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Original Article

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

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Abstract

Calcium hydroxide ($Ca(OH)_2$) is the gold standard material used for pulp-capping but still has a high failure rate. Thus, an alternative material is needed, one of which is propolis. The combination of $Ca(OH)_2$ propolis is expected to have better quality and to be biocompatible. The aim of this study is to investigate the viability of human pulp fibroblast after the administration of $Ca(OH)_2$, propolis, and its combination. Human pulp fibroblast culture derived from premolar teeth of 16-year-old patients, 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 (calcium hydroxide-propolis 1:2). They were placed in a 96 wells plate and put into incubator for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test was conducted to calculate the viability of human pulp fibroblasts. The data were analyzed statistically using Kolmogorov–Smirnov, Levene's test, one-way analysis of variance, and Tukey-honestly significant difference ($P < 0.05$). The number of living human pulp fibroblast after the administration of $Ca(OH)_2$ and propolis combination is greater than the application of $Ca(OH)_2$ or propolis with significant different between groups ($P < 0.05$). The viability of human pulp fibroblasts after the administration of $Ca(OH)_2$-propolis combination is greater than that of the application of $Ca(OH)_2$ and propolis alone.

Key words: Calcium hydroxide, cytotoxicity test, human pulp fibroblast, propolis, pulp capping

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.[1] The application of calcium hydroxide ($Ca(OH)_2$) on the surface of pulp tissue to stimulate the formation of dentine reparative.[2] 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.[3]

$Ca(OH)_2$ has alkali properties for antibacterial and ability to stimulate reparative dentin formation.[4] $Ca(OH)_2$ is the gold standard for direct pulp capping, but its high

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pH (12.5) can creates necrosis in the pulp tissue if contact directly to $Ca(OH)_2$ substances.[5] The application in a certain concentration cultivates fibroblast necrosis which can damage pulp tissue.[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 nondense 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.[4]

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, balm, 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.[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.[8] CAPE can stimulate and inhibit the cell proliferation in the certain concentrations.[7,9] 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.[10]

Propolis is a better natural material to increase the anti-microbial action in $Ca(OH)_2$.[10] $Ca(OH)_2$ combined with propolis has biocompatibility as evidenced in the previous studies. One of 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.[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.[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.[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/HRECC.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®, Darmstatd, 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 (Memmert 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,

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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-il)-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), propolis and its combination in each group

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

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

$$\% \text{ Cell Viability} = \frac{\text{OD Sample}}{\text{OD Control}} \times 100\%$$

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.[9] The fibroblast cell death increases at certain doses along with the increasing concentration of propolis.[7,9] CAPE at certain doses can induce fibroblast cells proliferation to a certain concentration level; since after

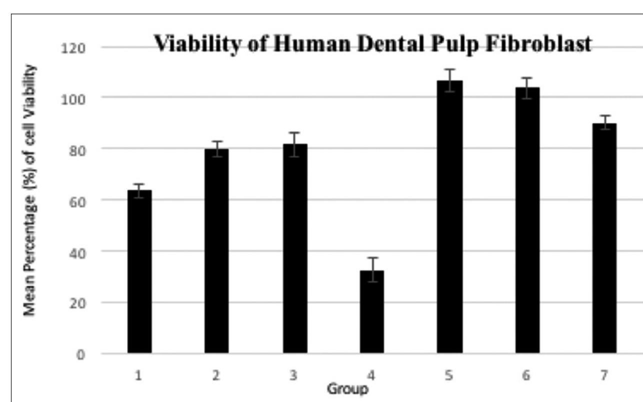

Figure 2: Mean and standard deviation of dental pulp fibroblast's viability after administration Ca (OH), propolis, and its combination in each group

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Table 2: Test results of pulp fibroblast cells' viability difference in the induction of calcium hydroxide-propolis combination

Group	Propolis 10 μg	Propolis 15 μg	Propolis 20 μg	Combination 1:1	Combination 1:1.5	Combination 1:2
Ca(OH)	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
Propolis 10 μg		0.094	0.000*	0.000*	0.000*	0.004*
Propolis 15 μg			0.000*	0.000*	0.000*	0.019*
Propolis 20 μg				0.000*	0.000*	0.000*
Combination 1:1					0.865	0.000*
Combination 1:1.5						0.000*

*Significant $P<0.05$

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 fibroblas 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] 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.

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