

## Comparison of Propolis and Calcium Hydroxide in terms of Mineralization and Cytotoxicity Using Dental Pulp Stem Cells

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### Abstract

**Objectives:** This study aimed to compare the in vitro cytotoxic activity of propolis, a bioactive material made by the honeybee, and calcium hydroxide (CH) and their effect on formation of mineralized nodules by human dental pulp stem cells (HDPSCs).

**Methods:** In this in vitro study, HDPSCs were obtained from the Cellular and Molecular Oral Biology Laboratory of School of Dentistry, Shahid Beheshti University of Medical Sciences. In order to evaluate the proliferative effect of propolis and CH, HDPSCs were incubated with different concentrations of propolis (0-32mg/mL) and CH (0-4.8 mg/mL). Twenty-four and 48 hours later, the methylthiazolyl diphenyl-tetrazolium bromide (MTT) assay was carried out to evaluate the proliferation potential and viability of HDPSCs treated with propolis and CH. The effect of propolis and CH on mineralization of HDPSCs was assessed by alizarin red staining.

**Results:** The MTT assay revealed that propolis at its highest concentration caused the greatest proliferation after 24 and 48 hours. Alizarin test showed that the lowest concentrations of CH and propolis at 14 days induced the formation of calcium nodules but at 21 days, propolis was deposited on the cells and calcification was not well recognizable.

**Conclusion:** Propolis led to higher cell vitality at all concentrations in comparison to CH. However, due to its deposition on the cells, its effects on mineralization at 48 hours could not be determined.

**Key Words:** Calcium Hydroxide; Dental Pulp; Stem Cells; Propolis; Tooth Calcification

### How to cite:

Ahangari Z, Tabatabaei FS, Hakimi N, Jalili M, Ghodsian B, Nakhaee M. Comparison of Propolis and Calcium Hydroxide in terms of Mineralization and Cytotoxicity Using Dental Pulp Stem Cells. *J Dent Sch* 2016; 34(2): 66-72.

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Received: 07.06.2016  
Accepted: 15.06.2016

## Introduction

Nearly all people are at risk of dental caries and subsequent pulpitis. In deep cavities, the carious lesion penetrates through the dentin towards the pulp tissue resulting in its irritation and subsequent exposure. In such cases, the patients have to undergo root canal therapy and accept the related potential risks. Therefore, measures for

preservation of pulp vitality are highly preferred.

Use of stem cells created a new era in tissue engineering. Stem cells are initial cells that can differentiate and replace degenerated cells in different parts of the body. Dental pulp is a rich source of stem cells. The most important property of DPSCs is their ability to regenerate dentin-pulp-like complex, which consists of mineralized matrix and

tubules covered with odontoblasts and fibrous tissue containing blood vessels (1).

Propolis is a resin compound derived from honeybee wax. In dentistry, propolis is used for its anti-microbial properties. Propolis protects teeth against caries. Also, 30% propolis solution has been recommended for root canal cleaning during endodontic therapy (2). Its resinous form has also been recommended for use as a pulp-capping agent in vital pulp therapy (3). Flavonoids of propolis may trigger the formation of reparative dentin and by stimulating the release of transforming growth factor beta, they may delay pulp inflammation and accelerate the synthesis of collagen by pulp cells (2).

Calcium hydroxide has long been used in root canal therapy to induce the formation of dentinal bridge, for apexification and as an intra-canal medicament. However, this material is potentially toxic due to its high pH. Its anti-microbial activity is temporary and its high solubility leads to subsequent microleakage (3). Its cytotoxicity against pulp and periodontal fibroblasts is almost 10 times more than that of propolis (4). It has been shown that CH is ineffective for in-vitro destruction of bacteria (5).

In the present study, proliferation of DPSCs and their mineralization under the effect of propolis were evaluated and compared with CH as the gold standard.

## Methods

Propolis was collected from the beehives in Azerbaijan Province of Iran and was kept desiccated pending its processing. The solid propolis (5g) was ground and transferred to

an Erlenmeyer flask containing 100 mL of 96° ethanol. The Erlenmeyer flask was placed on a shaker for 24 hours. Undissolved particles were filtered using a filter paper. Next, 100 mL of ethanol was added to the sample again and the solution was placed on a shaker for another 24 hours. This process was repeated for three times. The solution was transferred to a distillation machine under vacuum and the ethanolic extract was derived. This extract had a resinous consistency (6).

The stock solutions of propolis were prepared in dimethyl sulfoxide or ethyl alcohol and subsequently diluted in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co, St. Louis, MO, USA).

Human dental pulp stem cells obtained from the Cellular and Molecular Oral Biology Laboratory of School of Dentistry, Shahid Beheshti University of Medical Sciences were defrosted and grown as a confluent monolayer in DMEM containing 2mM L-glutamine, 1vol.% antibiotic/antimycotic solution, and 10 vol.% fetal bovine serum at 37°C under 5% CO<sub>2</sub> and 95% air. The fourth passage cells were detached from the culture flask using 0.5% trypsin, centrifuged and re-suspended in 1 mL of culture medium. After staining with trypan blue (1:1 v/v), the viable cells were counted using a Neubauer chamber and seeded in 96-well plates at a density of 3×10<sup>3</sup> cells /well for 24 hours to allow adhesion.

The MTT assay was conducted to evaluate the proliferation potential and viability of HDPCs treated with propolis or CH (Merck, Darmstadt, Germany). Four groups were designed. The two test groups were

treated with propolis and CH. These medicaments were prepared in serial concentrations of 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/mL for propolis and 0.035, 0.075, 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 mg/mL for CH. Finally, the two test groups were divided into eight subgroups for the prepared dilutions and each concentration was tested in 12 wells. The positive control group was treated with distilled water and the negative control group with DMEM. The MTT assay was carried out after 24 and 48 hours. Five mg/mL of MTT (Sigma Aldrich, St. Louis, MO, USA) solution was prepared by dissolving MTT in phosphate buffered saline, and this solution was filter-sterilized. The MTT was diluted (100  $\mu$ L into 900  $\mu$ L) in DMEM. The cells were treated with diluted MTT solution for 4 hours at 37°C. The solution was removed, and dimethyl-sulfoxide was added to dissolve the formazan crystals. The optical density of the metabolized MTT was measured by ELISA reader (Anthos 2020, Salzburg, Austria) at 570 nm.

The effect of propolis and CH on the mineralization of stem cells was assessed by alizarin red staining. A total of  $5 \times 10^4$  cells/well were seeded in two six-well plates, each containing the negative control group with normal culture medium, the positive control group with mineralization induction medium supplemented with 5mM  $\beta$ -glycerophosphate and 10 nM dexamethasone (Sigma, St. Louis, MO, USA), and four experimental groups with CH (at different concentrations) or propolis (at different concentrations).

The results were compared after 14 and 21 days. The experimental procedure included

the fixation phase consisting of growth medium removal, followed by phosphate buffered saline washing, and the final phase of placement in 4% paraformaldehyde solution (pH of 7.4) for 30 minutes. The fixed cells were washed with distilled water and stained with 2% alizarin red stain at a pH of 4.2–4.4 for 30 minutes. The remaining dye was washed with distilled water, and the cells were rinsed again. Finally, the stained cells were photographed.

In the propolis and CH groups, the proliferation potential and viability of HDPSCs were analyzed using independent samples t-test. This test was also used to compare the proliferation potential and viability of HDPSCs between propolis and CH groups. Paired t-test was used to assess the viability of HDPSCs in the propolis group and CH groups at 24 and 48 hours. The data were expressed as mean  $\pm$  standard deviation. Statistical significance of differences between the control and experimental groups was analyzed using SPSS version 16.0 (SPSS Inc., IL, USA).  $P < 0.05$  was considered statistically significant.

## Result

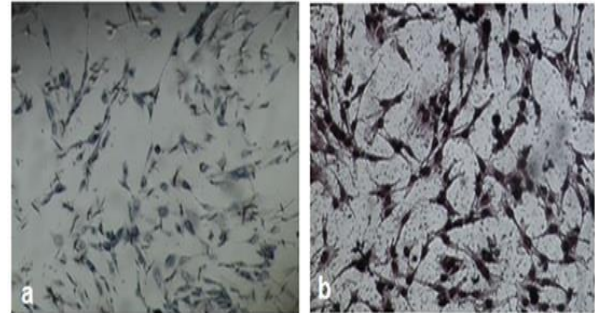
Before the MTT assay, samples were evaluated by a light microscope. None of the tested concentrations of propolis or CH led to cell necrosis, but all cells present in the positive control group had been necrotized. The concentrations of propolis and particularly the highest concentration caused more proliferation in comparison to the negative control group, which only contained normal culture medium (at both

24 and 48 hours). In the CH group, no difference was observed with the control group.

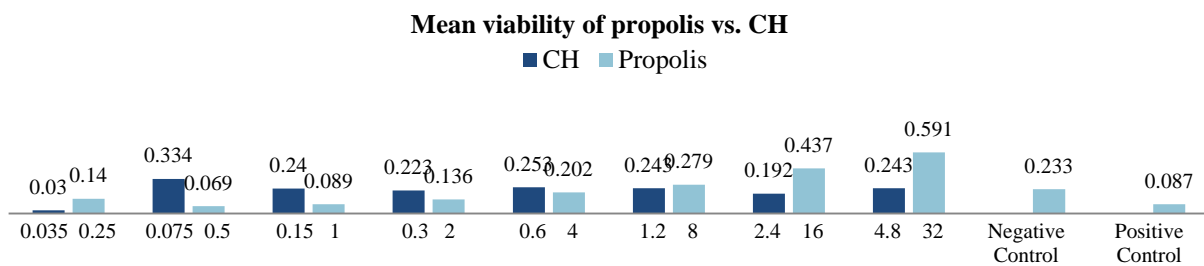
Microscopic observation of cells treated with propolis and CH after the MTT assay revealed the formation of violet formazan crystals after both time intervals in all groups (Figure 1). Therefore, none of the tested concentrations were toxic for the cells; whereas, all cells in the positive control group had been necrotized.

The results of the MTT assay showed that the highest concentration of propolis led to the highest amount of proliferation in DPSCs while lower concentrations led to

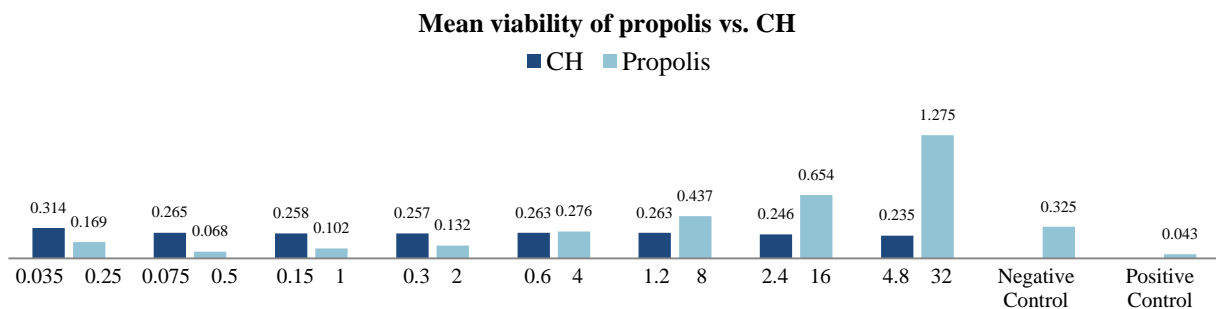
less proliferation. This difference was statistically significant ( $P < 0.05$  after 24 hours and  $P < 0.01$  after 48 hours; Diagrams 1 and 2)



**Figure 1- Formazan crystals under microscopic evaluation with low magnification (a) and high magnification (b).**



**Diagram1- MTT assay after 24 hours**



**Diagram 2- MTT assay after 48 hours**

Cells treated with propolis had significantly greater proliferation after 48 hours in comparison to 24 hours (the mean difference of cell viability and proliferation in propolis after 24 and 48 hours was 0.11357,  $P < 0.007$ ). However, in the CH group, no

significant difference was observed between the two time points (mean difference of 0.02525,  $P = 0.51$  by paired t-test). No statistically significant difference was observed between CH and propolis after 24 hours (mean difference of 0.00209,  $P = 0.94$ ).

After 48 hours, the mean cell vitality in the propolis group was significantly higher than that in the CH group (mean difference of 0.08623,  $P=0.08$ ).

In evaluation of mineralization, mineralized nodules were observed by alizarin red staining in the CH group with 0.035mg/mL concentration in osteogenic medium after 14 and 21 days and in the propolis group with 0.25mg/mL concentration in induction culture medium after 14 days. Calcium hydroxide with 4.8mg/mL concentration led to cell necrosis. Propolis in both concentrations deposited at 21 days and therefore cells or calcified nodules could not be observed.

## Discussion

In the present study, proliferation of DPSCs and their mineralization under the effect of propolis were evaluated and compared with CH as the gold standard. Desirable properties of CH include its antibacterial and biologic effects i.e. neutralization of bacterial lipopolysaccharides, antiresorptive activity and stimulation of hard tissue formation. Nonetheless, CH is potentially toxic due to its high pH (5). Its antimicrobial activity is temporary and it is highly soluble leading to microleakage (6).

Kaida et al. (7) in 2008 reported that Vitapex containing CH led to formation of dentinal bridge but it was very porous and carried the risk of bacterial infection. Therefore, this material is not favorable for this treatment. The material used for this purpose in the current study was propolis, which is derived from honeybees and is available at a very low cost. Although the effect of propolis on

DPSCs has not been studied before, various studies have shown the effect of propolis on other tissues and cell lines (8).

In the current study, the MTT assay was carried out to evaluate the proliferation potential and viability of HDPSCs treated with propolis or CH. The effect of propolis and CH on the mineralization of stem cells was assessed by alizarin red staining of HDPSCs.

Ahangari et al. (9) in 2012 stated that propolis resulted in formation of regular tubular dentin while Dycal enhanced osteodentin (low quality dentin) formation (3). In another study, CH and mineral trioxide aggregate were compared in terms of cytotoxicity and it was revealed that both materials decreased the number of fibroblasts.

On the other hand, Al-Shaher et al. (4) in 2004 compared the effects of propolis and CH on pulp and periodontal ligament fibroblasts and noted that very high concentrations of propolis in comparison to CH can preserve the viability of pulp and periodontal ligament cells indicating the fact that propolis is a biocompatible material. Some studies evaluated the effects of propolis on viability of cells in time durations less than 24 hours (4,10,11). In the current study, cell vitality was assessed after 24 and 48 hours and the obtained results revealed that cell proliferation after 48 hours was much more compared to 24 hours.

The current study showed that the vitality of HDPSCs in the propolis group was more than the value in the CH group. On the other hand, Al-Shaher et al. in 2004 showed the cytotoxicity of CH to be nearly 10 times more than that of propolis (4).

In this study different concentrations of propolis were used and since increasing the concentration of propolis led to increase in vitality and proliferation of cells, its biocompatibility was confirmed. In the current study assessment of mineralization was done at days 14 and 21, which were in accord with the study by Paola-Silva *et al.* who also investigated mineralization following exposure to CH at days 14 and 21 (12).

In the current study, standard culture methods were applied, and control samples were used for comparison of CH and propolis. The effect of propolis on mineralization cannot be definitely confirmed or refuted in this study because of the deposition of this material on the cell layer and masking of cells, which made the detection of cells almost impossible.

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## Conclusion

Evaluation of the effect of propolis on DPSCs revealed that exposure of samples to propolis in comparison to CH resulted in higher in-vitro proliferation and viability of HDPSCs after 24 and 48 hours. This effect was time- and dose-dependent and higher concentrations and longer exposure periods were associated with a better outcome. However, in the present study calcified nodule formation was not observed because of the deposition of propolis.

## Acknowledgments

Authors wish to acknowledge the financial support provided by the Iran Research Dental center.

**Conflict of Interest:** “None Declared”

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