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

**C. Septommy1, I. Nirwana2\* and I. Widjiastuti3**

1Doctoral Study Program, Faculty of Dental Medicine , Universitas Airlangga, Surabaya-60132, Indonesia,

2Department of Dental Materials, Faculty of Dental Medicine, Universitas Airlangga, Surabaya-60132, Indonesia,

3Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya-60132, Indonesia.

\*E-mail : [intan-n@fkg.unair.ac.id](mailto:intan-n@fkg.unair.ac.id)

**ABSTRACT**

Calcium hydroxide still has a failure rate of 13.64% after one year of pulp capping treatment. Calcium hydroxide in direct contact with exposed pulp can cause necrosis on the surface of the pulp tissue. Carbonate apatite is one of the biocompatible materials that has the ability to carry and release drug effects in phosphate buffer saline solutions that function to regulate the pH and osmolarity balance of cells. A mixture containing 3% EA in calcium hydroxide increased the survival and proliferation of fibroblast cells, with a viability percentage value of 91.9% 72 hours of treatment and 12.5% EA has been able to inhibit the growth of Enterococcus Faecalis bacteria. This study aims to develop a calcium hydroxide formula combined with various amounts of apatite carbonate with 12.5% EA solvent that can determine the cytotoxicity and proliferation of fibroblasts. Material samples were prepared by mixing Ca(OH)2 and CO3Ap dissolved in 12.5% EA solvent at a ratio of 1:1 (w/w). BHK-21cell line were prepared and incubated 96-well microplate for 24 h and 72 h. 100µl/well media that has been contaminated with samples according to the treatment group. The absorbance of each microplate was measured using an elisa reader. At 24 h Group 4 (75% CO3Ap) showed the highest percentage The highest percentage was found in group 3 (50% CO3Ap) after 72 h. Group 3 and 4 of did not have a significant difference (p>0,05). the combination ratio (50:50) and (25:75) wt% has non-toxic properties and able to stimulate fibroblast cells.

**Keywords:** Calcium hydroxide, carbonate apatite, ellagic acid, proliferation, fibroblast cells

**INTRODUCTION**

Calcium hydroxide [Ca(OH)2,Merck 102407] has been the gold standard pulp protection material since 1921, as it has good anti-bacterial properties. The use of calcium hydroxide as a pulp protection material still needs additional intervention to make up for the deficient properties of calcium hydroxide. Calcium hydroxide still has a failure rate of 13.64% after one year of pulp capping treatment. The failure was followed up with tooth extraction and root canal treatment.1 Calcium hydroxide in direct contact with exposed pulp can cause necrosis on the surface of the pulp tissue because this material has high alkaline properties.2

Carbonate apatite (CO3Ap,Balai Besar Keramik-Indonesia) is one of the biocompatible materials that has the ability to carry and release drug effects in phosphate buffer saline solutions that function to regulate the pH and osmolarity balance of cells.3 The carbonate content in hydroxyapatite will increase its solubility properties so that it will reduce its crystalline properties and change its crystal morphology to facilitate attachment between cells. Carbonate ions will increase its chemical reaction properties by replacing apatite anions when working in a biological environment, so that it can induce adhesion, proliferation, and metabolic activity in regenerated cells.3,4

Ellagic acid (EA,…) is a natural bioactive compound that belongs to the phenolic and flavonoid groups. Several studies have shown 1-3% EA has anti-oxidant, anti-inflammatory, immunodulatory, anti-tumourigenic, anti-cancer, neuroprotection, hepatoprotection, and cardioprotection potential.5–9 A mixture containing 3% EA in calcium hydroxide increased the survival and proliferation of fibroblast cells, with a viability percentage value of 91.9% 72 hours of treatment and 12.5% EA has been able to inhibit the growth of Enterococcus Faecalis bacteria.10,11

This study aims to develop a calcium hydroxide formula combined with various amounts of apatite carbonate with 12.5% EA solvent that can determine the cytotoxicity and proliferation of fibroblasts.

**EXPERIMENITAL**

**Materials and Methods**

**Calcium hydroxide combination preparations**

Material samples were prepared by mixing Ca(OH)2 and CO3Ap dissolved in 12.5% EA solvent at a ratio of 1:1 (w/w) according to Table 1. EA 12.5% solvent (w/w percentage) was prepared by adding 12.5 mg in 87.5 mg of solution (90% Aquades and 10% PEG 400). (Table 1)

Table 1. Combination of the amount of apatite carbonate on calcium hydroxide

with 12.5% EA solvent

|  |  |  |  |
| --- | --- | --- | --- |
| Group | Ca(OH)2 | CO3Ap | EA 12,5% |
| 1 | 100% | 0% | 100% |
| 2 | 60% | 40% | 100% |
| 3 | 50% | 50% | 100% |
| 4 | 25% | 75% | 100% |
| 5 | 12,5% | 87,5% | 100% |

**Fibroblast cell preparation**

The baby hamster kidney-21 (BHK-21, #CCL-10) cell line in the study has been the laboratory standard for observation of biological processes since 1961 and also has guidelines for maintaining or growing fibroblast cells.12 During the maintenance stage of fibroblast cell culture in roux culture bottles, the cells were washed with 10 ml phosphate buffer saline (PBS, Merck) and to detach the cells from the culture bottles by adding 5 ml tripsin-ethylenediaminetetraacetic acid (Tripsin-EDTA, PAN-Biotech) and kept at 37°C for 2-3 minutes for the cell incubation process. Inactivate trypsin-EDTA by adding 5 ml of minimal essential media non essential amino acid(MEM NEAA, PAN-Biotech) with 10% fetal bovine serum (FBS, PAN-Biotech). 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 from the conical tube and resuspended with 10 ml of MEM NEAA added 10% FBS to the cell culture flask and incubated for 72 h at 37°C and 5% CO2. Cell counting is done when cells are confluent in the flask, attached, and grow to the bottom of the flask. Cell suspension and trypan blue (Sigma-Aldrich) were pipetted into the haemocytometer using a micropipette (10µl cells/10µl trypan blue) to count the number of living cells with a binocular microscope.13 The number of viable cells was diluted to prepare a cell suspension with a density of 2x104cells/100µl.

**Toxicity test**

Modified and pure Ca(OH)2 were weighed at 1 mg to be put in 1ml MEM/ependorf, then the tube was kept for 24 hours at room temperature. Microplates (Biologix) were divided into 6 groups with 5 treatments based on table 1 and one group as cell control. BHK21 cell suspension was put in 96-well microplate as much as 50µl/well then adding 100µl/well media that has been contaminated with samples according to the treatment group. The microplate was incubated at 37°C for 24 and 72 h. MTT (PAN-Biotech) was added in the amount of 10µl/well and reincubation for 3 h and added dimethyl sulfoxide (DMSO, Sigma-Aldrich) 50µl/well. The absorbance of each microplate was measured using an elisa reader (biobase®) with a wavelength of 520 nm. Percentage of living cells using the formula:

OD = Optical Density

Cell Viability = Percentage of Living Cell after Test

**Statistical test**

Mean absorbance values were tested with one-way ANOVA and to determine significant differences between groups using Tukey's post hoc. Tests were analysed using IBM®SPSS® statistics 25.0

**RESULTS AND DISCUSSION**

**Results**

Group 4 (75% CO3Ap) showed the highest percentage of living fibroblast cells after 24 h treatment. The highest value of cell viability percentage was found in group 3 (50% CO3Ap) after 72 h treatment. 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 cell (BHK-21) after calcium hydroxide modified carbonate apatite treatment with 12.5% EA solvent

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Group | Absorbance | | Cell Viability | |
| 24 h | 72 h | 24 h | 72 h |
| cell control | 0,98 | 1,25 | 100 | 100 |
| 1 | 0,99 | 1.13 | 107,5 | 89 |
| 2 | 0,93 | 0,95 | 103,3 | 73,7 |
| 3 | 1,23 | 1,18 | 136,9 | 94,8 |
| 4 | 1,23 | 1,12 | 147,5 | 88,8 |
| 5 | 1,13 | 0,39 | 96,7 | 31,5 |

Based on the Tukey test, group 3 and 4 at 24 h of treatment did not have a significant difference (p>0,05). At 72 h treatment control cell, group 1, group 3, and group 4 did not have a significant difference (p>0,05)

**Discussion**

CO3Ap from Balai Besar Keramik Indonesia has a composition of oxygen (O), magnesium (Mg), aluminium (Al), silicate (Si), phosphor (P), and calcium (Ca). Calcium concentration was highest at 38,40 wt%, and the lowest silicate composition is 0,92 wt%. The Ca/P ratio had a value of 1.67.14 The source of carbonate ions is provided by adding calcium carbonate (CaCO3) and magnesium carbonate (MgCO3) to hydroxyapatite through dry mechanosynthesis. The combination of CaCO3 in hydroxyapatite can contribute to the excess of Ca.3 The combination of apatite carbonate with calcium hydroxide can stimulate dentin remineralization by forming reparative dentin with calcium and phosphate replacement to the damaged vital teeth.

Proangiogenic growth factors influence the regulation of nerve and vascular cell function for the proliferation, migration, and growth of new capillaries. Fibroblasts are called cells that have extra-cellular matrix to form and repair connective tissue to preserve anatomical integrity. The viability of fibroblast cells at 24 h observation showed that all groups of calcium hydroxide combined with apatite carbonate showed more than 50% living cells. These results showed that the material was not toxic, indicating that Ca/P was in a stable condition when immersed in the media for 24 h. CO3Ap in MTT media will decompose at 48 h after immersion.15

Ca/P variations in media can affect the chemical consistency of the biological response. The Ca/P level changes occurred 72 h after immersion, this can increase Ca and P ion levels in the media. High Ca levels will affect the amount of nitric oxide products in the cells.16 Cell viability decreased at 72 h that might be due to an increase in nitric oxide. High calcium ions values can cause stimulation of the endoplasmic reticulum to release Ca2+ ions in the intercellular so that it can disrupt its homeostasis and mitochondria will experience apoptosis.17

Powder with a combination of 12.5% Ca(OH)2 : 87.5% CO3Ap (wt%) had the highest toxicity value after 72 hours of treatment compared to other groups. The addition of carbonate can promote solubility and decrease the crystallinity of hydroxyapatite. The consequent solubility can lead to an increase in Ca and phosphate ions in fibroblast cells. Increasing the amount of inorganic phosphate in the extracellular has a wide impact on intracellular homeostasis, cell viability and cell death. An increase in the amount of inorganic phosphate occurs when the cell becomes acidic.18,19

The pH in cells can change because the cell buffer system is disturbed by a change in the amount of water and inorganic ions in the intracellular. Calcium ions 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 calcium ions (2mM) must be preserved in the cytoplasm, which is stored in the endoplasmic reticulum. Cell death can be induced by an excess of Ca ions that will disturb the electrolyte condition of the cell and cause damage to the cell membrane.20,21

The degree of crystallinity in causing cell death depends on the particle size and composition of the material. A smaller degree of crystallinity will make it easier for particles to enter fibroblast cells. The crystal size of carbonate apatite is smaller than pure hydroxyapatite due to the substitution of CO32- and Mg2+ into the HA structure through dry-mechano milling treatment.22,23

#### Conclusion

The combination of calcium hydroxide and carbonate apatite (50:50) and (25:75) wt% with 12.5% EA solvent showed high fibroblast cell viability and proliferation. This study confirms that this combination has non-toxic properties and able to stimulate dental pulp proliferation. This combination also has ideal characteristics as a new candidate for pulp capping material.

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**REFERENCES**

(1) Brizuela, C.; Ormeño, A.; Cabrera, C.; Cabezas, R.; Silva, C. I.; Ramírez, V.; Mercade, M. Direct Pulp Capping with Calcium Hydroxide, Mineral Trioxide Aggregate, and Biodentine in Permanent Young Teeth with Caries: A Randomized Clinical Trial. *J Endod* **2017**, *43* (11), 1776–1780. https://doi.org/10.1016/j.joen.2017.06.031.

(2) Goldberg, M.; Njeh, A.; Uzunoglu, E. Is Pulp Inflammation a Prerequisite for Pulp Healing and Regeneration? *Mediators Inflamm* **2015**, *2015*, 347649. https://doi.org/10.1155/2015/347649.

(3) Damayanti, H.; Wahyudi, K.; Noordiningsih, K.; Ratnasari, A.; Rianti, D. Dry Mechanosynthesis and Characterization of Carbonate Apatite Based on Indonesian Natural Sources. *AIP Conference Proceedings* **2021**, *2349* (1), 020072. https://doi.org/10.1063/5.0052812.

(4) Iafisco, M.; Ruffini, A.; Adamiano, A.; Sprio, S.; Tampieri, A. Biomimetic Magnesium-Carbonate-Apatite Nanocrystals Endowed with Strontium Ions as Anti-Osteoporotic Trigger. *Mater Sci Eng C Mater Biol Appl* **2014**, *35*, 212–219. https://doi.org/10.1016/j.msec.2013.11.009.

(5) Alves, M. M. de M.; Brito, L. M.; Souza, A. C.; Queiroz, B. C. S. H.; de Carvalho, T. P.; Batista, J. F.; Oliveira, J. S. de S. M.; de Mendonça, I. L.; Lira, S. R. de S.; Chaves, M. H.; Gonçalves, J. C. R.; Carneiro, S. M. P.; Arcanjo, D. D. R.; Carvalho, F. A. de A. Gallic and Ellagic Acids: Two Natural Immunomodulator Compounds Solve Infection of Macrophages by Leishmania Major. *Naunyn Schmiedebergs Arch Pharmacol* **2017**, *390* (9), 893–903. https://doi.org/10.1007/s00210-017-1387-y.

(6) Baradaran Rahimi, V.; Ghadiri, M.; Ramezani, M.; Askari, V. R. Antiinflammatory and Anti-Cancer Activities of Pomegranate and Its Constituent, Ellagic Acid: Evidence from Cellular, Animal, and Clinical Studies. *Phytotherapy Research* **2020**, *34* (4), 685–720. https://doi.org/10.1002/ptr.6565.

(7) Ceci, C.; Lacal, P. M.; Tentori, L.; De Martino, M. G.; Miano, R.; Graziani, G. Experimental Evidence of the Antitumor, Antimetastatic and Antiangiogenic Activity of Ellagic Acid. *Nutrients* **2018**, *10* (11), 1756. https://doi.org/10.3390/nu10111756.

(8) Kang, I.; Buckner, T.; Shay, N. F.; Gu, L.; Chung, S. Improvements in Metabolic Health with Consumption of Ellagic Acid and Subsequent Conversion into Urolithins: Evidence and Mechanisms. *Adv Nutr* **2016**, *7* (5), 961–972. https://doi.org/10.3945/an.116.012575.

(9) Ríos, J.-L.; Giner, R. M.; Marín, M.; Recio, M. C. A Pharmacological Update of Ellagic Acid. *Planta Med* **2018**, *84* (15), 1068–1093. https://doi.org/10.1055/a-0633-9492.

(10) Nirwana, I.; Munadziroh, E.; Yogiartono, R. M.; Thiyagu, C.; Ying, C. S.; Dinaryanti, A. Cytotoxicity and Proliferation Evaluation on Fibroblast after Combining Calcium Hydroxide and Ellagic Acid. *J Adv Pharm Technol Res* **2021**, *12* (1), 27–31. https://doi.org/10.4103/japtr.JAPTR\_154\_20.

(11) Prasetyo, E. P.; Saraswati, W.; Wahjuningrum, D. A.; Mooduto, L.; Rosidin, R. F.; Tjendronegoro, E. White Pomegranate (Punica Granatum) Peels Extract Bactericidal Potency on Enterococcus Faecalis. *Conservative Dentistry Journal* **2021**, *11* (2), 84–88. https://doi.org/10.20473/cdj.v11i2.2021.84-88.

(12) Hernandez, R.; Brown, D. T. Growth and Maintenance of Baby Hamster Kidney (BHK) Cells. *Current Protocols in Microbiology* **2010**, *17* (1), A.4H.1-A.4H.7. https://doi.org/10.1002/9780471729259.mca04hs17.

(13) Strober, W. Trypan Blue Exclusion Test of Cell Viability. *Current protocols in immunology* **2015**, *111*, A3.B.1. https://doi.org/10.1002/0471142735.ima03bs111.

(14) Rianti, D.; Kristanto, W.; Damayanti, H.; S. Putri, T.; Dinaryanti, A.; Syahrom, A.; Yuliati, A. The Characteristics and Potency of Limestone-Based Carbonate Hydroxyapatite to Viability and Proliferation of Human Umbilical Cord Mesenchymal Stem Cell. *RJPT* **2022**, 2285–2292. https://doi.org/10.52711/0974-360X.2022.00380.

(15) Rahyussalim, A. J.; Marsetio, A. F.; Kamal, A. F.; Supriadi, S.; Setyadi, I.; Pribadi, P. M.; Mubarok, W.; Kurniawati, T. Synthesis, Structural Characterization, Degradation Rate, and Biocompatibility of Magnesium-Carbonate Apatite (Mg-Co3Ap) Composite and Its Potential as Biodegradable Orthopaedic Implant Base Material. *Journal of Nanomaterials* **2021**, *2021*, e6615614. https://doi.org/10.1155/2021/6615614.

(16) Liu, H.; Yazici, H.; Ergun, C.; Webster, T. J.; Bermek, H. An in Vitro Evaluation of the Ca/P Ratio for the Cytocompatibility of Nano-to-Micron Particulate Calcium Phosphates for Bone Regeneration. *Acta Biomaterialia* **2008**, *4* (5), 1472–1479. https://doi.org/10.1016/j.actbio.2008.02.025.

(17) Panda, S.; Behera, S.; Alam, M. F.; Syed, G. H. Endoplasmic Reticulum & Mitochondrial Calcium Homeostasis: The Interplay with Viruses. *Mitochondrion* **2021**, *58*, 227–242. https://doi.org/10.1016/j.mito.2021.03.008.

(18) Alexander, R.; Debiec, N.; Razzaque, M. S.; He, P. Inorganic Phosphate-Induced Cytotoxicity. *IUBMB Life* **2022**, *74* (1), 117–124. https://doi.org/10.1002/iub.2561.

(19) Michigami, T.; Kawai, M.; Yamazaki, M.; Ozono, K. Phosphate as a Signaling Molecule and Its Sensing Mechanism. *Physiol Rev* **2018**, *98* (4), 2317–2348. https://doi.org/10.1152/physrev.00022.2017.

(20) Cao, N.; Chen, X. B.; Schreyer, D. J. Influence of Calcium Ions on Cell Survival and Proliferation in the Context of an Alginate Hydrogel. *International Scholarly Research Notices* **2012**, *2012*, e516461. https://doi.org/10.5402/2012/516461.

(21) Sugimoto, N. Chapter Eight - Noncanonical Structures and Their Thermodynamics of DNA and RNA Under Molecular Crowding: Beyond the Watson–Crick Double Helix. In *International Review of Cell and Molecular Biology*; Hancock, R., Jeon, K. W., Eds.; New Models of the Cell Nucleus: Crowding, Entropic Forces, Phase Separation, and Fractals; Academic Press, 2014; Vol. 307, pp 205–273. https://doi.org/10.1016/B978-0-12-800046-5.00008-4.

(22) Damayanti, H.; Noordiningsih, K.; Wahyudi, K.; Hernawan; Maryani, E.; Yuliati, A.; Rianti, D.; Asri, L. A. T. W.; Septawendar, R. Synthesis of Carbonate Hydroxyapatite Contained Magnesium Cations by Dry Mechano-Milling. *Materials Letters* **2023**, *338*, 133999. https://doi.org/10.1016/j.matlet.2023.133999.

(23) Wang, M.; Qian, R.; Bao, M.; Gu, C.; Zhu, P. Raman, FT-IR and XRD Study of Bovine Bone Mineral and Carbonated Apatites with Different Carbonate Levels. *Materials Letters* **2018**, *210*, 203–206. https://doi.org/10.1016/j.matlet.2017.09.023.