



RESEARCH ARTICLE

Combinations of propolis and Ca(OH)₂ in dental pulp capping treatment for the stimulation of reparative dentin formation in a rat model [version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

Background: Caries in the dental pulp result in inflammation and damage to the pulp tissue. During inflammation of the pulp, various inflammatory mediators and growth factors are released, including IL-8, IL-10, TLR-2, VEGF and TGF-β through the NF-κB pathway. In the present study, therapy for pulpal caries was performed through pulp capping by giving a combination of propolis and calcium hydroxide (Ca(OH)₂). This treatment was expected to stimulate the formation of reparative dentin as an anti-inflammatory material to prevent pulp tissue damage.

Methods: 28 Wistar rats were divided into four groups and treated with Ca(OH)₂ with or without the addition of propolis for either 7 or 14 days. Immunohistochemical examination was used to determine the expression of IL-8, IL-10, TLR-2, VEGF, TGF-β in the four treatment groups.

Results: The group treated with a combination of propolis and Ca(OH)₂ for 7 days showed that the expression of IL-10, IL-8, TLR-2, VEGF, TGF-β increased significantly compared to the treatment group treated with only Ca(OH)₂. The expression of IL-10, TLR-2, TGF-β, VEGF increased in the treatment group treated with propolis and Ca(OH)₂ for 14 days, while the expression of IL-8 in the decreased significantly.

Conclusions: Administration of a combination of propolis and Ca(OH)₂ has efficacy in the pulp capping treatment process because it has anti-bacterial and immunomodulatory properties. The results show that it is able to stimulate the process of pulp tissue repair through increased expression of IL-10, TGF-β, VEGF, TLR -2 and decreased expression of IL-8.

Keywords

Propolis, Ca (OH)₂, IL-10, IL-8, TGF-β, VEGF dan TLR-2

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Introduction

Caries in the dental pulp result in inflammation and damage to the pulp tissue. During inflammation of the pulp, inflammatory mediators, including cytokines, regulate the activity of the immune response to local and systemic inflammation against the external stimuli¹⁻³. NF- κ B activation due to injury causes transcription and translational processes in cells, which results in pro-inflammatory cytokine production, such as IL-1 β , IL-6 and TNF- α , initiating the immunological process that aids pulp tissue in overcoming inflammation³. NF- κ B is considered as a prototypical inflammatory pathway and is used to target anti-inflammatory drugs, thus the expression of NF- κ B can be investigated to measure the inhibition of proinflammatory cytokine production^{3,4}.

Calcium hydroxide (Ca(OH)₂) is the gold standard for pulp capping treatment in tooth pulp inflammation but has a high pH (pH 12.5), which causes pulp tissue necrosis in direct contact with Ca(OH)₂⁵. Ca(OH)₂ plays a role in the formation of reparative dentine, apexification, and intracanal medicament. It has anti-microbial properties (able to neutralize microbial products), inhibits root resorption and induces the formation of hard tissue⁶. The application of Ca(OH)₂ with a certain concentration causes fibroblast cell death resulting in damage to the pulp tissue⁷. In addition, use of Ca(OH)₂ in long term can cause physical changes; reparative dentine formation is not dense enough due to dentin bridge discontinuity leading to an area of necrosis called a "tunnel defect", which causes dentists to search for alternative materials, including natural herbal-based ingredients⁸. Propolis is a natural material produced by bees, consisting of balsam resin, beeswax, essential oils, and pollen from other organic materials. It may have a role in the process of pulp capping treatment and the healing process, due to its anti-bacterial, anti-fungal, anti-virus, anti-tumor, anti-oxidation, and immunomodulatory properties⁹. It has been reported that toxicity of propolis to fibroblast cells is low and is able to inhibit apoptosis and stimulate fibroblast cell proliferation¹⁰. In addition, propolis may be used as an alternative herbal ingredient for pulp capping treatment because it can reduce inflammation in pulp exposed to cariogenic material¹¹. The inhibition of NF- κ B by the active component of propolis causes a decrease in the expression of proinflammatory cytokine genes, such as IL-1, IL-6, IL-8, and TNF- α , leading to downregulation of TLR-2 in dental pulp^{12,13}. The combination of Ca(OH)₂ with propolis does not cause a toxic reaction, which was shown when evaluating the biocompatibility of Ca(OH)₂ and propolis in the subcutaneous tissue of rats. It was found that both materials were able to reduce inflammation significantly through the role of IL-10, TGF- β , VEGF and was biocompatible with rat connective tissue^{11,14}. Propolis contains caffeic acid phenethyl ester (CAPE), showing that propolis can function as an anti-inflammatory. However, to date research on pulp capping treatment for the stimulation of reparative dentin formation with a combination of Ca(OH)₂ and propolis in an immunopathological study has not yet been performed. Therefore, this study aimed to find out if there was increased expression of IL-10, VEGF, TGF- β and TLR-2 as well as a decrease in pro-inflammatory cytokines IL-8 when using Ca(OH)₂ in combination with propolis.

Methods

Ethics statement

All procedures performed in this study were ethical, and the study was approved by the Ethics Commission of the Dental and Oral Hospital of the Faculty of Dentistry, Airlangga University (002/HRECC.DHAU/XI/2019) with principal researcher as RPR. The study took place at the Department of Biochemistry, Medical Faculty, Airlangga University and Department of Biochemistry, Medical Faculty, Brawijaya University, which were certified to be ethical. All efforts were made to ameliorate any suffering of animals with the use of local anesthesia (lidocaine with Citoject syringe) to decrease pain in this treatment.

Animals

Adult male Wistar rats aged 16–18 weeks, weight 150–250 grams, with healthy teeth and no caries, were kept in cages at room temperature (26°C) in light conditions, with food (Phokphan Hi-Provite) and water to drink available *ad libitum*. The rats were obtained from Biokimia Laboratories, Medical Faculty, Airlangga University. The number of rats obtained was calculated using the formula by Frederer: Sample = (r-1) (t-1), so that the total number of rats was 48. In each cage there were 7 rats and the rats were acclimatised to their cages for 1 week. The rats were kept in cages containing clean husk cushions and covered with a cover made of tenuous wire to function air circulation. Chaff was replaced three times a day to keep the cage clean.

Treatment

The 48 Wistar strain rats were divided into eight groups (6 rats/group/time point): Control group, treated with aquadest (no drilling); negative control group, drilling only with no treatment; P1, treated with 0.625 μ g Ca(OH)₂ (Dycal from Dentsply); and P2, treated with a combination of propolis extract (natural raw bee) propolis extract powder (from *Mellifera apis*; concentration of 0.937 μ g) with Ca(OH)₂ (0.781 μ g) in a ratio of 1:1.5.

Animals were randomly allocated to each group with random number generator. For each group, rats were given a number between 1 and 48. The first 6 numbers generated were in the positive control group, the next 6 in the negative control group and so forth.

On day 1 of the experiment, all groups of experimental animals were prepared for grade 1 cavity with a low speed round bur (0.84 mm diameter) to reach the right mandibular M1 pulp chamber. Rats were anesthetized using ketamin (ket A) dissolved in a sterile isotonic saline solution (0.2 ml/87 mg bb). The treatments were applied to the molar teeth of the rats as follows: group P1, Ca(OH)₂ base was mixed with catalyst until it formed a paste-like consistency and this was applied to the molar teeth of the animal when it received a plastic filling; group P2, propolis extract powder was mixed with Ca(OH)₂ powder and liquid until it formed a paste-like consistency.

On day 7, half the animals from each group (6/group) were anesthetised with ether and decapitated. Their jaws were removed

and placed in a sample pot with 10% formalin buffer (pH 7.4). On day 14, the remaining animals (6/group) underwent the same procedures.

Immunohistochemistry

The dental tissue removed from the rats were kept in the sample pot with 10% formalin buffer (pH 7.4) for 24 hours. Subsequently, the tissue was decalcified using EDTA 10% at 37°C, which was replaced every day until the soft tissue broke down (this took up to 2 months). If the teeth were soft enough to be pricked with needle, then it is processed to make a paraffin block through the stages of dehydration, clearing, impregnation and embedding, as follows: dehydration, the dental tissue is kept in various percentages of alcohol (70, 80, 95, 96, 96, 96%) for 2 hours each percentage; clearing, dental tissue is placed in n-xylol for 4 hours; impregnation, the dental tissue is placed in solid paraffin for 4 hours; embedding, the dental tissue in the solid paraffin is placed into a mould base using tweezers. The mould base is filled with tissue on the cold plate and paraffin block ready to be cut. Using a microtome, tissue slices at a thickness of 0.5 cm were cut. These were stained with haematoxylin to look for macrophages, and were then prepared to check expressions of cytokines using the following kits: IL-10 (sc-52560 from Santa Cruz Biotechnology (Europe), VEGF (sc-7279 from Santa Cruz Biotechnology (Europe), IL-8 (BS3479 from Bioworld Technology), TGF- β (ab27969 from Abcam) and TLR-2 (pAb anti-TLR2 NB 200–536 from Novus Biologicals). Expression was looked at under a light binocular microscope (Merk Olympus) at 400x magnification. For macrophages expressing the cytokines, cells that were brown were counted as positive.

Data analysis

Data analysis was completed with *SPSS 16.0 version*. Normality testing was done using Kolmogorov-Simrnov test, and a homogeneity test using Levene's test. One-way ANOVA test

used to assess the difference between groups, followed by post-hoc least significant difference tests used for homogeneous data and Tamhane test used for non-homogeneous data. P value >0.05 was considered significant^{39,40}.

Results

The control group that was not treated with either Ca(OH)₂ or propolis had higher VEGF expression than IL-10, TGF- β , IL-8, and TLR-2, while the negative control group (drilled but no treatment) had higher IL-8 expression than IL-10, TGF- β , VEGF, TLR-2. In the P1 group (Ca(OH)₂ only) had higher VEGF expression than IL-8, TLR-2, IL-10, TGF- β , while in group P2 (Ca(OH)₂ and propolis combination) had higher IL-10 expression compared to VEGF, IL-8, TGF- β , and TLR-2 (Table 1).

Data were normally distributed, and homogeneity testing showed that IL-10 and VEGF were not homogeneous, while IL-8, TGF- β , TLR-2 were homogeneous. To see a significant difference using the One-way ANOVA and post-hoc testing showed that there were significant differences between the expression of IL-10, VEGF, IL-8, TGF- β and TLR-2 in the negative control group and the P2 group (combination of propolis extract with Ca(OH)₂) (p = 0.00).

The control group (not treated) had IL-10 expression higher than VEGF, TGF- β , IL-8, and TLR-2, while the negative control group (drilled but not treated) had a higher expression of IL-8 than IL-10, TGF- β , VEGF, or TLR-2. In group P1 (Ca(OH)₂ only) had higher IL-8 expression than VEGF, TLR-2, IL-10, TGF- β , while in P2 group (Ca(OH)₂ and propolis combination) had higher VEGF expression compared to IL-10, IL-8, TGF- β , and TLR-2 (Table 2).

Data were normally distributed, and homogeneity testing showed that showed that VEGF and IL-8 expression were not homogeneous and IL-10, TGF- β and TLR-2 were homogenous.

Table 1. IL-10, VEGF, TGF- β and TLR-2 expression in rats treated with various concentrations of propolis and calcium hydroxide for 7 days.

Group (mean \pm SD)	IL-10	VEGF	IL-8	TGF- β	TLR-2
Control (aquadest)	8.83 \pm 0.75	9.16 \pm 2.71	6.16 \pm 0.98	6.33 \pm 0.81	3.50 \pm 1.04
Drilling only (negative control)	4.66 \pm 1.63	4.00 \pm 1.89	14.16 \pm 2.78	4.00 \pm 0.89	5.50 \pm 1.04
Ca(OH) ₂ only (P1)	8.67 \pm 1.86	10.50 \pm 0.54	9.00 \pm 1.67	7.83 \pm 1.16	8.16 \pm 0.75
Ca(OH) ₂ and propolis (P2)	15.00 \pm 2.36	14.83 \pm 1.47	9.83 \pm 0.98	10.66 \pm 1.36	11.16 \pm 1.16

Table 2. IL-10, VEGF, TGF- β and TLR-2 expression in rats treated with various concentrations of propolis and calcium hydroxide for 14 days.

Group	IL-10	VEGF	IL-8	TGF- β	TLR-2
Control (aquadest)	9.00 \pm 1.41	8.50 \pm 2.07	7.16 \pm 2.13	8.33 \pm 0.81	2.50 \pm 0.54
Drilling only (negative control)	4.67 \pm 2.58	4.67 \pm 2.42	16.50 \pm 2.25	5.67 \pm 1.03	4.50 \pm 1.04
Ca(OH) ₂ only (P1)	9.83 \pm 0.75	10.83 \pm 0.75	11.83 \pm 1.32	9.83 \pm 1.1	7.5 \pm 0.54
Ca(OH) ₂ and propolis (P2)	16.00 \pm 2.19	17.16 \pm 3.06	8.85 \pm 0.75	13.16 \pm 1.72	10.5 \pm 1.04

One-way ANOVA and post-hoc testing showed that there were significant differences between IL-10, VEGF, IL-8, TGF- β , and TLR-2 expression in the negative control group compared with the P2 group (combination of propolis extract with Ca(OH)₂) ($p = 0.00$).

Discussion

This study used a combination of propolis and Ca(OH)₂ as ingredients for the formation of reparative dentin. Propolis has advantages as an anti-inflammatory ingredient as it contains CAPE^{13,14}. The anti-inflammatory properties possessed by propolis can reduce inflammation in the pulp chamber and can induce reparative dentin¹⁵. To find out whether propolis can be used as a bioproduct for stimulation of reparative dentin formation, this study tested the expression of the following cytokines: TLR-2, a membrane receptor that facilitates signaling pathway in the reparative dentin process¹⁶; IL-10, an anti-inflammatory cytokine^{17,18}; VEGF, a protein that helps in the process of angiogenesis^{19,21}; IL-8, a pro-inflammatory cytokine that can induce the formation of reparative dentin through the recruitment of neutrophils to repair damaged tissue damage^{22,23}; and TGF- β , a regulator of cell proliferation, differentiation and reparative dentinogenesis²⁴.

This study showed that on day 7, the group treated with a combination of propolis and Ca(OH)₂ showed significantly increased expression of IL-10, VEGF, IL-8, TGF- β , and TLR-2 compared with the negative control. IL-10 expression was the highest among all the cytokines. Propolis has anti-inflammatory components, such as caffeic acid, which can inhibit the synthesis of eicosanoids from arachidonic acid and suppress the activity of COX-1 and COX-2 enzymes. Therefore, it inhibits the release of inflammatory mediators such as PGE-2 (prostaglandin), leukotrienes and thromboxanes, leading to the increasing expression of IL-10 expression with increasing dose of propolis^{23,24}. The decrease in prostaglandin is caused by the inhibition of prostaglandin synthesis from arachidonic acid which is catalyzed by the transformation of the cyclooxygenase enzyme, which is produced by galangin compounds from flavonoids and CAPE. In addition, galangin from flavonoids are also able to inhibit leukotrienes from arachidonic acid through the enzyme lipoxygenase^{24,25}.

The study showed that on day 14, there were changes in the expression of IL-10, TGF- β , and TLR-2 in the combination treatment group compared with the negative control group. The expression of IL-10, TGF- β , and TLR-2 in the treatment group treated with Ca(OH)₂ alone without propolis was more significant compared with the negative control, whereas the expression of VEGF and IL-8 in the treatment group treated with a combination of propolis and Ca(OH)₂ was significantly increased compared with the negative control. The combination treatment group showed the highest VEGF expression among other variables, while the Ca(OH)₂ group showed that the expression of IL-10 was highest among the other variables. The 14th day is probably when the process of proliferation and angiogenesis has been initiated. In this research combination therapy of propolis and calcium hydroxide increases VEGF at 14 days. This could be due to the role of PDGF (platelet-derived

growth factor), which is known as a promoter of proteoglycan and collagen formation. As local fibroblasts respond to PDGF by producing collagen and transforming it into myofibroblasts to increase wound contraction, fibroblasts also emit keratinocyte growth factor (KGF), which stimulate epithelialization from keratinocytes, and produce VEGF endothelial cell growth factors, and fibroblast growth factors (bFGF) to promote blood vessel growth²⁷. IL-10 expression has also increased because Ca(OH)₂ has high alkaline properties, which means that it will increase the pH for pulp healing if damaged as a high pH can neutralize acids in the inflammatory site²⁸.

Ca(OH)₂ is used as an intracanal medicament and is antibacterial. Ca(OH)₂ is used because it is stable for a long time and can kill bacteria^{26,29}. In previous studies, endodontic therapy using Ca(OH)₂ showed that IL-1 α as an indicator of pro-inflammatory cytokines decreased on day 7, indicating that there is an immune system response to reduce the inflammatory process so that the expression of IL-10 as an anti-cytokine -inflammation will increase^{26,30}.

Calcium hydroxide can induce odontoblast formation through increased VEGF, this is due to the occurrence of differentiation and viability of endothelial cells^{31,32}. VEGF is also expressed by odontoblasts and sub-odontoblasts by large numbers of endothelial cells³³. VEGF also has a key role in the physiological and pathological development of dentinogenesis and angiogenesis in healthy dental pulp^{33,34}. In previous studies, odontoblast-like cells and undifferentiated pulp cells express high amounts of VEGF in vitro³⁵. In the inflammatory process, day 7 shows an increase in VEGF after the formation of granulation tissue and decreases until day 14^{35,36}. The VEGF mechanism is as an angiogenesis process through odontoblasts; LPS stimulated from bacteria present in the pulp can increase the permeability of the blood vessels so as to facilitate the process of neutrophil, lymphocyte and monocyte diapedesis and recruit new blood vessels around the carious tissue to increase immune defense³⁷.

The reparative dentin stimulation process occurs after pulp damage which is then treated using pulp capping material in the form of calcium hydroxide Ca(OH)₂ which is influenced by the nature of cytotoxicity and cytokine production ability^{34,35}. Inflammatory cytokines such as IL-8 found in dental pulp will recruit neutrophils in case of infection from bacteria, which will cause acute inflammation. IL-8 also plays a role in determining the duration of the inflammatory process²¹.

In the combination treatment of propolis with Ca(OH)₂ from the 7th day to the 14th day, the expression of IL-10, VEGF, TGF- β increased while IL-8 and TLR-2 decreased. Propolis activated the initial steps of the immune response by upregulating TLR-2 expression and the production of pro-inflammatory cytokines in the rats, modulating the mechanisms of innate immunity. TGF- β 1 may be involved in the healing/regeneration processes of dental pulp in response to injury by stimulation of collagen and TIMP-1 production³⁷. These events are associated with activin receptor-like kinase-5, Smad2/3 and MEK/ERK signaling and TGF- β will inhibit TLR-2 mediated odontoblast

pathway when inflammation occurs in the pulp chamber^{37,38}. TGF- β is a signaling molecule that induces cell proliferation, chemotaxis and apoptosis in monocyte, epithelial, mesenchymal and neuron tissues. In the formation of reparative dentin, TGF- β has an important role in regulating cell proliferation, differentiation and reparative dentinogenesis²³. The release of TGF- β from the dentinal matrix requires diffusion, which generally passes through the dentinal tubules to the pulp cell to activate the signaling pathway through TLR-2. Activation of TLR-2 will increase the effector of the innate immune system, including pro-inflammatory cytokines, chemokines, and anti-inflammatory cytokines to repair tissue damage³⁹. This is consistent with the results of this research showing that with the combination of propolis with Ca(OH)₂ TGF- β expression is higher than in the treatment group with only Ca(OH)₂. TGF- β is secreted by odontoblasts, and its expression is increased in carious lesions. TGF- β is proinflammatory during the initial stages of inflammation, and anti-inflammatory in later stages. Proinflammatory effects of TGF- β include immune cell recruitment and induction of matrix metalloproteinase secretion, and stimulation of the accumulation of immature dendritic cells in odontoblast and subodontoblast layers of the pulp horn close to the lesion in strategic locations to encounter foreign antigens entering the dentinal tissue^{14,39}.

IL-8 is expressed continuously by odontoblast cells. IL-8 plays a role in neutrophil recruitment in cases of inflammation in the pulp to support the healing process³⁹. In this study, IL-8 was expressed at a lower in the propolis and Ca(OH)₂ combination treatment group compared with the group treated with Ca(OH)₂ only. This illustrates that the administration of a combination of propolis and Ca(OH)₂ can repair pulp tissue damage through the formation of reparative dentine. On the 14th day with propolis and Ca(OH)₂ combination, VEGF expression was high so that it can help the process of angiogenesis in the dental pulp to be faster, while the group with only Ca(OH)₂ has anti-inflammatory properties and high IL-10 expression so as to reduce inflammation which occurs in the pulp chamber.

Conclusion

Combination therapy of propolis with Ca(OH)₂ could be considered as a product for pulp capping treatment that can stimulate the formation of reparative dentin.

Data availability

Underlying data

Harvard Dataverse: Combinations of propolis and Ca(OH)₂ in dental pulp capping treatment for the stimulation of reparative dentin formation in a rat model, <https://doi.org/10.7910/DVN/SLV60A>⁴¹

This project contains the following underlying data:

- Values for immunohistochemistry for all groups for 7-day treatment
- Values for immunohistochemistry for all groups for 14-day treatment
- **Figure S1. Mean of IL-10, VEGF, IL-8, TGF- β and TLR-2 expressions on the 7th day.**
- **Figure S2. Mean of IL-10, VEGF, IL-8, TGF- β and TLR-2 expressions on the 14th day.**
- **Figure S3. Immunohistochemical examination results from VEGF.** A. Control, B. Negative Control, C. Therapy with Ca(OH)₂, D. Therapy with a combination of propolis and Ca(OH)₂.
- **Figure S4. Results of immunohistochemical examination of IL-10.** A. Control, B. Negative Control, C. Therapy with Ca(OH)₂, D. Therapy with a combination of propolis and Ca(OH)₂.
- **Figure S5. Immunohistochemical examination results from TGF- β .** A. Control, B. Negative Control, C. Therapy with Ca(OH)₂, D. Therapy with a combination of propolis and Ca(OH)₂.
- **Figure S6. Results of immunohistochemical examination of TLR-2.** A. Control, B. Negative Control, C. Therapy with Ca(OH)₂, D. Therapy with a combination of propolis and Ca(OH)₂.
- **Figure S7. Results of immunohistochemical examination of IL-8.** A. Control, B. Negative Control, C. Therapy with Ca(OH)₂, D. Therapy with a combination of propolis and Ca(OH)₂.

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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Noengki Prameswari 

Orthodontic laboratory Staff, Dentistry Faculty, Hang Tuah University, Surabaya, Indonesia

1. The sample size mentioned in abstract methods and full-text methods are very vague. They should be rewritten for better clarity.
2. Methods: you mention that this treatment for stimulating the formation of reparative dentine. What is the clinical parameter for showing that your treatment (dental pulpcapping) has an effect on the formation of reparative dentine?
3. Discussion: The explanation about the role of natural ingredient in Propolis and its combination with $\text{Ca}(\text{OH})_2$ as dental pulpcapping to stimulate the formation of reparative dentine is not clear.
4. Conclusion: you should state the difference result of pulpcapping treatment in 7 days and 14 days, and also the mechanism.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Bone remodeling, herbal medicine, physiology, orthodontic

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 06 July 2020

<https://doi.org/10.5256/f1000research.24725.r65377>

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Mohd Farhan Hanif Reduan 

Department of Paraclinical Studies, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Kota Bharu, Malaysia

Reviewer comment:

Combinations of propolis and Ca(OH)₂ in dental pulp capping treatment for the stimulation of reparative dentin formation in a rat model.

Reviewer comments:

In general, the research is well conducted and the findings are worth considering for publication. The paper needs a minor revision as below:

- Conclusion of the abstract, please omit the “antibacterial property” as the research conducted not based on the antibacterial study.
- Paragraph 1: “*NF-kB activation due to injury causes transcription and translational processes in cells, which results in pro-inflammatory cytokine production, such as IL-1 β , IL-6 and TNF- α , initiating the immunological process that aids pulp tissue in overcoming inflammation*” - the reviewer suggest confirming this statement as pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α are promoting inflammation, not overcome the inflammation, so need clarification on this statement.
- Methodology – in the animal section, the authors need to replace the “biokimia” term into the English term.
- Methodology – in the treatment section, the authors need to highlight the experiment is separated into two periods of time; 7 and 14 days at the initial, easier for the reader to capture, the authors need to explain why two different concentrations of calcium hydroxide were selected in the discussion.

- Methodology – in the immunohistochemistry section, please use a past tense to describe the terms, few in the present tense, the authors need to explain in detail on the evaluation of the expression of cytokines like the intensity of the brownish expression and etc. If possible, include references on the method of immunohistochemistry. It is because immunohistochemistry is a major part of this study.
- Results – The authors are suggested to include the statistical indication in Tables 1 and 2. It will help the reader to understand the table faster rather than read in the sentences. Please put arrows in the figures.
- Discussion – Paragraph 2; *“In addition, galangin from flavonoids are also able to inhibit leukotrienes from arachidonic acid through the enzyme lipoxygenase”*- authors need to state what is the source of galangin, is it from propolis?
- Discussion – Paragraph 3; *“The 14th day is probably when the process of proliferation and angiogenesis has been initiated”*, need to improvise the statement as the proliferation and angiogenesis are at the peak in the 14th day.
- Discussion – Paragraph 5: *“Ca(OH)₂ is used as an intracanal medicament and is antibacterial. Ca(OH)₂ is used because it is stable for a long time and can kill bacteria”*. The authors are suggested to further discuss the details on the effect of calcium hydroxide as caries always related to bacterial infection.
- Discussion – Paragraph 8: The authors need to add elaboration on the anti-inflammatory activity of TGF at the late stage.
- Conclusion – the mechanism on the stimulation of the formation of reparative dentin needs to be addressed based on the present findings.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My research on the wound healing and toxicological study using an animal model in particular to pathological evaluation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 07 May 2020

<https://doi.org/10.5256/f1000research.24725.r62925>

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Guang Hong

Liaison Center for Innovative Dentistry, Graduate School of Dentistry, Tohoku University, Sendai, Japan

This paper needs minor revision. I would make the following comments.

Introduction:

1. Please add the hypothesis of your study in this section.

Methods:

1. In your study, you use the 16-18 weeks rat. I think it is a little bit old, how do you think? Please add the reason why you choose this age rat in the discussion section.

Discussion:

1. The answer to the hypothesis of this study should be included in the discussion section.
2. Natural ingredients are difficult to obtain stable properties. Please add the discussion regarding this issue in the discussion section.
3. Please add the clinical implications of your study and the clinical significance of the findings in the discussion section.
4. What is the limitation of this study? You should indicate in this section.

Figure:

1. Fig. 1 and Fig. 2: Please add the result of the statistical analysis.
2. Fig. 3 to Fig. 7: Please use arrows to indicate the point that you want to explain.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biomaterials and tissue engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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