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# The Effect of the Combination of Calcium Hydroxide, Carbonate Apatite, and Ellagic Acid on The Viability and Proliferation of Fibroblast Cells

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## Abstract

The failure rate of calcium hydroxide [Ca(OH)2] as a pulp protection material is 13.64% one year after treatment. Carbonate apatite [CO3Ap] in phosphate-buffered saline solution has the ability to carry and release drugs that function to regulate the pH and osmolarity balance of cells. This study aims to develop a formula for Ca(OH)2 combined with CO3Ap, with ellagic acid (EA) solvent, and investigate its effects on the cytotoxicity and proliferation of fibroblasts. Material samples were prepared by mixing various proportions of Ca(OH)2 and CO3Ap dissolved in 12.5% EA solvent. Baby hamster kidney-21 cells were prepared and incubated in a 96-well microplate for 24 h and 72 h with media containing the material samples. Cell viability was monitored by measuring the absorbance of each microplate using an ELISA reader. After 24 h, group 3 (50% CO3Ap wt%) showed the highest percentage of viable cells; after 72 h, the highest percentage was found in group 3 (50% CO3Ap wt%). Group 5 (87.5% CO3Ap) had the lowest percentage value of living cells after 24 and 72 h treatment. The difference between groups 3 and 5 at 24 hours was significant (p<0.05). The combinations in groups 1,2,3 and 4 are nontoxic and able to stimulate the proliferation of fibroblast cells and thus have ideal characteristics as a new candidate material for pulp capping.

## Introduction

Calcium hydroxide [Ca(OH)2, Merck, Germany] has been the gold-standard pulp protection material since 1921, as it has good antibacterial properties. However, treatment interventions such as tooth extraction and root canal treatment are often necessary because the failure rate of Ca(OH)2 one year after pulp capping treatment is 13.64% [1]. Ca(OH)2 in direct contact with exposed pulp can cause necrosis on the surface of the tissue because of its high alkalinity [2].

Carbonate apatite (CO3Ap, Balai Besar Keramik, Indonesia) is a biocompatible material that has the ability in phosphate-buffered saline (PBS) solution to carry and release drugs that function to regulate the pH and osmolarity balance of cells [3]. Increased carbonate increases hydroxyapatite solubility, reducing its crystalline properties and changing the crystal morphology. Carbonate ions replace apatite anions in a biological environment, inducing adhesion, proliferation, and metabolic activity in ~~regenerated~~ cell regeneration [3, 4]. Treatment with the combination of CO3Ap and Ca(OH)2 can stimulate dentin remineralization by forming reparative dentin, replacing calcium and phosphate in damaged vital teeth.

Ellagic acid (EA) is a natural, bioactive phenolic and flavonoid compound. Several studies have shown 1–3% EA to have antioxidant, anti-inflammatory, immunomodulatory, antitumorigenic, anticancer, neuroprotective, hepatoprotective, and cardioprotective potential [5–9]. A mixture containing 3% EA in Ca(OH)2 increased the survival and proliferation of fibroblast cells, with a viability of 91.9% after 72 hours of treatment, and 12.5% EA has been shown to inhibit the growth of *Enterococcus faecalis* [10, 11].

This study aimed to combine Ca(OH)2 and CO3Ap in varying proportions with 12.5% EA solvent and to determine the effect of the material on the cytotoxicity and proliferation of fibroblasts.

## Materials and Methods

### Calcium Hydroxide/Carbonate Apatite Preparations

Materials were prepared according to Table 1 by mixing Ca(OH)2 and CO3Ap dissolved in 12.5% EA solvent at a ratio of 1:1 (w/w). The 12.5% EA solvent (w/w percentage) was prepared by adding 12.5 mg to 87.5 mg of a solution of 90% distilled water and 10% polyethylene glycol 400.

Table 1: Combinations of powder mixture Ca(OH)2 and CO3Ap

|  |  |  |
| --- | --- | --- |
| Group | Ca(OH)2 | CO3Ap |
| 1 | 100% | 0% |
| 2 | 60% | 40% |
| 3 | 50% | 50% |
| 4 | 25% | 75% |
| 5 | 12.5% | 87.5% |

### Fibroblast Cell Preparation

The baby hamster kidney-21 (BHK-21, #CCL-10) fibroblast cell line used in the study has been the laboratory standard for observation of biological processes since 1961 and is suitable for maintaining or growing [12]. During the maintenance stage of fibroblast cell culture, in Roux culture bottles, the cells were washed with 10 ml PBS (Merck) and detached from the culture bottles by the addition of 5 ml trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, PAN-Biotech, Germany) then kept at 37°C for 2–3 minutes for the cell incubation process. The trypsin-EDTA was then inactivated by adding 5 ml minimal essential media nonessential amino acid solution (MEM NEAA, PAN-Biotech, Germany) with 10% fetal bovine serum (FBS, PAN-Biotech, Germany). The cell suspension was moved from the flask into a sterile 15 ml conical tube and centrifuged for 5 minutes at room temperature. The centrifuged supernatant was discarded and the cells resuspended with 10 ml MEM NEAA / 10% FBS, placed in the cell culture flask, and incubated for 72 h at 37°C and 5% CO2.

Cells were counted when confluent, attached, and grown to the bottom of the flask. Cell suspension and trypan blue (Sigma-Aldrich, United States) were pipetted into the hemocytometer using a micropipette (10 µl cells / 10 µl trypan blue) and the number of living cells counted using a binocular microscope [13]. The cells were diluted to prepare a cell suspension with a density of 2 × 104 viable cells / 100 µl.

### Toxicity Test

~~Modified and pure Ca(OH)~~~~2~~~~, 1 mg, was weighed into a 1 ml Eppendorf tube containing MEM,~~ 1 mg of sample of Ca(OH)2/CO3Ap preparations was put into 1 ml of MEM in the Eppendorf tube which was kept at room temperature for 24 hours. Microplates (Biologix, Germany) were divided into six groups: five treatments (Table 1) and one control group. The BHK-21 cell suspension was put in a 96-well microplate (50 µl/well) to which was then added media containing the treatment combinations (100 µl/well). The microplate was incubated at 37°C for 24 and 72 h. After incubation, the microplate was rinsed with PBS and then MTT medium (PAN-Biotech, Germany) was added at 10 µl/well and the plates reincubated for 3 h. Finally dimethyl sulfoxide (Sigma-Aldrich; 50 µl/well) was added to terminate the reaction. The absorbance of each microplate was measured using an ELISA reader (Biobase, China) with a wavelength of 520 nm. The percentage of living cells (% cell viability) was calculated using the formula

where OD is optical density.

### Statistical Tests

Mean absorbance values were tested with one-way ANOVA and significant differences between groups determined using Tukey’s post hoc test. Tests were analyzed using IBM® SPSS® statistics 25.0.

## Results

Group 3 (50% CO3Ap) showed the highest percentage of living fibroblast cells after 24 h treatment. The highest cell viability percentage after 72 h treatment was found in group 3 (50% CO3Ap). Group 5 (87.5% CO3Ap) had the lowest percentage value of living cells after 24 and 72 h treatment (Table 2).

Table 2: Percentage of living fibroblast cells (BHK-21) after incubation with combinations of calcium hydroxide and carbonate apatite with 12.5% EA solvent.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Group | Absorbance | | Cell viability | |
| 24 h | 72 h | 24 h | 72 h |
| Cell control | 0.97 | 1.23 | 100 | 100 |
| 1 | 1.02 | 1.16 | 105.1 | 91.5 |
| 2 | 0.92 | 1.09 | 100.8 | 86.2 |
| 3 | 1.19 | 1.30 | 124.8 | 109.3 |
| 4 | 1.22 | 1.16 | 117.7 | 91.4 |
| 5 | 0.97 | 0.30 | 92.3 | 23.9 |

Figure 1: Eﬀects of combinations of calcium hydroxide and carbonate apatite with 12.5% EA solvent on fibroblast cell viability after 24 h incubation (a) and 72 h incubation (b). Data are shown as mean ± S.E.M. (n = 5). ∗P<0.05 versus Cell control (one-way ANOVA, followed by Tukey’'s post hoc test).

We evaluated the effect of combinations calcium hydroxide and carbonate apatite with 12,5% EA solvent on fibroblast cell viability by comparing treatment groups with control cells {Figure 1 (a) and (b)}. Based on the Tukey test, ~~the difference between groups 3 and 5 at 24 h of treatment was not significant (p>0.05).~~ After 24 h incubation, group 3 has a significant difference with group 5 (p<0,05) After 72 h incubation, group 5 significantly decreased cell viability (p<0,05) compared with the control group. ~~differences between the control group, group 1, group 3, and group 4 were not significant (p>0.05).~~

## Discussion

The CO3Ap used in this study is composed of oxygen (O), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), and calcium (Ca). Calcium concentration was highest, at 38.40 wt%, and silicon the lowest at 0.92 wt%. The Ca/P ratio was 1.67 [14]. Carbonate ions are provided by calcium carbonate (CaCO3) and magnesium carbonate (MgCO3), combined with hydroxyapatite through dry mechanosynthesis. However, the use of CaCO3 can contribute to an excess of Ca2+ ~~Ca~~ [3].

~~Proangiogenic growth factors influence the regulation of nerve and vascular cell function for the proliferation, migration, and growth of new capillaries.~~ Fibroblast cells in the extracellular matrix form and repair connective tissue to preserve anatomical integrity. Fibroblasts serve as stem cells for specialised mesenchymal cell types, such as bone-forming osteoblasts or lipid-filled adipocytes, during embryonic development, homeostasis, repair and remodelling. The viability of fibroblast cells at 24 h observation was more than 50% in all treatment groups. These results show that combinations calcium hydroxide and carbonate apatite (40%, 50%, and 75% CO3Ap) with 12,5% EA solvent ~~CO~~~~3~~~~Ap~~ is not toxic.

Ca/P variations in media can affect the biological response. ~~The Ca/P ratio remained stable when CO~~~~3~~~~Ap was immersed in the media for 24 h~~. The Ca/P ratio has been shown to remain stable when CO3Ap is immersed in MTT media for 24 h; CO3Ap decomposes after 48 h of immersion in MTT medium and levels of Ca and P ions have been observed to increase 72 h after immersion [15]. The Ca/P changes occurred 72 h after immersion, with levels of Ca and P ions increasing. High Ca levels will affect the amount of nitric oxide products in the cells [16]: cell viability decreased at 72 h, which might be due to an increase in nitric oxide. Also, high concentrations of Ca2+ can stimulate the endoplasmic reticulum to release Ca2+ ions into the intercellular fluid so that it can disrupt its homeostasis and mitochondria will experience apoptosis [17].

The combination in group 5, which contained the lowest amount of carbonate [12.5% Ca(OH)2 : 87.5% CO3Ap (wt%)], had the highest toxicity after 72 h of treatment compared with other groups. Carbonate can promote the solubility of hydroxyapatite, which can lead to an increase in Ca2+ and phosphate ions (PO43-) in fibroblast cells. ~~Increasing the amount~~ ~~of inorganic phosphate in the extracellular fluid has a wide impact~~ The amount of inorganic phosphate in the extracellular fluid affects on intracellular homeostasis, cell viability, and cell death. Inorganic phosphate increases when the cell becomes acidic [18, 19].

The pH in cells can change if the cell buffer system is disturbed by a change in the amount of water and inorganic ions in the intracellular fluid. Ca2+ play an important role in eukaryotic cell culture as they are required for important processes such as enzyme activity, cell attachment, motility, tissue formation, cell metabolism, signal transmission, replication, and electrochemical responses. A low concentration of Ca2+ (2 mM) must be preserved in the cytoplasm, being stored in the endoplasmic reticulum. Cell death can be induced by an excess of Ca2+ that will disturb the electrolyte condition of the cell and cause damage to the cell membrane [20, 21].

The effect of the degree of crystallinity in causing cell death depends on the particle size and composition of the material. Smaller crystals find it easier to enter fibroblast cells. CO3Ap crystals are smaller than pure hydroxyapatite crystals because of the substitution of CO32- and Mg2+ into the structure [22, 23].

## Conclusions

The combination of Ca(OH)2 and CO3Ap (50:50) and (25:75) wt% with 12.5% EA solvent showed high fibroblast cell viability and proliferation. This study confirms that this combination is nontoxic and able to stimulate dental pulp proliferation. This combination has ideal characteristics as a new candidate material for pulp capping.

## Conflicts of Interest

The authors declared that there is no conﬂict of interests.

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