D) NGF expression after perforation at 24 h; (E) NGF expression after $Ca(OH)_2$ application at 24 h; (F) NGF expression after application of the combination of $Ca(OH)_2$ and propolis at 24 h.

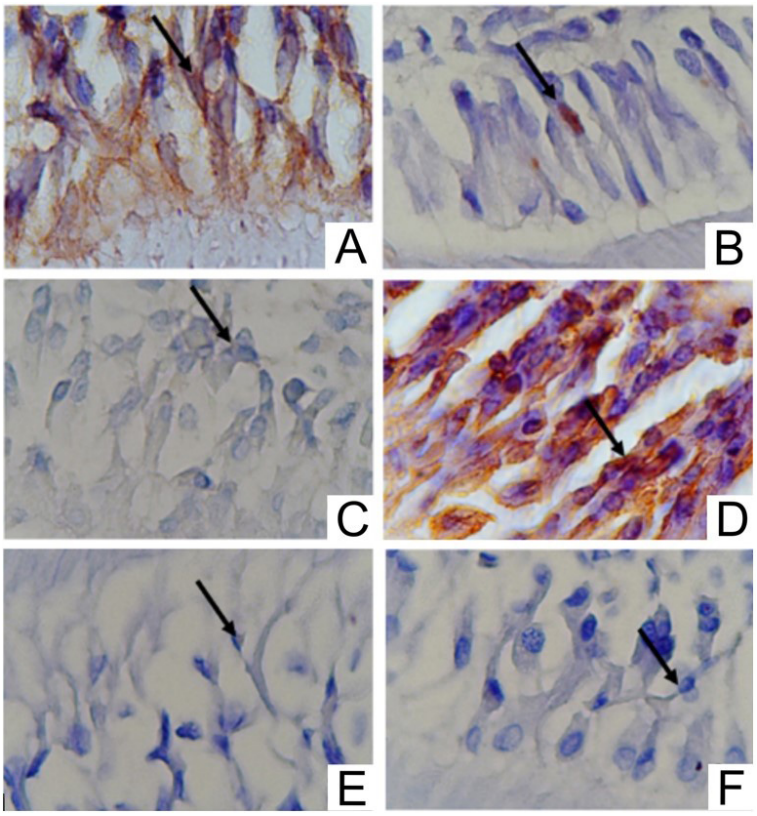

Figure 3 - Immunohistochemistry pulp nerve cells of rat lower incisor that express SP at 1000x magnification. Black arrows indicate NGF expression at 12 h and 24 h: (A) SP expression after perforation at 12 h; (B) SP expression after $Ca(OH)_2$ application at 12 h; (C) SP expression after application of the combination of $Ca(OH)_2$ and propolis at 12 h; (D) SP expression after perforation at 24 h; (E) SP expression after $Ca(OH)_2$ application at 24 h; (F) SP expression after application of the combination of $Ca(OH)_2$ and propolis at 24 h.

Table III - *Multiple Comparison* Tukey HSD between groups on NGF expression at 12 h

Group	$Ca(OH)_2$ + Propolis Extract	$Ca(OH)_2$	Control
$Ca(OH)_2$ + Propolis Extract			
$Ca(OH)_2$	0.225		
Control	< 0.001	0.001	

Table IV - *Multiple Comparison* Tukey HSD between groups on NGF expression at 24 h

Group	$Ca(OH)_2$ + Propolis Extract	$Ca(OH)_2$	Control
$Ca(OH)_2$ + Propolis Extract			
$Ca(OH)_2$	0.372		
Control	< 0.001	< 0.001	

Table V - *Multiple Comparison* Tukey's HSD between groups on SP expression at 12 h

Group	$Ca(OH)_2$ + Propolis Extract	$Ca(OH)_2$	Control
$Ca(OH)_2$ + Propolis Extract			
$Ca(OH)_2$	< 0.001		
Control	< 0.001	0.968	

Table VI - *Multiple Comparison* Tukey's HSD between groups on SP expression at 24 h

Group	$Ca(OH)_2$ + Propolis Extract	$Ca(OH)_2$	Control
$Ca(OH)_2$ + Propolis Extract			
$Ca(OH)_2$	< 0.001		
Control	< 0.001	0.901	

DISCUSSION

This study used Wistar rats as animal models because apart from being easy to handle and relatively economical compared to primates, the rat's dental pulp reaction to a substance is, in principle, similar to the reaction in human dental pulp [19]. The selection of lower incisors in rats was based on the consideration that the structure of the rat incisors was the same as the rat molar teeth. The molars' innervation differs from the incisors' innervation only in that they project toward the roof of the pulp chamber and the pulp horn, where the radicular and coronal structures are formed. In addition, the nerve innervation of the rat incisor teeth is straighter and does not form a woven nerve plexus in the sub-odontoblastic area. In contrast, the rat molar tooth's nerve innervation does form a woven nerve plexus in the sub-odontoblast area [20]. It is considered easier to read and make histology with the incisors' simpler structure and larger morphology than the molars.

In this study, observations were made about the expression of NGF and SP because NGF is a neurotrophic factor neuropeptide, a well-known mediator for persistent pain, and has a role in inflammatory hyperalgesia [21,22]. SP is considered a significant mediator of neurogenic inflammation and associated hyperalgesia and is a promising target for therapy aimed at controlling pain and minimizing the adverse consequences of tissue injury [7].

The control group showed significant differences compared with the $Ca(OH)_2$ group and the $Ca(OH)_2$-propolis extract group. This means that the release of the neuropeptides NGF and SP was mainly observed in the control group. Cention restorative materials are alkasite restorative materials, a new category of filling material such as compomer, a subgroup of the composite material class. This new category of materials uses alkaline fillers that can release acid-neutralizing ions. Cention N is a self-cure, tooth-colored direct restorative filling material with an additional light-curing option.

Cention can release fluoride ions, calcium ions, and hydroxide ions. The reactive parts of resin-based restorative materials are monomers, along with initiators, catalysts, and other additives. The monomers contained in Cention N are urethane dimethacrylate (UDMA), tricyclodecan-dimethanol dimethacrylate, etramethyl-xylylen-diurethane dimethacrylate and polyethylene glycol 400 dimethacrylate. Dimethacrylate contains an irritant that can cause inflammation [23]. UDMA exposure has the potential to induce inflammation and toxicity in pulp cells, where it can cause cell cycle dysregulation and trigger the accumulation of reactive oxygen species (ROS). An imbalance between ROS and antioxidants causes oxidative stress, which in turn will trigger tissue inflammation [24]. This tissue inflammation is thought to be related to the high expression of NGF and SP in the control group.

The study's results on NGF expression at 12 h showed a significant difference between the control group with $Ca(OH)_2$ and the control group with the combination of $Ca(OH)_2$ and propolis. This was also the case with NGF expression at 24 h. The results of this study indicate that the application of $Ca(OH)_2$ can reduce NGF expression because $Ca(OH)_2$ can dissolve NGF per the opinion of Tomson et al. [25], who state that $Ca(OH)_2$ can dissolve several growth factors including stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), insulin-like growth factor-binding proteins-1 (IGFBP-1), NGF and glial cell line-derived neurotrophic factor (GDNF). Rosa et al. [26], share this opinion and note that pulp capping materials such as mineral trioxide aggregate and $Ca(OH)_2$ can dissolve dentin bioactive molecules such as TGF-β1, NGF, and GDNF, stimulating tertiary dentinogenesis.

The flavonoid content in propolis, in addition to inhibiting the COX_2 enzyme so that it suppresses the synthesis of PGE_2 and reduces NGF expression, also has a Caffeic acid effect that can induce NGF [27,28]. Consequently, there was no significant difference between the 12- and 24-h measures of NGF expression in the $Ca(OH)_2$ group compared with the $Ca(OH)_2$-propolis combination group. In addition, the ingredients in propolis, such as quercetin and artepillin C, also have a neuroprotective effect and can induce NGF. These results are consistent with Ni et al. [29], which indicated that the content of artepillin C in propolis could increase the production of NGF, and also supported by Xu et al.'s [30], study which noted that 33 identified kinds of flavonoids could induce the synthesis and secretion of neurotrophic factors including NGF, GDNF and brain-derived neurotrophic factor (BDNF).

The expression of SP at 12- and 24-h results showed there were significant differences between the control group and the $Ca(OH)_2$-propolis combination group, as well as with the comparison between the $Ca(OH)_2$ group and the $Ca(OH)_2$-propolis combination group. These results establish that the administration of propolis creates the most significant results in reducing pain, as evidenced by the drastic decrease in SP expression. This decrease in SP occurs because the acidic environment (acidosis) produced by the inflammatory reaction is inhibited, so sensitization to TRPV1, which triggers the release of SP, is also inhibited. TRPV1 is a cation permeable channel expressed in nociceptive fibers and is responsible for detecting noxious stimuli from the periphery such as low pH, temperature rise (> 42 °C), changes in osmolality, AA metabolites, second inflammatory messengers and capsaicin (an irritant in chili peppers) [31]. Acidosis conditions up to pH 6 make TRPV1 sensitive to agonists, including heat and capsaicin, whereas acidosis, when pH < 6, can directly open the receptor gate [32]. However, the presence of OH^- ions, which have a high pH, can neutralize the acidic environment produced by the inflammatory reaction so that TRPV1 activation is inhibited [33,34]. With inhibition of TRPV1 activation, the release of SP will also decrease. The decrease in SP expression was also due to the content of propolis ingredients. Propolis has anti-inflammatory components, such as flavonoids and caffeic acid phenethyl ester (CAPE), which can inhibit eicosanoid synthesis from arachidonic acid and suppress the activity of COX_1 and COX_2 enzymes. This occurs because it inhibits the release of inflammatory mediators such as PGE_2, leukotrienes, and thromboxane, causing an increase in IL-10 expression with an increasing propolis dose. The decrease in prostaglandins is caused by the inhibition of prostaglandin synthesis from arachidonic acid, catalyzed by the transformation of the cyclooxygenase enzyme produced by galangin compounds from flavonoids and CAPE. Furthermore, galangin from flavonoid materials can also inhibit leukotrienes from arachidonic acid lipoxygenase (LOX) enzymes [35], and the quercetin component in propolis can inhibit LOX enzymes [27,36].

There was no significant difference in SP expression in the $Ca(OH)_2$ control group at 12 and 24 h. From this analysis, it can be concluded that the $Ca(OH)_2$ administration cannot significantly suppress pain. This is because when pure $Ca(OH)_2$ is applied to the pulp, it will destroy some of the pulp tissue causing continuous inflammation, according to Dwiandhono et al. [37], Inflammation that occurs can trigger the release of SP so that SP expression in the $Ca(OH)_2$ group was not different from the control group. The SP expression that did not experience a significant decrease due to the administration of $Ca(OH)_2$ may have occurred because the continuous $Ca(OH)_2$ solubility process made the Ca^{2+} concentration high. Calcium receptors on cells can thus act as sensors for increased concentration and activate chemotaxis from deeper tissues to the affected site. Increasing the concentration of Ca^{2+} ions may increase calcium influx through the VGCC gate and increase intracellular Ca_{2+}, which can increase SP release from nerves [38]. It is expected that this research can be used as the basis for the development of a combination of calcium hydroxide and propolis extract as an alternative material for pulp capping processing in humans. This research is limited by the the lack of investigation into the antimicrobial properties of propolis and the inadequacy of considering different propolis extraction techniques.

CONCLUSION

SP expression in the combination of $Ca(OH)_2$ with propolis extract exhibit a significant decrease compared to the application of $Ca(OH)_2$. NGF expression in the combination of $Ca(OH)_2$ with propolis extract did not show a significant decrease compared to the application of $Ca(OH)_2$.

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Author's Contribution

IW: Conceptualization, Methodology, Writing – Original Draft Preparation, Supervision, Validation. S: Writing – Review & Editing, Supervision, Validation. GAS: Writing – Review & Editing, Software, Formal Analysis, Resources, Data Curation. KFB: Writing – Review & Editing, Visualization, Investigation, Data Curation, Project Administration, Funding Acquisition.

Conflict of Interest

No conflicts of interest were declared concerning the publication of this article.

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Regulatory Statement

Before the commencement of research, the ethical approval for the current study was obtained from the Health Research Ethical Clearance Commission of the Universitas Airlangga Faculty of Dental Medicine. The approval code for this study is No. 259/HRECC.FODM/V/2020.

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