Original Article

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

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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

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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 ($P < 0.05$)

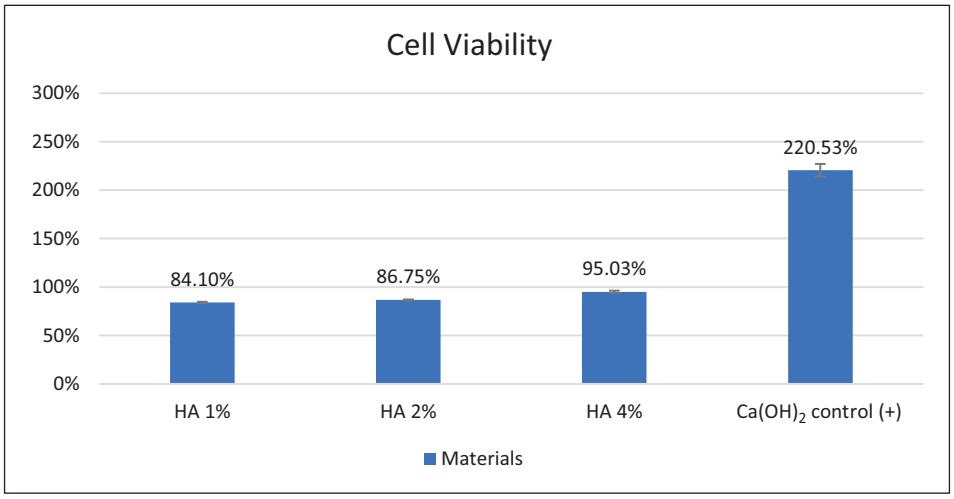

Figure 1: The human dental pulp stem cell viability chart (%) showed that eggshell hydroxyapatite was nontoxic at all tested concentrations and could induce cell proliferation

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.

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Conflicts of interest

There are no conflicts of interest.

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