The cytotoxicity test of calcium hydroxide, propolis, and calcium hydroxide-propolis combination in human pulp fibroblast

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INTRODUCTION

Pulp capping is frequently used in dental restoration to prevent dental pulp from necrosis after being exposed or nearly exposed during a cavity preparation. The application of calcium hydroxide (Ca(OH)₂) on the surface of pulp tissue to stimulate the formation of dentine reparative. The aim of pulp capping is to maintain pulp vitality by protecting it from bacterial penetration and enhance the formation of dentine bridges. The success of direct pulp capping depends on its ability to close the dentinal tubules, not to irritate the pulp, but to protect the pulp from mechanical, chemical, and bacterial irritation.

Ca(OH)₂ has alkali properties for antibacterial and ability to stimulate reparative dentin formation. Ca(OH)₂ is the gold standard for direct pulp capping, but its high

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pH (12.5) can create necrosis in the pulp tissue if contact directly to Ca(OH)$_2$ substances.\textsuperscript{[5]} The application in a certain concentration cultivates fibroblast necrosis which can damage pulp tissue.\textsuperscript{[6]} Ca(OH)$_2$ applied to the dental pulp cells in a few hours initiates necrosis due to its alkaline pH. While in a long term, it undergoes changes in physical features leading to non-dense dentine reparative formation due to the occurrence of dentine bridge discontinuities in the area of necrosis called "tunnel defects." This can trigger the penetration of bacteria into dental pulp, leading an irritation to the dental pulp.\textsuperscript{[8]}

Therefore, many researchers investigate other alternative materials, one of which is natural ingredients. In recent years, many studies have developed alternative materials from nature to use in the medical field. Propolis is one of natural products created by bees, which consists of resin, bal, beeswax, essential oils, pollen, and other organic ingredients. Propolis known as an anti-bacterial, anti-fungal, anti-viral, anti-tumor, anti-oxidant, immunomodulatory, and plays an important role in the healing process.\textsuperscript{[7]}

In the dental restorative field, propolis can reduce dentin permeability and be used as direct pulp capping material because it can form dentine reparative. In addition to reducing inflammatory reactions, bacterial infections and necrosis, it can also produce denser dentine layers through the stimulation of endogenous stem cells due to caffeic acid phenethyl ester (CAPE), which is one of the active ingredients in propolis.\textsuperscript{[8]} CAPE can stimulate and inhibit the cell proliferation in the certain concentrations.\textsuperscript{[7,8]} The use of Ca(OH)$_2$ combined with propolis is highly recommended because Ca(OH)$_2$ is able to dissociate into calcium and hydroxyl ions, thus, they can diffuse well into the dentinal tubules.\textsuperscript{[10]}

Propolis is a better natural material to increase the anti-microbial action in Ca(OH)$_2$.\textsuperscript{[10]} Ca(OH)$_2$ combined with propolis has biocompatibility as evidenced in the previous studies. One of the studies evaluated the inflammatory process in rat connective tissue in the combination treatment of Ca(OH)$_2$ and propolis with a ratio of 1:2. It was found that Ca(OH)$_2$ and propolis are able to reduce inflammation significantly and biocompatible with rat connective tissue.\textsuperscript{[11]} Other studies expressed that the combination of Ca(OH)$_2$ and propolis with a ratio of 1:1 is proven to be effective in treating pulpotomy in hard-tissue formation.\textsuperscript{[12]} This combination is expected to obtain better quality than Ca(OH)$_2$ alone, because of its anti-bacterial features and the ability of propolis in increasing the fibroblast cell proliferation. In Ca(OH)$_2$-propolis combination, it contains CAPE as an anti-oxidant that can bind free radicals, mostly are hydroxyl ions (OH), which can prevent the occurrence of lipid peroxidation and cell death.\textsuperscript{[8,13]}

Several studies have been conducted on the biocompatibility of Ca(OH)$_2$ and propolis material, but no studies have measured the viability of human pulp fibroblasts after the administration of Ca(OH)$_2$-propolis combination. The determination of dose in the combination of Ca(OH)$_2$ and propolis in this study was obtained through a preliminary research that had been done previously. It found that the nontoxic dose of Ca(OH)$_2$, which can be used in the human pulp fibroblast culture is 10 μg and for propolis are 10 μg, 15 μg, and 20 μg. The aim of this study was to investigate the viability of human pulp fibroblast after the administration of Ca(OH)$_2$ propolis, and its combination.

**MATERIALS AND METHODS**

This study was approved by the Ethics Committee of Faculty of Dentistry in Airlangga University with reference number 174/HREC/FODM/IX/2017. This research was a laboratory experimental study with posttest only controlled group design. The sample was human pulp fibroblast culture derived from premolar teeth of 16-year-old patients.

Ca(OH)$_2$ used was in pure dosage form, whereas propolis was utilized from raw propolis collected from Apis sp. of a bee farm in Lawang, Indonesia, which then was extracted with 96% alcohol and filtered with filter paper.

The pulp obtained from premolar tooth were cultured in petri dish with complete media consisted of 10% Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich®, Darmstadt, Germany), 10% Fetal Bovine Serum (FBS, Gibco™, South America Origin), trypsin 1%, fungizone 0.5 μg/ml, and pen strep 1%-2% (Gibco™, South America Origin). Place petri dish in 5% CO$_2$ incubator (Mammert GmbH+Co.KG, Schwabach, Germany). Media was replaced every 3- or 4-day until fibroblast cells appeared on the edges of dental pulp tissue in form of a long and spindle shape.

After 80% confluent cells, the fibroblast was harvested and being trypsinization, then centrifuged. Insert the cells into the flask, replaced by the complete media every 3-4 days until the 3rd passage. Then, wash out the medium. Add 1–2 ml trypsin-EDTA 0.25% (Gibco™, E.U-approved, South America Origin) to release the cells, place the cells into the centrifuge tube, add medium, then centrifuged for 10 min at 1500 rpm. Remove the supernatant, added 1 ml complete medium, homogenized, and then cell calculation was carried out.

The samples were divided into seven groups; Group 1 (10 μg Ca(OH)$_2$); Group 2 (10 μg propolis); Group 3 (15 μg propolis); Group 4 (20 μg propolis); Group 5 (Ca(OH)$_2$-propolis 1:1); Group 6 (Ca(OH)$_2$-propolis 1:1.5); and Group 7 (Ca(OH)$_2$-propolis 1:2). Put into 96 wells plate (SPL Life Sciences Co., Ltd., Gyeonggi-do, Korea) for 100 μl each with a density of 2 × 10$^4$/20,000 cells/well, left it for 1–2 h. Incubate the cells in incubator for at least 4 h. Then, add 100 μl of Ca(OH)$_2$ propolis extract,
and a combination of Ca(OH)$_2$ and propolis extract, incubate again in incubators for 24 h with 5% CO$_2$ levels, 37°C, and 98% humidity. After 24 h, put 100 ml of 5 mg 3-(4,5-dimethylthiazol-2-ⅱ)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen’s Vybrant® M, Carlsbad, California, USA) + 1 ml phosphate buffer saline (PBS, Merck®, Darmstadt, Germany) + 9 ml DMEM in each well and incubated for 4 h until formazan was formed. Then, 100 μl sodium dodecyl sulfate 10% (SDS, Merck®, Darmstadt, Germany) stopper solution was integrated into 0.01 N HCl in all wells, incubated again for overnight.

The cell calculation was conducted using ELISA Reader (Bio-Rad® 680 XR Microplate Reader, USA) with 550-nm wavelength. The living pulp fibroblast cells colored in formazan blue. The calculation of cell viability percentage using formula is shown in Figure 1.

The Kolmogorov–Smirnov test was conducted to find the normality of data followed by Levene’s test to determine the homogeneity ($P > 0.05$). From the results of data analysis, it was obtained data with normal and homogeneous distribution. Thus, one-way analysis of variance test and Tukey-honest significant difference (HSD) test were performed to determine differences between groups ($P < 0.05$).

**RESULTS**

The highest viability human dental pulp fibroblast showed in Group 5, meanwhile, the lowest viability showed in Group 4 [Figure 2 and Table 1]. There was significant difference between groups ($P = 0.000$; $P < 0.05$). While Tukey-HSD result showed significant difference between groups with exception Group 2 and Group 3; Group 5 and Group 6 [Tables 1 and 2].

**Table 1: The mean and standard deviation value of dental pulp fibroblast’s viability after administration Ca(OH)$_2$, propolis and its combination in each group**

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 μg Ca(OH)$_2$</td>
<td>63.64±2.714</td>
</tr>
<tr>
<td>2</td>
<td>10 μg propolis</td>
<td>80.03±3.013</td>
</tr>
<tr>
<td>3</td>
<td>15 μg propolis</td>
<td>81.63±4.615</td>
</tr>
<tr>
<td>4</td>
<td>20 μg propolis</td>
<td>32.65±4.841</td>
</tr>
<tr>
<td>5</td>
<td>Ca(OH)$_2$-propolis 1:1</td>
<td>106.63±40.81</td>
</tr>
<tr>
<td>6</td>
<td>Calcium hydroxide-propolis 1:1.5</td>
<td>103.64±3.773</td>
</tr>
<tr>
<td>7</td>
<td>Calcium hydroxide-propolis 1:2</td>
<td>90.12±2.532</td>
</tr>
</tbody>
</table>

SD: Standard deviation, Ca(OH)$_2$: Calcium hydroxide

**Figure 1:** Cell viability percentage formula. OD: Optical density, % cell viability: Percentage of living cells after testing

**DISCUSSION**

In this study, we found that the viability of human dental pulp fibroblast after application of Ca(OH)$_2$ in Group 1 is smaller compared to the other groups. This is probably due to a greater number of OH-ions in Group 1 than the rest of the groups.

OH-ion can lead to lipid peroxidase, protein denaturation, and interference with DNA as OH-ions are highly reactive free radicals. This cause damage of cell membrane, protein breakdown, and DNA damage which can trigger cell death.[6] In Ca(OH)$_2$-propolis combination group, the number of OH-ions is less than Ca(OH)$_2$ group. It probably because Ca(OH)$_2$-propolis combination contains CAPE, a powerful anti-oxidant that chain free radicals, mostly hydroxyl ions (OH$^-$), thus, preventing the occurrence of lipid peroxidation and cell death.[13] Propolis can bind free radicals by releasing hydrogen ions to bind hydroxyl ions, thus, free radicals are reduced, preventing the occurrence of oxidation reactions in the cells.[14] Ca(OH)$_2$-propolis combination may change pH after combining with Ca(OH)$_2$. This pH change supports the fibroblast proliferation activity, as it has been known that fibroblast proliferation increases significantly at pH 8.5–9.5.[15] This study exhibits 6% increase of proliferation in combination groups with 1:1 ratio and 3% with 1:1.5 ratio. Thus, the viability of fibroblast cells in Ca(OH)$_2$ group with pH 12 is smaller than 10 μg and 15 μg propolis group, and all combination groups.

The viability of fibroblast in Ca(OH)$_2$ induction is >20 μg propolis induction. At this dose, there is a possibility that CAPE can interfere cell hemostasis, because it can trigger the occurrence of apoptosis/cell death through expression of p53 protein which can activate the apoptotic cell component and cause cell death.[19] The fibroblast cell death increases at certain doses along with the increasing concentration of propolis.[7,14] CAPE at certain doses can induce fibroblast cells proliferation to a certain concentration level; since after

**Figure 2:** Mean and standard deviation of dental pulp fibroblast’s viability after administration Ca (OH)$_2$, propolis, and its combination in each group
passing the concentration limit and it triggers fibroblast cell death along with the increasing doses of propolis.\[7\] In this study, this might cause the viability of 20 μg propolis group is smaller than the Ca(OH)\(_2\) group.

The cell viability of 10 μg propolis group is similar with 15 μg propolis group due to CAPE concentration limit in triggering fibroblast proliferation is at the doses between groups 10 μg and 15 μg propolis, in 20 μg propolis group, an increase of CAPE is able to trigger fibroblast cell death which lead to the least viability. The cell viability of Ca(OH)\(_2\)-propolis combination group at 1:1 ratio is similar with 1:1.5 ratio, possibly considering that CAPE triggers fibroblast's proliferation at combination groups with 1:1 and 1:1.5 ratio. At low doses, CAPE can increase cell proliferation activity by increasing transforming growth factor β, a cell growth regulator which can stimulate fibroblast cell proliferation.\[16,17\] The fibroblast cell death increases at the certain doses as the concentration of flavonoids rises in propolis.\[7,9\]

The viability of fibroblasts induced by 10 μg, 15 μg, and 20 μg propolis is smaller than Ca(OH)\(_2\)-propolis combination with 1:1, 1:1.5, and 1:2 ratios. In this study, there is 6% increase of proliferation in combination group with 1:1 ratio and 3% increase in 1:1.5 ratio. It may be due to the hydroxyl ions in the combination groups with 1:1 and 1:1.5 ratio is lesser. In addition, the pH in the combination group may change after it combine with Ca(OH)\(_2\),\[3\] this pH change supports fibroblast cell proliferation. CAPE can trigger fibroblast cell’s proliferation at the doses of combination groups from all ratios. The viability of fibroblast cells in the Ca(OH)\(_2\)-propolis combination groups is greater than the 10 μg, 15 μg, and 20 μg propolis group.

The Ca(OH)\(_2\)-propolis combination group with 1:1 and 1:1.5 ratio have greater viability than the 1:2 ratio. The combination group with 1:1 and 1:1.5 ratio contain the optimal CAPE concentration to trigger fibroblast cell proliferation. The increasing of CAPE in 1:2 ratio already can trigger fibroblast cells death. Thus, the viability of 1:2 ratio is smaller due to the increasing cell death which goes along with the improving propolis concentration. The CAPE’s active ingredient in propolis can interfere the cell hemostasis, since it can lead to the occurrence of cell death through the expression of p53 protein which activates the apoptotic cell component and eventually fosters the cell death.\[7\] As demonstrated in Ca(OH)\(_2\)-propolis combination group with 1:2, ratio, there are more fibroblast death occur, therefore, the viability is smaller.

**CONCLUSIONS**

The viability of human pulp fibroblasts after administration of Ca(OH)\(_2\)-propolis combination with ratio 1:1 is greater than that of the application of Ca(OH)\(_2\) and propolis alone with various ratios.

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**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**


