Abstract Page

Cytotoxicity Test of Eggshell-Based Hydroxyapatite on Human Dental Pulp Cells

Contributors
1. Utama Jeremy
2. Dr. Elline E
3. Dr. Subrata A
4. Dr. Prahasti A.E

Department(s) and institution(s)
Department of Conservative Dentistry, Faculty of Dentistry, Universitas Trisakti, Indonesia
Corresponding Author:
Name : Elline Elline
E-mail address : elline@trisakti.ac.id

Abstract:
Background: Materials such as $Ca(OH)_2$ are commonly used for vital pulp therapy in dentistry, but they have some limitations. Hydroxyapatite (HA) is able to induce reparative dentine; therefore, it can be used as an alternative to $Ca(OH)_2$ for pulp treatment. However, pulp treatment materials should have some ideal characteristics, including low toxicity. The toxicity test is essential to ensure the biological safety of pulp treatment materials. **Objective:** To determine the toxicity of various concentrations of HA derived from eggshell waste to human dental pulp stem cells (hDPSCs). **Method:** We determined the viability of the hDPSCs after exposure to 1%, 2%, or 4% HA by the MTT assay method and using an ELISA reader to calculate the optical density. **Results:** The viability values of the hDPSCs exposed to 1%, 2%, and 4% HA were 84.1%, 86.75%, and 95.03%, respectively. HA concentration has no significant effect in hDPSC proliferation. **Conclusion:** Chicken eggshell HA is a nontoxic material that has the potential to support human dental pulp cell proliferation, which is one of the essential criteria for a pulp treatment material.

Keywords: Eggshell Based Hydroxyapatite, toxicity, Human Dental Pulp Cell Culture

Commented [A1]: This abstract was very rambling and wordy, making it rather ineffective as a summary of the work. Consider the revisions shown for a clearer overview of the study.

Commented [A2R1]: The author has simplified the some of the sentences

Commented [A3]: Once you have defined an abbreviation, there is no need to redefine it. Just use the abbreviation here.

Commented [A4]: When you have a heading "Conclusion", there is no need to use wording like "it can be concluded that…" Just state your conclusion.

Text

BACKGROUND

Vital pulp treatments are often used to avoid further pulp infection, which can lead to pulp necrosis. Pulp treatment materials are applied to exposed pulp to induce reparative dentine.[1] Calcium hydroxide (Ca(OH)2 is one commonly used material in vital pulp treatment;[2] however, previous studies have reported that Ca(OH)2, which is soluble, can cause a necrosis layer because of its high pH (as high as pH 12.5). It can also cause imperfect dentine bridges, as well as tunnel defects that allow repenetration of bacteria into the dental pulp.[3]

An alternative material for dental pulp treatment is hydroxyapatite (HA) Ca10(PO4)6(OH)2, the main mineral in bones and teeth.[4] In dentistry, HA has been applied for various uses, such as bone tissue regeneration.[5] HA has also been considered for use as a vital pulp treatment material because it can induce the formation of hard tissue.[6] HA has the potential to form reparative dentine without tunnel defect, and it has lower inflammation than $Ca(OH)_2$. This might be because HA has lower alkaline pH (8-9) .[7]

One underutilized source of HA, especially in Indonesia, is eggshell waste, which can be processed into HA powder.[8] Chicken eggshells contain 94% calcium carbonate (CaCO3) and have been used in dentistry as bone graft material.[9] The calcium carbonate content is higher in the shells of chicken eggs than in eggshells of other poultry.[10] Hydroxyapatite can be synthesized from eggshell calcium carbonate by several methods, such as wet precipitation, dry precipitation, and hydrothermal methods.[11]

Previous research has shown that HA has the potential to induce reparative dentine, but studies testing the toxicity of HA derived from chicken eggshells are lacking. The toxicity test is an important characterization to ensure the biological safety of an intended dental material, as the material should not elicit a rejection response from the body's cells or cause cell death.[12] The novelty of the present study is that it addresses the potential toxicity of various concentrations of chicken eggshell-based hydroxyapatite (pro-Db) on human dental pulp stem cells (hDPSCs) to evaluate the potential of using chicken eggshell waste as a source of pulp treatment material.

Commented [A5]: Citations should be superscripts in square brackets.

Commented [A6]: You need some type of transitional wording that connects the ideas of dental pulp treatment and HA. Consider something like the wording shown.

Commented [A7]: Try to avoid using wording like "several studies" when you only provide one citation number.

Commented [A8]: Consider providing the actual pH value here.

Commented [A9R8]: The author has added the pH value of HA on page 2, 2nd paragraph

Commented [A10]: Again, there is no logical flow here.

Commented [A11R10]: The author has edited the sentence on page 2, 2nd paragraph at the last sentence.

Commented [A12]: Once you have introduced an abbreviation, you should use it consistently throughout the rest of the text.

Commented [A13]: This is a generic chemical, so it does not need to be capitalized.

Subjects and Methods

This was an experimental laboratory study with a posttest-only control group design. The research was conducted at the ProSTEM Laboratory (PT. Prodia StemCell Indonesia). The samples from this study were human pulp cells (cell lines) cultured in the laboratory, so the research did not require ethical approval.

The pulp cells were cultured in a 100 mm petri dish (7 mL of growth medium + 1 mL of cell suspension) and incubated for ± 5 days until a confluence of >80% was reached. The cells were then harvested by adding 3 mL of TrypLe (Trypsin) solution and incubating at 37°C; 5% CO2 for 7 min. The petri dish was then tapped gently to completely release the cells, and the TrypLe was neutralized with PBS (1:1). The solution containing the released cells was transferred from the petri dish to a 15 mL conical tube and centrifuged for 5 minutes at 300g. The supernatant was discarded, and the cells were resuspended in 1 mL of growth medium and counted using a microscope.

Commented [A14]: The methods section should not be presented as instructions. It is meant to describe the methods that were used to generate the results that are about to be presented and should be written in past tense.

The cells were divided into four treatment groups: 1%, 2%, and 4% HA (Pro DB, PT Aleesha Berkah Utama, Berkasi, Jawa Barat, Indonesia) and a Ca(OH)2 positive control. Each sample group had 6 repetitions, so the total sample number was 24.

HA powder prepared from eggshell waste was ready made by PT Aleesha Berkah Utama using the wet-dry (chemical) precipitation method. The eggshells were cleaned, dried at 110 degrees, and then calcined to produce CaO. Ammonium hydroxide (NH4OH) was used to raise the solution pH to 10. A phosphate solution was prepared using the same procedure. The calcium solution was added dropwise to the phosphate solution to form the HA, and it was mixed in 40°C temperature and stirred at 300 rpm. After the HA precipitation was complete, it was aged for 24 h. The precipitate was then filtered through Whatman 42 filter paper and washed with distilled water to remove the remaining ammonium nitrate. The precipitate was then dried at 110°C, followed by calcining at 900°C for 5 h. The desired concentrations of HA (1%, 2%, and 4%) were prepared by dissolving HA in deionized water. Calcium hydroxide (PT Pudak Scientific, Jakarta, Indonesia) for the control solution was mixed with deionized water at a ratio of 1:1 and then allowed to set.

Commented [A15]: Use consistent SI units throughout the paper.

The hydroxyapatite and calcium hydroxide were dissolved in Dulbecco's modified Eagle's medium (DMEM) and then added to each cell sample, and the cells were cultured for 24 h. Toxicity to the pulp after HA exposure was then determined by measuring the cell viability/number of living cells using the MTT colorimetric method and calculated as percentages using an ELISA reader (Sigma Aldrich, St Louis, Missouri, United States). The MTT solution was prepared by mixing 5 mg of MTT powder and 1 mL of 0.9% NaCl. A 50 µL volume of the cell solution was added to each well of a 96-well microplate using a micropipette and incubated for 4 h. The wells were then washed with PBS (Lonza, Basel, Switzerland) and the MTT reaction was stopped by adding 100 µL of acidified isopropanol per well. The 96-well microplate was placed in an orbital shaker for 60 min, and then absorbance was measured using an ELISA reader at a wavelength of 595 nm. The optical density (OD) was calculated by averaging the readings from the ELISA reader for each repeat. A viability formula was used to calculate cell viability; we used ISO 10993-5 to determine cell viability. A material was considered nontoxic if the cell viability was greater than 70%.[13]

RESULTS

A significant difference in cell viability values was observed between the HA groups and the Ca(OH)2 group ($p < 0.05$). The cell viability was greater than 70% for every sample (Table 1) (Figure 1). The cell proliferation values, from highest to lowest, were as follows: $Ca(OH)_2$ group (220.3%), HA 4% (95.03%), 2% HA (86.75%), and 1% HA (84.1%), as shown in Figure 1.

Table 1. The mean and and standart deviation result of the hDPSc viability in different concentrations of eggshell hydroxyapatite

HA Concentrations	Cell Viability (%) ±SD	p
1%	84,1 ± 0,001	<0.001*
2%	86,75 ± 0,004	
4%	95,03 ± 0,014	

*Tukey Test ($p<0.05$)

Commented [A16]: This Table caption is very vague. A reader should be able to look at the table, read the caption, and understand what is shown without having to read the main text of your paper. You should try to expand on this a bit. Consider something like the revision shown.

Commented [A17R16]: The caption has been edited so the reader able to understand

Commented [A18]: Please kindly translate to english

Commented [A19R18]: The text has been translated to english

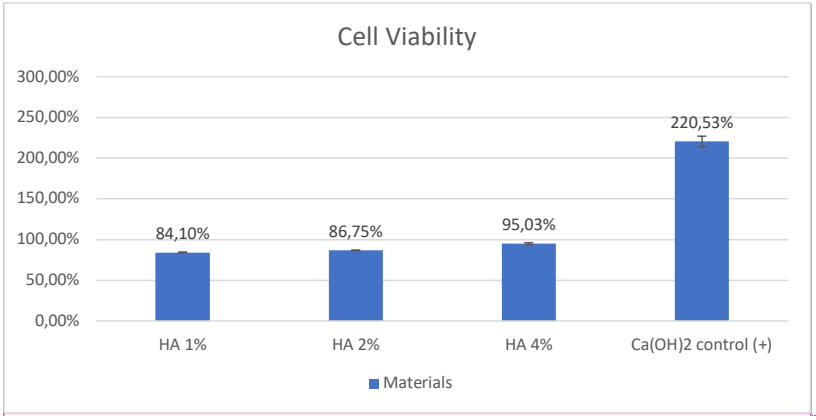


Figure 1. The human dental pulp stem cell viability chart (%) showed that eggshell hydroxyapatite was nontoxic at all tested concentrations and could induce cell proliferation.

Commented [A20]: Please translate to English "Viabilitas Sel" and "Bahan"

Commented [A21R20]: Viabilitas and bahan has been translated to english

DISCUSSION

The findings of this study confirmed that hydroxyapatite from chicken eggshell waste can induce human dental pulp cell proliferation. Therefore, this source of HA has the potential for use as an alternative for pulp treatment, but other characterization studies are still required. The eggshell-derived HA was biocompatible, and it may have other benefits by reducing allergies and inflammation reactions after contact with human tissues.[14]

This study was conducted using precultured hDPSCs because it presents a high capacity for proliferation and are considered representative of the original dental pulp cells in human teeth.[15] Pulp cells are cultured in two forms: as primary cell cultures and as cell line cultures. Primary cell culture is done with cells obtained directly from the organism, whereas cell line cultures are carried out with cells obtained from primary cell cultures. In this study, cell line culture was chosen because of its ease of use, unlimited cell production, and lack of ethical concerns regarding the use of human and animal tissue.[16] The MTT test was chosen because it is easy to perform, requires a relatively short time, and has good accuracy. The principle of the MTT method is the reduction of the yellow tetrazolium salt into purple formazan due to

succinate dehydrogenase enzyme activity from living cell mitochondria.[17] The MTT test results can be measured by light absorbance at a wavelength of 550–600 nm. Dead cells lose the ability to reduce the tetrazolium salt, so they do not elicit a color change to purple; therefore, a greater intensity of purple color indicates that more cells are alive.[18]

According to ISO 10993-5, bioactive materials are considered biocompatible when the cell viability is greater than 70%.[19] All the samples tested in this study showed cell viability above 70%, indicating that the test materials were biocompatible. These results also indicated a correlation between cell viability and the level of HA exposure. As the HA concentration increased, the cell viability also increased. No significant differences were found between the viability values of the HA groups ($p > 0.05$), suggesting that HA at 1%, 2%, and 4% will not generate different effects in clinical application, but further study is needed.

HA may have the potential to serve as an alternative material for vital pulp treatment, as it can support dental pulp cell proliferation. In previous studies, HA was shown to have a lower pH compared to Ca(OH)2.[13] The higher alkalinity of $Ca(OH)_2$ causes the formation of imperfect dentine bridges, resulting in tunnel defects that allow the possibility of repenetration of bacteria into the pulp.[20] A lower pH can reduce these tunnel defects, as well as inflammation reactions in the dental pulp. In the control group in the present study, $Ca(OH)_2$ was shown to support high cell viability because of the high concentration of calcium, which will induce cell proliferation.[21] The cell proliferation rate was not high in the first 24 h of HA treatment; however, a previous study has shown that cell viability at 72 h is higher after HA treatment than in a negative control.[22] This is because the pH of HA is pH 8–9, which makes it less irritating to pulp cells.[13] A previous study comparing the rate of necrosis of cells exposed to Ca(OH)2 and HA also showed that HA treatment led to less necrosis.[23] This could also occur because of the lower pH of HA than of Ca(OH)2.

HA, as a dental material, can overcome the limitations of Ca(OH)2, which mainly arise due to the generation of tunnel defects in the reparative dentine that is formed. Previous studies have reported that HA did not cause tunnel defects, while also producing less inflammation than Ca(OH)2.[23] The limitation of the present study was that cell proliferation was only assessed after 24 h. Assessment after longer exposure

Commented [A22]: Use a consistent abbreviation throughout the paper.

times may be needed in a future study. However, the findings presented here suggest that HA is nontoxic to dental pulp cells and can support human dental pulp cell proliferation.

CONCLUSION

The novelty of this study is using chicken eggshell as the induction material for supporting hDPSc proliferation. Chicken eggshell hydroxyapatite with 1%, 2%, and 4% concentrations was proved to be non-toxic, which showed viability values of more than 70% after HA exposure for 24 h. Further characterization studies are needed to explore the ability of HA to support the dentine pulp reaction that induces the healing mechanism.

Commented [A23]: The novelty of your study is the use of eggshell HA, so consider including that point in your conclusion.

REFERENCES

1. Yoshida S, Sugii H, Itoyama T, Kadowaki M, Hasegawa D, Tomokiyo A, et al. Development of a novel direct dental pulp-capping material using 4-META/MMA-TBB resin with nano hydroxyapatite. Mater Sci Eng C Mater Biol Appl. 2021;130:e112426.
2. Li Z, Cao L, Fan M, Xu Q. Direct Pulp Capping with Calcium Hydroxide or Mineral Trioxide Aggregate: A Meta-analysis. J Endod. 2015;41(9):1412–7.
3. Widjiastuti I, Dewi M, Prasetyo E, Pribadi N, Moedjiono M. The cytotoxicity test of calcium hydroxide, propolis, and calcium hydroxide-propolis combination in human pulp fibroblast. J Adv Pharm Technol Res. 2020;11(1):20–4.
4. Sadat-Shojai M, Khorasani MT, Dinpanah-Khoshdargi E, Jamshidi A. Synthesis methods for nanosized hydroxyapatite with diverse structures. Acta Biomater. 2013;9(8):7591–621.
5. Okamoto M, Matsumoto S, Sugiyama A, Kanie K, Watanabe M, Huang H, et al. Performance of a Biodegradable Composite with Hydroxyapatite as a Scaffold in Pulp Tissue Repair. Polymers (Basel). 2020;12(4):937.
6. Nasution DFR. Perbedaan Pembentukan Dentinal Bridge Antara Karbonat Apatit dan Kalsium Hidroksida Setelah Dilakukan Direct Pulp Capping pada Molar Satu Maksila Tikus Wistar (Pengamatan 6 Minggu) [Disertasi]. Medan: Universitas Sumatera Utara; 2020.
7. Elline, Fibryanto E, Amanda H. Characterization of nano-hydroxyapatite–collagen and epigallocatechin-3-gallate (EGCG) composites by scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy. Sci Dent J. 2022;6(2):80–6.
8. Suprianto K, Hidayati H, Nilam C, Khairiyah N, Amelia R, Rahmadita S. Hidroksiapatit dari cangkang telur sebagai bone graft yang potensial dalam terapi periodontal. MKGK Clin Dent. 2021;5(3):76–87.
9. Sitohang F, Azis Y, Zultiniar Z. Sintesis Hidroksiapatit Dari Precipitated Calcium Carbonate (PCC) Kulit Telur Ayam Ras Melalui Metode Hidrotermal. jom FTEKNIK. 2016;3(2):1–7.
10. Elline E, Ismiyatin K. Nanohydroxiapatite Using Chicken Eggshell Waste and Its Characterization. Mal J Med Health Sci. 2021;17:83–6.

11. Gago J, Ngapa YD. Pemanfaatan Cangkang Telur Ayam Sebagai Material Dasar Dalam Sintesis Hidroksiapatit Dengan Metode Presipitasi Basah. CAKRA KIMIA (Indonesian E-Journal of Applied Chemistry). 2021;9(1):29–34.
12. Komala D, Amin MN, Rahayu YC. Uji Sitotoksisitas Hidroksiapatit Cangkang Telur Ayam Ras (Gallus gallus) terhadap Sel Fibroblas Ligamen Periodontal Manusia. STOMATOGNATIC. 2022;19(1):49–54.
13. Elline I, Ismiyatin K, Indah Budhy T, Bhardwaj A. The potential of eggshell hydroxyapatite, collagen, and EGCG (HAp-Col-EGCG) scaffold as a pulp regeneration material. Saudi Dent J. 2022 Dec;34(8):715–22.
14. Modena KC da S, Calvo AM, Sipert CR, Colombini-Ishikiriama BL, Dionísio TJ, Navarro MF de L, et al. Molecular Response of Pulp Fibroblasts after Stimulation with Pulp Capping Materials. Braz Dent J. 2020;31(3):244–51.
15. Takeo W. Dental Pulp Stem Cells: Advances to Applications. Stem Cells Cloning: Adv Appl. 2020;13:33–42.
16. Kaur G, Dufour JM. Cell lines. Spermatogenesis. 2012;2(1):1–5.
17. Abdulrahman I, Tijani HI, Mohammed BA, Saidu H, Yusuf H, Ndejiko Jibrin M, et al. From Garbage to Biomaterials: An Overview on Egg Shell Based Hydroxyapatite. J Mater. 2014;2014:e802467.
18. Salzmann JA. Craig's Restorative dental materials. Vol. 71. 14th edn. Elsevier Inc 1977: p. :228.
19. Sirait M, Sinulingga K, Siregar N, Doloksaribu ME, Amelia. Characterization of hydroxyapatite by cytotoxicity test and bending test. J Phys: Conf Ser. 2022;2193(1):e012039.
20. Mohammadi Z, Shalavi S, Yazdizadeh M. Antimicrobial Activity of Calcium Hydroxide in Endodontics: A Review. Chonnam Med J. 2012;48(3):133–40.
21. Chen L, Zheng L, Jiang J, Gui J, Zhang L, Huang Y, et al. Calcium Hydroxide–induced Proliferation, Migration, Osteogenic Differentiation, and Mineralization via the Mitogen-activated Protein Kinase Pathway in Human Dental Pulp Stem Cells. J Endod. 2016;42(9):1355–61.
22. Haniastuti T, Susilowati H, Rinastiti M. Viability and Alkaline Phosphatase Activity of Human Dental Pulp Cells after Exposure to Yellowfin Tuna Bone-Derived Hydroxyapatite In Vitro. Int J Dent. 2020;2020:e8857534.
23. Swarup SJ, Rao A, Boaz K, Srikant N, Shenoy R. Pulpal response to nano hydroxyapatite, mineral trioxide aggregate and calcium hydroxide when used as a direct pulp capping agent: an in vivo study. J Clin Pediatr Dent. 2014;38(3):201–6.

Acknowledgment: none

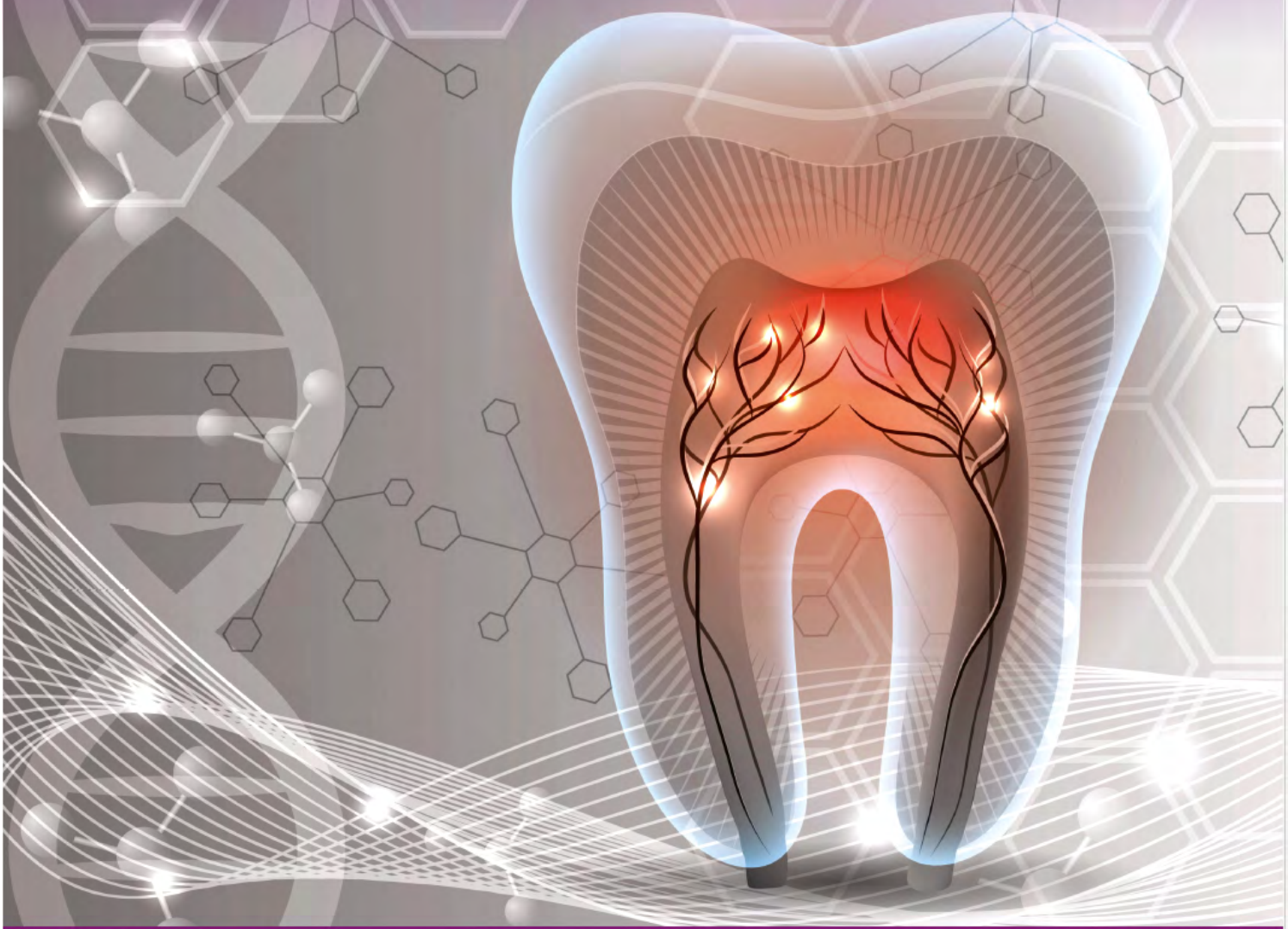
ISSN (Print) : 2580–6548
ISSN (Online): 2541–321X

Volume 7 • Issue 1 • January–April 2023

SCIENTIFIC DENTAL JOURNAL

Official Journal of Faculty of Dentistry, Trisakti University, Jakarta, Indonesia

www.scidentj.com



Wolters Kluwer

Medknow

Editorial Board

Original Articles

Chronic Osteomyelitis of the Jaws: A 7-Year Retrospective Clinico-Surgical Evaluation in a Tertiary Hospital in Northwest Nigeria

Bala, Mujtaba; Braimah, Ramat Oyebunmi; Taiwo, Abdulrazaq Olanrewaju; More

Scientific Dental Journal. 7(1):1-5, Jan-Apr 2023.

Abstract Favorite PDF Permissions

OPEN

In Vitro Evaluation of the Compressive Strength of Glass Ionomer Cement Modiied with Propolis in Different Proportions

Azalia, Advita; Pratiwi, Deviyanti; Hasan, Akhmad Endang Zainal; More

Scientific Dental Journal. 7(1):6-10, Jan-Apr 2023.

Abstract Favorite PDF Permissions

OPEN

The Potential of Cogon Grass (*Imperata cylindrica*) Ethanol Extract in Inhibiting Nitric Oxide Secretion in Fibroblast

Mangkulion, Sherlyn; Roeslan, Moehamad Orliando; Monthanpisut, Paopanga

Scientific Dental Journal. 7(1):11-14, Jan-Apr 2023.

Abstract Favorite PDF Permissions

OPEN

Periodontitis as a Risk Factor for Stroke: A Hospital-based Case-Control Study

Kalpdev, Mayank; Gupta, Rashmi; Singh, Kusum; More

Scientific Dental Journal. 7(1):15-21, Jan-Apr 2023.

Abstract Favorite PDF Permissions

OPEN

Cytotoxicity Test of Chicken Eggshell-Based Hydroxyapatite on Human Dental Pulp Cells

Utama, Jeremy; Elline, Elline; Subrata, Aryadi; More

Scientific Dental Journal. 7(1):22-25, Jan-Apr 2023.

Abstract Favorite PDF Permissions

OPEN

Effects of *Hibiscus sabdariffa* L. extract on multispecies *Porphyromonas gingivalis* and *Treponema denticola* Biofilms *In Vitro*

Winson, Angela; Suwandi, Trijani; Komala, Olivia Nauli

Scientific Dental Journal. 7(1):26-32, Jan-Apr 2023.

Table of Contents Outline | Back to Top

⊟ Case Reports

Case Series of Low-Level Laser Therapy for Minor Ulceration: Diverse Outcomes Related to Predisposing Factors

Indrastiti, Ratna Kumala; Wardhany, Indriasti Indah; Mandasari, Masita; More

Scientific Dental Journal. 7(1):33-38, Jan-Apr 2023.

Management of Early Infancy Tooth Following Natal Tooth Extraction: An Unusual Case Report

Samal, Santoshni; Baliarsingh, Ratna Renu; Ray, Prayas; More

Scientific Dental Journal. 7(1):39-41, Jan-Apr 2023.

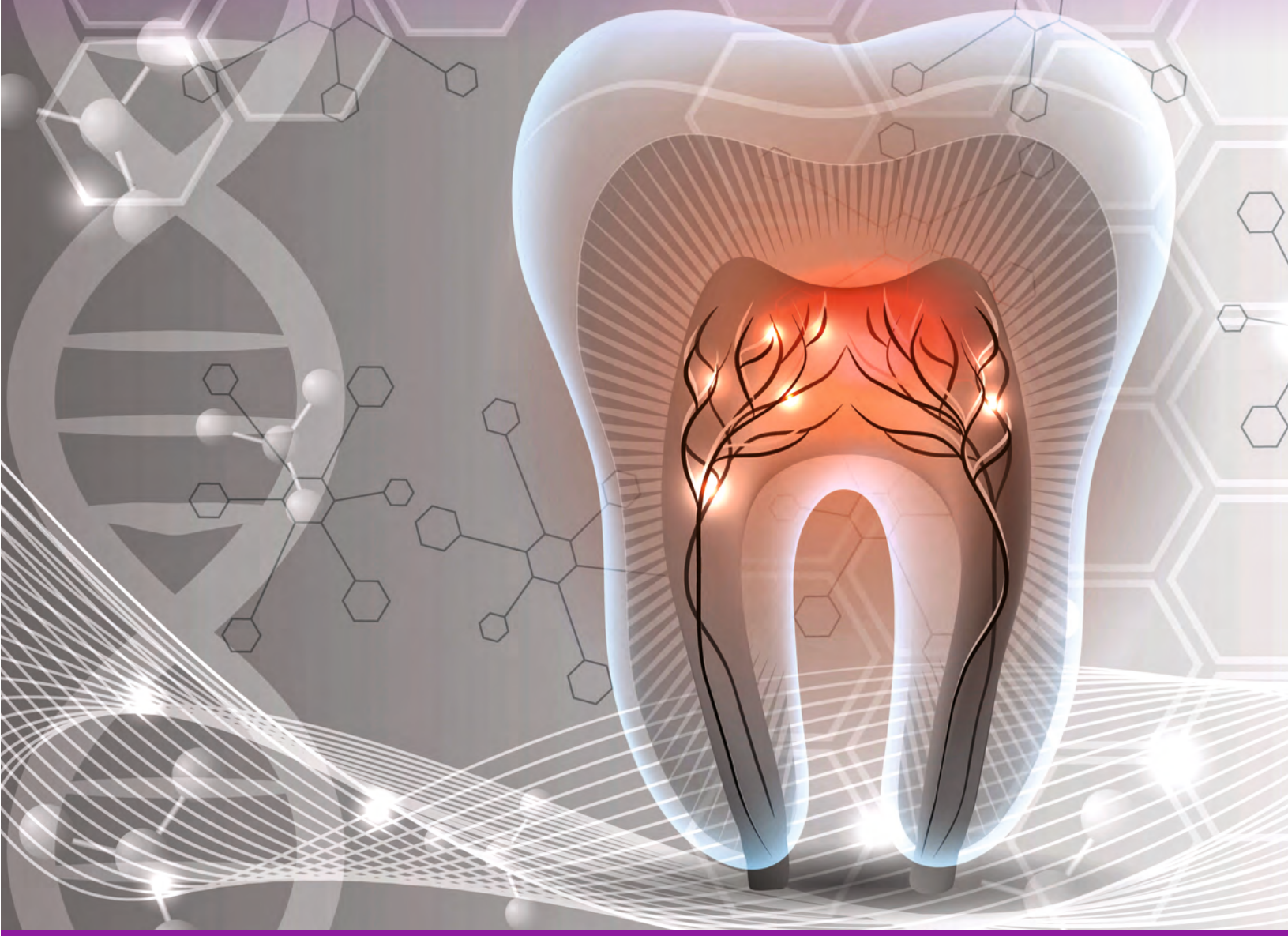
ISSN (Print) : 2580–6548
ISSN (Online): 2541–321X

Volume 7 • Issue 1 • January–April 2023

SCIENTIFIC DENTAL JOURNAL

Official Journal of Faculty of Dentistry, Trisakti University, Jakarta, Indonesia

www.scidentj.com



Wolters Kluwer

Medknow

Original Article

Cytotoxicity Test of Chicken Eggshell-Based Hydroxyapatite on Human Dental Pulp Cells

Jeremy Utama, Elline Elline, Aryadi Subrata, Anastasia Elsa Prahasti, Syuwari Azhar Azman[1]

Department of Conservative Dentistry, Faculty of Dentistry, Universitas Trisakti, Jakarta Barat, Indonesia, [1]Department of Restorative Dentistry, Kulliyyah of Dentistry, International Islamic University, Malaysia, Kuantan Campus, Kuantan, Malaysia

Received: 07-12-22
Revised: 11-01-23
Accepted: 15-02-23
Published Online: 17-05-23

ABSTRACT

Background: Materials such as calcium hydroxide [$Ca(OH)_2$] are commonly used for vital pulp therapy in dentistry, but they have some limitations. Hydroxyapatite (HA) is able to induce reparative dentin; therefore, it can be used as an alternative to $Ca(OH)_2$ for pulp treatment. However, pulp treatment materials should have some ideal characteristics, including low toxicity. The toxicity test is essential to ensure the biological safety of pulp treatment materials. **Objective:** To determine the toxicity of various concentrations of HA derived from eggshell waste to human dental pulp stem cells (hDPSCs). **Methods:** We determined the viability of the hDPSCs after exposure to 1%, 2%, or 4% HA by the diphenyl-2H-tetrazolium bromide assay method to measure cell viability and using an enzyme-linked immunosorbent assay reader to calculate the optical density. **Results:** The viability values of the hDPSCs exposed to 1%, 2%, and 4% HA were 84.1%, 86.75%, and 95.03%, respectively. HA concentration had no significant effect on hDPSC proliferation. **Conclusion:** Chicken eggshell HA is a nontoxic material that has the potential to support human dental pulp cell proliferation, which is one of the essential criteria for a pulp treatment material.

Keywords: *Eggshell-based hydroxyapatite, human dental pulp cell culture, toxicity*

Background

Vital pulp treatments are often used to avoid further pulp infection, which can lead to pulp necrosis. Pulp treatment materials are applied to exposed pulp to induce reparative dentin.[1] Calcium hydroxide [$Ca(OH)_2$] is one commonly used material in vital pulp treatment[2]; however, previous studies have reported that $Ca(OH)_2$, which is soluble, can cause a necrosis layer because of its high pH (as high as pH 12.5). It can also cause imperfect dentin bridges, as well as tunnel defects that allow repenetration of bacteria into the dental pulp.[3]

An alternative material for dental pulp treatment is hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$, the main mineral in bones and teeth.[4] In dentistry, HA has been applied for various uses, such as bone tissue regeneration.[5] HA has also been considered for use as a vital pulp treatment material because it can induce the formation of hard tissue.[6] HA has the potential to form reparative dentin without tunnel defect, and it has lower inflammation than $Ca(OH)_2$. This might be because HA has lower alkaline pH (8–9).[7]

One underutilized source of HA, especially in Indonesia, is eggshell waste, which can be processed into HA powder.[8] Chicken eggshells contain 94% calcium carbonate ($CaCO_3$) and have been used in dentistry as bone graft material.[9] $CaCO_3$ content is

Address for correspondence: Dr. Elline Elline, Department of Conservative Dentistry, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa no. 260, Grogol, Jakarta Barat 11440, Indonesia.
E-mail: elline@trisakti.ac.id

Access this article online

Quick Response Code:

Website: www.scidentj.com

DOI: 10.4103/SDJ.SDJ_6_23

How to cite this article: Utama J, Elline E, Subrata A, Prahasti AE, Azman SA. Cytotoxicity test of chicken eggshell-based hydroxyapatite on human dental pulp cells. Sci Dent J 2023;7:22-5.

higher in the shells of chicken eggs than in eggshells of other poultry.[10] HA can be synthesized from eggshell $CaCO_3$ by several methods, such as wet precipitation, dry precipitation, and hydrothermal methods.[11]

Previous research has shown that HA has the potential to induce reparative dentin, but studies testing the toxicity of HA derived from chicken eggshells are lacking. The toxicity test is an important characterization to ensure the biological safety of an intended dental material, as the material should not elicit a rejection response from the body's cells or cause cell death.[12] The novelty of the present study is that it addresses the potential toxicity of various concentrations of chicken eggshell-based HA (pro-Db) on human dental pulp stem cells (hDPSCs) to evaluate the potential of using chicken eggshell waste as a source of pulp treatment material.

Materials and Methods

This was an experimental laboratory study with a posttest-only control group design. The research was conducted at the ProSTEM Laboratory Jakarta Pusat, Indonesia (PT Prodia StemCell Indonesia). The samples from this study were human pulp cells (cell lines) cultured in the laboratory, so the research did not require ethical approval.

The pulp cells were cultured in a 100 mm petri dish (7 mL of growth medium + 1 mL of cell suspension) and incubated for ±5 days until a confluence of >80% was reached. The cells were then harvested by adding 3 mL of TrypLe (Trypsin) solution and incubating at 37°C; 5% CO_2 for 7 min. The petri dish was then tapped gently to completely release the cells, and the TrypLe was neutralized with phospate buffer saline (1:1). The solution containing the released cells was transferred from the petri dish to a 15 mL conical tube and centrifuged for 5 min at 300*g*. The supernatant was discarded, and the cells were resuspended in 1 mL of growth medium and counted using a microscope.

The cells were divided into four treatment groups: 1%, 2%, and 4% HA (Pro-DB, PT Aleesha Berkah Utama, Berkasi, Jawa Barat, Indonesia) and a $Ca(OH)_2$ positive control. Each sample group had six repetitions, so the total sample number was 24.

HA powder prepared from chicken eggshell waste was made ready by PT Aleesha Berkah Utama using the wet-dry (chemical) precipitation method. The eggshells were cleaned, dried at 110ºC, and then calcined to produce CaO. Ammonium hydroxide (NH_4OH) was used to raise the solution pH to 10. A phosphate solution was prepared using the same procedure. The calcium solution was added dropwise to the phosphate solution to form the HA, and it was mixed in 40°C temperature and stirred at 300 rpm. After the HA precipitation was complete, it was aged for 24 h. The precipitate was then filtered through Whatman 42 filter paper and washed with distilled water to remove the remaining ammonium nitrate. The precipitate was then dried at 110°C, followed by calcining at 900°C for 5 h. The desired concentrations of HA (1%, 2%, and 4%) were prepared by dissolving HA in deionized water. $Ca(OH)_2$ (PT Pudak Scientific, Jakarta, Indonesia) for the control solution was mixed with deionized water at a ratio of 1:1 and then allowed to set.

The HA and $Ca(OH)_2$ were dissolved in Dulbecco's modified Eagle's medium and then added to each cell sample, and the cells were cultured for 24 h. Toxicity to the pulp after HA exposure was then determined by measuring the cell viability/number of living cells using the diphenyl-2H-tetrazolium bromide (MTT) colorimetric method and calculated as percentages using an enzyme-linked immunosorbent assay reader (Sigma-Aldrich, St Louis, Missouri). The MTT solution was prepared by mixing 5 mg of MTT powder and 1 mL of 0.9% NaCl. A 50 µL volume of the cell solution was added to each well of a 96-well microplate using a micropipette and incubated for 4 h. The wells were then washed with phospate buffer saline (Lonza, Basel, Switzerland) and the MTT reaction was stopped by adding 100 µL of acidified isopropanol per well. The 96-well microplate was placed in an orbital shaker for 60 min, and then absorbance was measured using an enzyme-linked immunosorbent assay reader at a wavelength of 595 nm. The optical density (OD) was calculated by averaging the readings from the enzyme-linked immunosorbent assay reader for each repeat. A viability formula was used to calculate cell viability; we used ISO 10993-5 to determine cell viability. A material was considered nontoxic if the cell viability was greater than 70%.[13]

Results

A significant difference in cell viability values was observed between the HA groups and the $Ca(OH)_2$ group ($P < 0.05$). The cell viability was greater than 70% for every sample [Table 1; Figure 1]. The cell

Table 1: The mean and standard deviation result of the hDPSc viability in different concentrations of eggshell hydroxyapatite

HA concentrations	Cell viability (%) ± SD	*P*
1%	84.1 ± 0.001	<0.001*
2%	86.75 ± 0.004	
4%	95.03 ± 0.014	

*Tukey test ($P < 0.05$)

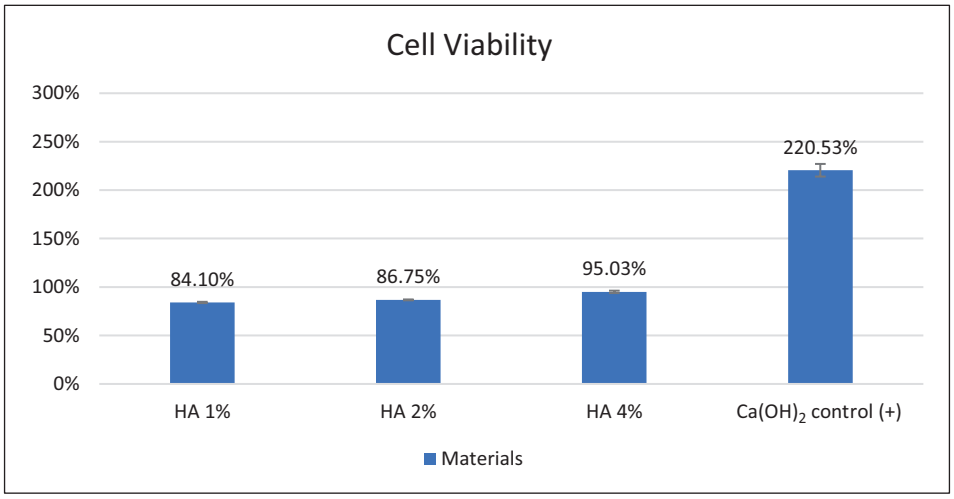


Figure 1: The human dental pulp stem cell viability chart (%) showed that eggshell hydroxyapatite was nontoxic at all tested concentrations and could induce cell proliferation

proliferation values, from highest to lowest, were as follows: $Ca(OH)_2$ group (220.3%), HA 4% (95.03%), 2% HA (86.75%), and 1% HA (84.1%), as shown in Figure 1.

Discussion

The findings of this study confirmed that HA from chicken eggshell waste can induce human dental pulp cell proliferation. Therefore, this source of HA has the potential for use as an alternative for pulp treatment, but other characterization studies are still required. The eggshell-derived HA was biocompatible, and it may have other benefits by reducing allergies and inflammation reactions after contact with human tissues.[14]

This study was conducted using precultured hDPSCs because it presents a high capacity for proliferation and are considered representative of the original dental pulp cells in human teeth.[15] Pulp cells are cultured in two forms: as primary cell cultures and as cell line cultures. Primary cell culture is done with cells obtained directly from the organism, whereas cell line cultures are carried out with cells obtained from primary cell cultures. In this study, cell line culture was chosen because of its ease of use, unlimited cell production, and lack of ethical concerns regarding the use of human and animal tissue.[16] The MTT test was chosen because it is easy to perform, requires a relatively short time, and has good accuracy. The principle of the MTT method is the reduction of the yellow tetrazolium salt into purple formazan due to succinate dehydrogenase enzyme activity from living cell mitochondria.[17] The MTT test results can be measured by light absorbance at a wavelength of 550–600 nm. Dead cells lose the ability to reduce the tetrazolium salt, so they do not elicit a color change to purple; therefore, a greater intensity of purple color indicates that more cells are alive.[18]

According to ISO 10993-5, bioactive materials are considered biocompatible when the cell viability is greater than 70%.[19] All the samples tested in this study showed cell viability above 70%, indicating that the test materials were biocompatible. These results also indicated a correlation between cell viability and the level of HA exposure. As the HA concentration increased, the cell viability also increased. No significant differences were found between the viability values of the HA groups ($P > 0.05$), suggesting that HA at 1%, 2%, and 4% will not generate different effects in clinical application, but further study is needed.

HA may have the potential to serve as an alternative material for vital pulp treatment, as it can support dental pulp cell proliferation. In previous studies, HA was shown to have a lower pH compared to $Ca(OH)_2$.[13] The higher alkalinity of $Ca(OH)_2$ causes the formation of imperfect dentin bridges, resulting in tunnel defects that allow the possibility of repenetration of bacteria into the pulp.[20] A lower pH can reduce these tunnel defects, as well as inflammation reactions in the dental pulp. In the control group in the present study, $Ca(OH)_2$ was shown to support high cell viability because of the high concentration of calcium, which will induce cell proliferation.[21] The cell proliferation rate was not high in the first 24 h of HA treatment; however, a previous study has shown that cell viability at 72 h is higher after HA treatment than in a negative control.[22] This is because the pH of HA is pH 8–9, which makes it less irritating to pulp cells.[13] A previous study comparing the rate of necrosis of cells exposed to $Ca(OH)_2$ and HA

also showed that HA treatment led to less necrosis.[23] This could also occur because of the lower pH of HA than of $Ca(OH)_2$.

HA, as a dental material, can overcome the limitations of $Ca(OH)_2$, which mainly arise due to the generation of tunnel defects in the reparative dentin that is formed. Previous studies have reported that HA did not cause tunnel defects, while also producing less inflammation than $Ca(OH)_2$.[23] The limitation of the present study was that cell proliferation was only assessed after 24 h. Assessment after longer exposure times may be needed in a future study. However, the findings presented here suggest that HA is nontoxic to dental pulp cells and can support human dental pulp cell proliferation.

Conclusion

The novelty of this study is using chicken eggshell as the induction material for supporting hDPSc proliferation. Chicken eggshell HA with 1%, 2%, and 4% concentrations was proved to be nontoxic, which showed viability values of more than 70% after HA exposure for 24 h. Further characterization studies are needed to explore the ability of HA to support the dentin pulp reaction that induces the healing mechanism.

Acknowledgment

None.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Yoshida S, Sugii H, Itoyama T, Kadowaki M, Hasegawa D, Tomokiyo A, *et al*. Development of a novel direct dental pulp-capping material using 4-META/MMA-TBB resin with nano hydroxyapatite. Mater Sci Eng C Mater Biol Appl 2021;130:e112426.
2. Li Z, Cao L, Fan M, Xu Q. Direct pulp capping with calcium hydroxide or mineral trioxide aggregate: A meta-analysis. J Endod 2015;41:1412-7.
3. Widjiastuti I, Dewi M, Prasetyo E, Pribadi N, Moedjiono M. The cytotoxicity test of calcium hydroxide, propolis, and calcium hydroxide-propolis combination in human pulp fibroblast. J Adv Pharm Technol Res 2020;11:20-4.
4. Sadat-Shojai M, Khorasani MT, Dinpanah-Khoshdargi E, Jamshidi A. Synthesis methods for nanosized hydroxyapatite with diverse structures. Acta Biomater 2013;9:7591-621.
5. Okamoto M, Matsumoto S, Sugiyama A, Kanie K, Watanabe M, Huang H, *et al*. Performance of a biodegradable composite with hydroxyapatite as a scaffold in pulp tissue repair. Polymers (Basel) 2020;12:937.
6. Nasution DFR. Perbedaan Pembentukan Dentinal Bridge Antara Karbonat Apatit dan Kalsium Hidroksida Setelah Dilakukan Direct Pulp Capping pada Molar Satu Maksila Tikus Wistar (Pengamatan 6 Minggu) [Disertasi]. Medan: Universitas Sumatera Utara; 2020.
7. Elline, Fibryanto E, Amanda H. Characterization of nano-hydroxyapatite–collagen and epigallocatechin-3-gallate (EGCG) composites by scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy. Sci Dent J 2022;6:80-6.
8. Suprianto K, Hidayati H, Nilam C, Khairiyah N, Amelia R, Rahmadita S. Hidroksiapatit dari cangkang telur sebagai bone graft yang potensial dalam terapi periodontal. MKGK Clin Dent 2021;5:76-87.
9. Sitohang F, Azis Y, Zultiniar Z. Sintesis hidroksiapatit dari precipitated calcium carbonate (PCC) kulit telur ayam ras melalui metode hidrotermal. Jom FTEKNIK 2016;3:1-7.
10. Elline E, Ismiyatin K. Nanohydroxiapatite using chicken eggshell waste and its characterization. Mal J Med Health Sci 2021;17:83-6.
11. Gago J, Ngapa YD. Pemanfaatan cangkang telur ayam sebagai material dasar dalam sintesis hidroksiapatit dengan metode presipitasi basah. CAKRA KIMIA 2021;9:29-34.
12. Komala D, Amin MN, Rahayu YC. Uji sitotoksisitas hidroksiapatit cangkang telur ayam ras (*Gallus gallus*) terhadap sel fibroblas ligamen periodontal manusia. Stomatognatic 2022;19:49-54.
13. Elline I, Ismiyatin K, Indah Budhy T, Bhardwaj A. The potential of eggshell hydroxyapatite, collagen, and EGCG (HAp-Col-EGCG) scaffold as a pulp regeneration material. Saudi Dent J 2022;34:715-22.
14. Modena KC da S, Calvo AM, Sipert CR, Colombini-Ishikiriama BL, Dionísio TJ, Navarro MF de L, *et al*. Molecular response of pulp fibroblasts after stimulation with pulp capping materials. Braz Dent J 2020;31:244-51.
15. Takeo W. Dental pulp stem cells: Advances to applications. Stem Cells Cloning: Adv Appl 2020;13:33-42.
16. Kaur G, Dufour JM. Cell lines. Spermatogenesis 2012; 2:1-5.
17. Abdulrahman I, Tijani HI, Mohammed BA, Saidu H, Yusuf H, Ndejiko Jibrin M, *et al*. From garbage to biomaterials: An overview on egg shell based hydroxyapatite. J Mater 2014;2014:1e802467-6.
18. Salzmann JA. Craig's Restorative Dental Materials. Vol. 71. 14th ed. St. Louis, Missouri, USA: Elsevier Inc.; 1977. p. 228.
19. Sirait M, Sinulingga K, Siregar N, Doloksaribu ME, Amelia. Characterization of hydroxyapatite by cytotoxicity test and bending test. J Phys Conf Ser 2022;2193:e012039.
20. Mohammadi Z, Shalavi S, Yazdizadeh M. Antimicrobial activity of calcium hydroxide in endodontics: A review. Chonnam Med J 2012;48:133-40.
21. Chen L, Zheng L, Jiang J, Gui J, Zhang L, Huang Y, *et al*. Calcium hydroxide-induced proliferation, migration, osteogenic differentiation, and mineralization via the mitogen-activated protein kinase pathway in human dental pulp stem cells. J Endod 2016;42:1355-61.
22. Haniastuti T, Susilowati H, Rinastiti M. Viability and alkaline phosphatase activity of human dental pulp cells after exposure to yellowfin tuna bone-derived hydroxyapatite in vitro. Int J Dent 2020;2020:e8857534.
23. Swarup SJ, Rao A, Boaz K, Srikant N, Shenoy R. Pulpal response to nano hydroxyapatite, mineral trioxide aggregate and calcium hydroxide when used as a direct pulp capping agent: An in vivo study. J Clin Pediatr Dent 2014;38:201-6.

jeremy

by Elline Istanto

Submission date: 10-Mar-2025 07:39PM (UTC+0700)
Submission ID: 2610627016
File name: revisi_jeremy_2_docx.docx (80.9K)
Word count: 2742
Character count: 14678

Title of the article: Cytotoxicity Test Of Eggshell-Based Hydroxyapatite On Human Dental Pulp Cell

Abstract:
Background: Nowadays, pulp treatment materials are necessary to preserve pulp vitality. The materials such as Ca(OH)2 are commonly used, but there are some limitations. Hydroxyapatite has been proven to be able to induce reparative dentin so it can be used as an alternative for pulp treatment. However, pulp treatment materials should have some ideal characteristics. The toxicity test is one of the essential tests to control the biological safety of material. **Purpose:** To determine the toxicity of hydroxyapatite from eggshell waste with various concentrations to human dental pulp cells (hDPSCs). **Method:** In the present research, we used the MTT assay method followed by an ELISA reader on the human dental pulp stem cells (hDPSCs) to see the cell viability.The study was conducted by testing HA with various concentrations, 1%, 2%, and 4%. The results are seen by calculating the Optical Density to determine the viability of the pulp cells. **Result:** The sequential values of the viability of the 1%, 2%, and 4% HA are 84.1%, 86.75%, and 95.03% and all groups performed insignificant human dental pulp cells proliferation value. **Conclusion:** It can be concluded that HA material is nontoxic material and it has the potential to support human dental pulp cell proliferation which was one of the essential criteria as a pulp treatment material.

Key-words: Eggshell Based Hydroxyapatite, toxicity, Human Dental Pulp Cell Culture

Text

Introduction:

Currently, vital pulp treatments are often used to avoid further pulp infection which can lead to pulp necrosis. Pulp treatment materials are applied to an exposed pulp to induce reparative dentine.[1] Calcium hydroxide (Ca(OH)2 are commonly used material in vital pulp treatment.[2] Several previous studies stated that Ca(OH)2 is soluble and can cause necrosis layer because of its high pH achieve 12,5, and it can cause imperfect dentine bridges. It can perform tunnel defects that cause repenetration of bacteria into the dental pulp.[3]

Hydroxyapatite (HA) has the chemical formula Ca10(PO4)6(OH)2 and is the main mineral in the preparation of bones and teeth.[4] In dentistry, the application of hydroxyapatite itself has been used for various uses such as bone tissue regeneration materials[5], and has also been considered for its function as a vital pulp treatment material because this material can induce hard tissue forming.[6] According to several studies, HA had a lower alkaline pH and has the potential in forming reparative dentine without tunnel defect, and it can lower the inflammation.[7]

Eggshell waste in Indonesia is very high and has not been utilized, therefore, one option for reducing eggshell waste is to process it into hydroxyapatite powder.[8] Chicken egg shells contain 94% Calcium Carbonate (CaCO3), and in dentistry, some of them are used as bone graft material.[9] The content of Calcium Carbonate in the shell of Chicken eggs is higher than eggshells of other poultry.[10] Hydroxyapatite can be synthesized using several methods, such as wet, dry precipitation, and hydrothermal methods.[11]

Former study showed that HA had the potential to induce reparative dentin but there are still insufficient studies performing the toxicity test of HA made from the chicken eggshell. The toxicity test is an important characterization to consider the biological safety of materials, it should have no rejection response from the body's cells or cause cell death.[12] The novelty of this study is to determine the toxicity of various concentrations of chicken eggshell-Based hydroxyapatite (pro-Db) on human dental pulp stem cells (hDPSCs) that may have the potential to be used as a pulp treatment material.

Subjects and Methods:

This is an experimental laboratory study with a posttest-only control group design. The research was conducted at the ProSTEM Laboratory (PT. Prodia StemCell Indonesia). The samples from this study were human pulp cells (cell lines) cultured in the laboratory, so we did not use ethical clearance.

Polyp cells were cultured in a 100 mm petri dish (7 ml of growth medium + 1 ml of cell suspension) incubated for ± 5 days until a confluence of >80% was reached. When confluent, cells were harvested by adding 3 ml of TrypLe (Trypsin) solution and incubated at 370C; 5% CO2 for 7 minutes. Gently tap the petri dish so that the cells are completely released. Neutralize TrypLe with PBS (1:1). Collect all the solution on the petri dish into a 15 mL conical tube. Centrifuge for 5 minutes at 300g speed, then discard the supernatant (TrypLe and PBS), add 1 ml of growth medium and count using a microscope.

The research group was divided into 4 groups: hydroxyapatite (Pro DB, PT Aleesha Berkah Utama, Berkasi, Jawa Barat, Indonesia) with a concentration of 1%, 2%, 4%, and positive control with Ca(OH)2 with 6 repetitions in each sample group so that the total sample was 24.

Hydroxyapatite powder from eggshell waste ready-made by PT Aleesha Berkah Utama that was synthesized using the wet-dry (chemical) precipitation method. The eggshells are cleaned, dried at 110 degrees, and then calcined to produce CaO. Ammonium hydroxide (NH4OH) was used to raise the solution's pH to 10. The phosphate solution was made using the same procedure. The calcium solution was added dropwise to the phosphate solution to perform the synthesis. Synthesis was carried out by dripping the calcium solution into the phosphate solution. The heating was adjusted to a temperature of 40°C (kept constant) with a stirring speed of 300 rpm. After the precipitation is complete, it is aged for 24 hours. The precipitate was then filtered to separate it from the supernatant using Whatman 42 filter paper and then washed with distilled water to remove the remaining ammonium nitrate. The precipitate was then dried at 110°C and then calcined at 900°C for 5 hours.

To make several concentrations (1%, 2%, and 4%), hydroxyapatite was dissolved using deionized water. As a control group, we used calcium hydroxide (PT Pudak *Scientific,* Jakarta, Indonesia) mixed with deionized water at a ratio of 1:1 and then allowed to be set. Preparation was carried out with each hydroxyapatite and calcium hydroxide placed in a dish and dissolved with cell medium Dulbecco's modified eagle medium (DMEM) before being added to each cell sample. Observations with MTT Assay were made 24 hours after the pulp sample was given the test material. Toxicity to the pulp was determined by measuring cell viability/number of living cells after exposure to hydroxyapatite using the MTT colorimetric method and calculating it using an ELISA reader with percent units (Sigma Aldrich, St Louis, Missouri, United States). MTT solution was prepared by mixing 5 mg of MTT powder and 1 mL of 0.9% NaCl. 50 μL of the solution was added to each well using a micropipette. 96-well microplate incubation for 4 hours. Each well was washed with PBS (Lonza, Basel, Switzerland). MTT activity was stopped with 100 μl of acidified isopropanol per well. The 96-well microplate was placed in an orbital shaker for 60 minutes. The absorbance was measured using an ELISA reader with a wavelength of 595 nm. The optical density (OD) is calculated by averaging the reading outcomes acquired with the ELISA reader for each repeat. The viability formula is used to calculate cell viability. We used ISO 10993-5 to determine cell viability presentation. The material was nontoxic if the cell viability more than 70%.[13]

$$Relative\ OD = \frac{OD\ of\ test\ group}{OD\ of\ control} \times 100$$

Results:

Based on the test results, there was a significant difference in cell viability values between HA groups to Ca(OH)2 groups ($p<0,05$). The viability cell of each sample is higher than 70% (Table 1)(Figure 1). The highest to the lowest cells proliferation valus was as follows, first was in Ca(OH)2 groups (220,3%), HAp 4% (95,03%) , 2% (86,75%) and 1% (84,1%). The data are shown in graphs (figure 1). There are no significant differences found between the viability value of the HA groups (Table 2).

Discussion:

According to this study, hydroxyapatite is proven to be able to induce human dental pulp cell proliferation. It has the potential to be used as an alternative for pulp treatment, but it has to be followed with other characterization studies. HA was shown to be biocompatible. It may reduce allergies and inflammation reactions after contact with human tissues.[14] In present study, we used hDPSCs because these cells present a high capacity for proliferation and it considered to be representative of original dental pulp cells in human teeth.[15]

This study was conducted using pre-cultured hDPSCs. Pulp cell culture is divided into 2, primary cell culture and cell line culture. Primary cell culture is done with cells obtained directly from the organism. Cell line cultures are carried out with cells obtained from primary cell cultures. This study used cell culture lines because of the ease of use, can produce unlimitedly, and ignore ethical concerns about the use of human and animal tissue.[16] MTT test was chosen because it is easy to perform, requires a relatively short time, and has good accuracy. The principle of the MTT method is the reduction of the yellow tetrazolium salt into formazan which is purple due to succinate hydrogenase enzyme activity from living cell mitochondria.[17] MTT test results can be measured with the absorbance of light at a wavelength of 550-600 nm. Dead cells will lose the ability to reduce the tetrazolium salt so it will not change color to purple. So the greater the changes of the color becomes purple indicating more cells are alive.[18]

According to ISO 10993-5 bioactive materials are considered to be biocompatible when the cell viability is greater than 70%.[19] All the samples show viability cells above 70% and are considered biocompatible. These results also indicate that the vitality of the cells and HA levels are correlated. As HA concentration rose, cell viability rose as well. The study found that there are no significant differences found between the viability value of the HA groups ($p>0,05$). It showed that 1%,2%, and 4% maybe do not have many different effects in clinical application, but further study should be conducted as well.

HA maybe has the potential to be an alternative material for vital pulp treatment. It can support dental pulp cell proliferation. In previous studies HAp proved to have a lower pH compared to Ca(OH)2.[13] The high alkaline of Ca(OH)2 causes imperfect dentine bridges resulting to tunnel defect which makes repenetration of bacteria is highly possible.[20] Lower pH can reduce tunnel defect and inflammation reaction to the dental pulp. In the present study, the $Ca(OH)_2$ performed high percent cell viability. It is because of the high concentration of calcium contained in the control group. The higher calcium contained will induce more proliferation of the cell.[21] Even though HA proliferation rate was not high in the first 24 hours, a previous study shows that in 72 hours the viability cell is higher than the negative control.[22] It is because HA has a pH of 8-9 which makes it less irritating to pulp cells.[13] Previous study compares the necrosis rate of cells with Ca(OH)2 and HA also shows that HA has less necrosis rate.[23] It may be because of the pH of HA is less than Ca(OH)2

This material can cover the limitations of Ca(OH)2 where the main drawback of Ca(OH)2 material is the presence of tunnel defects in the reparative dentin that is formed. Previous studies reported that HA did not cause tunnel defects and the resulting inflammation was less than Ca(OH)2.[23] The limitation of this study was the cell proliferation value was only observed in 24 hours, maybe more times were needed in a future study. This study showed that HA is non-toxic to dental pulp cells and it supports human dental pulp cell proliferation.

Conclusion:

The HA with 1%,2%, and 4% concentrations proven was non-toxic to human dental pulp cells with a viability value more than 70%. Future characterization study needs to be performed to explore the ability of HA to support the dentin pulp reaction to induce healing mechanism.

References:

1. Yoshida S, Sugii H, Itoyama T, Kadowaki M, Hasegawa D, Tomokiyo A, et al. Development of a novel direct dental pulp-capping material using 4-META/MMA-TBB resin with nano hydroxyapatite. Mater Sci Eng C Mater Biol Appl. 2021;130:e112426.
2. Li Z, Cao L, Fan M, Xu Q. Direct Pulp Capping with Calcium Hydroxide or Mineral Trioxide Aggregate: A Meta-analysis. J Endod. 2015;41(9):1412–7.
3. Widjiastuti I, Dewi M, Prasetyo E, Pribadi N, Moedjiono M. The cytotoxicity test of calcium hydroxide, propolis, and calcium hydroxide-propolis combination in human pulp fibroblast. J Adv Pharm Technol Res. 2020;11(1):20–4.
4. Sadat-Shojai M, Khorasani MT, Dinpanah-Khoshdargi E, Jamshidi A. Synthesis methods for nanosized hydroxyapatite with diverse structures. Acta Biomater. 2013;9(8):7591–621.
5. Okamoto M, Matsumoto S, Sugiyama A, Kanie K, Watanabe M, Huang H, et al. Performance of a Biodegradable Composite with Hydroxyapatite as a Scaffold in Pulp Tissue Repair. Polymers (Basel). 2020;12(4):937.
6. Nasution DFR. Perbedaan Pembentukan Dentinal Bridge Antara Karbonat Apatit dan Kalsium Hidroksida Setelah Dilakukan Direct Pulp Capping pada Molar Satu Maksila Tikus Wistar (Pengamatan 6 Minggu) [Disertasi]. Medan: Universitas Sumatera Utara; 2020.
7. Elline, Fibryanto E, Amanda H. Characterization of nano-hydroxyapatite–collagen and epigallocatechin-3-gallate (EGCG) composites by scanning electron microscopy-energy dispersive spectroscopy (SEM-EDS), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy. Sci Dent J. 2022;6(2):80–6.
8. Suprianto K, Hidayati H, Nilam C, Khairiyah N, Amelia R, Rahmadita S. Hidroksiapatit dari cangkang telur sebagai bone graft yang potensial dalam terapi periodontal. MKGK Clin Dent. 2021;5(3):76–87.
9. Sitohang F, Azis Y, Zultiniar Z. Sintesis Hidroksiapatit Dari Precipitated Calcium Carbonate (PCC) Kulit Telur Ayam Ras Melalui Metode Hidrotermal. jom FTEKNIK. 2016;3(2):1–7.
10. Elline E, Ismiyatin K. Nanohydroxiapatite Using Chicken Eggshell Waste and Its Characterization. Mal J Med Health Sci. 2021;17:83–6.
11. Gago J, Ngapa YD. Pemanfaatan Cangkang Telur Ayam Sebagai Material Dasar Dalam Sintesis Hidroksiapatit Dengan Metode Presipitasi Basah. CAKRA KIMIA (Indonesian E-Journal of Applied Chemistry). 2021;9(1):29–34.
12. Komala D, Amin MN, Rahayu YC. Uji Sitotoksisitas Hidroksiapatit Cangkang Telur Ayam Ras (Gallus gallus) terhadap Sel Fibroblas Ligamen Periodontal Manusia. STOMATOGNATIC. 2022;19(1):49–54.
13. Elline I, Ismiyatin K, Indah Budhy T, Bhardwaj A. The potential of eggshell hydroxyapatite, collagen, and EGCG (HAp-Col-EGCG) scaffold as a pulp regeneration material. Saudi Dent J. 2022 Dec;34(8):715–22.
14. Modena KC da S, Calvo AM, Sipert CR, Colombini-Ishikiriama BL, Dionísio TJ, Navarro MF de L, et al. Molecular Response of Pulp Fibroblasts after Stimulation with Pulp Capping Materials. Braz Dent J. 2020;31(3):244–51.
15. Takeo W. Dental Pulp Stem Cells: Advances to Applications. Stem Cells Cloning: Adv Appl. 2020;13:33–42.
16. Kaur G, Dufour JM. Cell lines. Spermatogenesis. 2012;2(1):1–5.
17. Abdulrahman I, Tijani HI, Mohammed BA, Saidu H, Yusuf H, Ndejiko Jibrin M, et al. From Garbage to Biomaterials: An Overview on Egg Shell Based Hydroxyapatite. J Mater. 2014;2014:e802467.
18. Salzmann JA. Craig's Restorative dental materials. Vol. 71. 14th edn. Elsevier Inc 1977: p. :228.
19. Sirait M, Sinulingga K, Siregar N, Doloksaribu ME, Amelia. Characterization of hydroxyapatite by cytotoxicity test and bending test. J Phys: Conf Ser. 2022;2193(1):e012039.

20. Mohammadi Z, Shalavi S, Yazdizadeh M. Antimicrobial Activity of Calcium Hydroxide in Endodontics: A Review. Chonnam Med J. 2012;48(3):133–40.
21. Chen L, Zheng L, Jiang J, Gui J, Zhang L, Huang Y, et al. Calcium Hydroxide–induced Proliferation, Migration, Osteogenic Differentiation, and Mineralization via the Mitogen-activated Protein Kinase Pathway in Human Dental Pulp Stem Cells. J Endod. 2016;42(9):1355–61.
22. Haniastuti T, Susilowati H, Rinastiti M. Viability and Alkaline Phosphatase Activity of Human Dental Pulp Cells after Exposure to Yellowfin Tuna Bone-Derived Hydroxyapatite In Vitro. Int J Dent. 2020;2020:e8857534.
23. Swarup SJ, Rao A, Boaz K, Srikant N, Shenoy R. Pulpal response to nano hydroxyapatite, mineral trioxide aggregate and calcium hydroxide when used as a direct pulp capping agent: an in vivo study. J Clin Pediatr Dent. 2014;38(3):201–6.
Acknowledgment: none

Tables:

	Control (-)	HA 1%	HA 2%	HA 4%	$Ca(OH)_2$
% cell viability	100%	84,1%	86,75%	95,03%	220,53%

Table 1. Human dental pulp cell viability (%) against hydroxyapatite and calsium hydroxide

Medium	
1% : 2%	0.989
1% : 4%	0.241
2% : 4%	0.479
1% : control (+)	<0.001
2% : control (+)	<0.001
4% : control (+)	<0.001

*Uji Tukey ($p<0,05$)

Table 2. Significancy score between the samples

Tabel 5.8 Tabel rata – rata viabilitas sel pulpa gigi pada pemeriksaan *MTT Assay*

Konsentrasi HAp	Viabilitas sel (%) Rerata±SD	p
1%	84,1±0,05	<0.001*
2%	86,75±0,04	
4%	95,03±	
$Ca(OH)_2$	220,53±	

*Uji Tukey (p<0,05)

Tabel atas hapus

Buat begini aja

Figure Legends

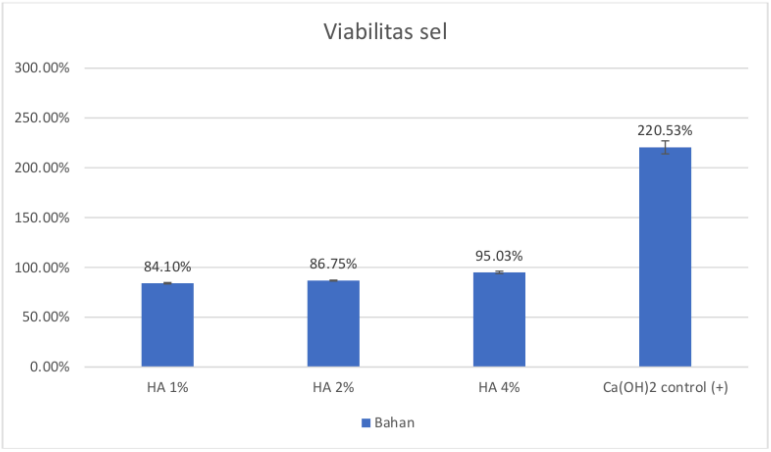


Figure 1. The human dental pulp cell viability chart (%) showed that all the HA groups were nontoxic and can induce the proliferation of the cell

jeremy

ORIGINALITY REPORT

17%	13%	9%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	doaj.org Internet Source	5%
2	Submitted to Universitas Islam Indonesia Student Paper	2%
3	www.mdpi.com Internet Source	1%
4	repositorio.ufrn.br Internet Source	1%
5	P Manu Preetha, G Radha, K Thanigai Arul, J Ramana Ramya. "Enhanced biocompatibility and antibacterial efficacy of CuO-HAp nanocomposite for hard tissue regeneration and repair", Inorganic Chemistry Communications, 2025 Publication	1%
6	repository.unair.ac.id Internet Source	1%
7	www.researchgate.net Internet Source	1%
8	www.jioh.org Internet Source	1%
9	Deok Hyo Yoon, Changwoo Han, Yuanying Fang, Shankariah Gundeti et al. " Inhibitory Activity of Extract on LPS-induced Inflammation in RAW 264.7 Cells by Suppressing NF-κB Activation ", Natural Product Sciences, 2017 Publication	1%

10	www.ncbi.nlm.nih.gov Internet Source	1 %
11	Tetiana Haniastuti, Heni Susilowati, Margareta Rinastiti. "Viability and Alkaline Phosphatase Activity of Human Dental Pulp Cells after Exposure to Yellowfin Tuna Bone-Derived Hydroxyapatite In Vitro", International Journal of Dentistry, 2020 Publication	<1 %
12	Atia Nurul Sidiqa, Myrna Nurlatifah Zakaria, Arief Cahyanto, I Made Joni, Ani Melani Maskoen. "Carbonation inhibitor by polyethylene glycol encapsulation of calcium hydroxide fine particles to improve antimicrobial and root canal penetration properties", Heliyon, 2023 Publication	<1 %
13	JM de Araújo Lopes, F Benetti, GC Rezende, MT Souza, LC Conti, E Ervolino, RC Jacinto, ED Zanotto, LTA Cintra. "Biocompatibility, induction of mineralisation and antimicrobial activity of experimental pastes based on glass and glass-ceramic materials", International Endodontic Journal, 2020 Publication	<1 %
14	Shihabudheen M.Maliyekkal, , Gayathri Pullangott, Satyakam Patnaik, Pamarthi Amesh, Nikita Shraogi. "Synergetic action of bicarbonate ions on chitosan-silver nanocomposite leading to enhanced water disinfection", Open Science Framework, 2023 Publication	<1 %
15	ecommons.aku.edu Internet Source	<1 %
16	japtr.org Internet Source	

		<1%
17	link.springer.com Internet Source	<1%
18	Elline Elline, Kun Ismiyatin, Theresia Indah Budhy, Anuj Bhardwaj. "The potential of eggshell hydroxyapatite, collagen, and EGCG (HAp-Col-EGCG) scaffold as a pulp regeneration material", The Saudi Dental Journal, 2022 Publication	<1%

Exclude quotes	On	Exclude matches	Off
Exclude bibliography	On		