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RESEARCH ARTICLE

The Toxicity Assessment of Hydroxyapatite with Theobromine Formulation on Human Dental Pulp Stem Cells (hDPSC)

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ABSTRACT

Background: *Deep caries not treated immediately can cause more severe damage and loss of tooth vitality. Ca(OH)2 and MTA are often used as vital pulp treatment materials, but both have limitations. Therefore, alternative materials are needed. Hydroxyapatite is a major component of bones and teeth forms reparative dentin without tunnel defects, while theobromine has been shown to have a better anti-cariogenic effect than fluoride. The combination of both can be a choice for vital pulp treatment. Therefore, conducting cytotoxicity and cell proliferation tests is important to ensure safety and determine whether the material can induce cell proliferation.* ***Objective:*** *To determine the toxicity and proliferation of hydroxyapatite theobromine on human dental pulp stem cells.* ***Materials and Methods:*** *The toxicity assessment of hydroxyapatite with theobromine formulation on human dental pulp stem cells (hDPSC) was a laboratory experimental study (in vitro) carried out using a posttest control group design with the method used being the MTT assay.* ***Results:*** *The highest cell viability value was Ca(OH)2 122.08%, control cells 113.70%, HA/TB 2:1 108.03%, HA/TB 1:1 103.23%, HA/TB 1:2 102.09%, and the lowest was negative control 100%. The highest proliferation value was in control cells 138.00%, HA/TB 2:1 135.00%, HA/TB 1:2 129.64%, Ca(OH)2 128.38%, and HA/TB 1:1 122.11%, and the lowest cell proliferation value was in the negative control at 100%. This shows that the higher the hydroxyapatite value compared to theobromine, the greater the percentage value.* ***Conclusion:*** *Theobromine hydroxyapatite formulation is non-toxic, so it has potential as a vital pulp treatment ingredient, but it is less effective in inducing cell proliferation.*

Keywords: *Cytotoxicity, Proliferation, Hydroxyapatite, Theobromine, MTT Assay.*

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INTRODUCTION

Caries is a disease of the teeth and mouth experienced by almost the entire population of the world.[1] If not treated immediately, it will worsen caries and cause loss of tooth vitality.[2] To maintain the vitality of the pulp in young permanent teeth, treatments can be carried out, including pulpotomy and pulp capping.[3]

$Ca(OH)_2$ and MTA are materials used in pulp capping and pulpotomy treatments.[4] These materials are used in vital pulp therapy treatments.[5] One of the gold standard materials in pulp capping treatments is $Ca(OH)_2$. This material has good antibacterial properties because it has a pH of around 12, but this can also be a limitation because it causes necrosis in tissues that come into direct contact with the material.[6] Previous studies have found that $Ca(OH)_2$ causes damage to tunnel defects, causing the potential for bacterial infection and necrosis in the superficial part of the pulp layer.[7] Compared to $Ca(OH)_2$, MTA is more effective and superior as a pulp coating material in pulp capping and pulpotomy treatments, showing a higher success rate with good results in maintaining tooth vitality in the long term.[8] There are limitations to MTA, including requiring a long setting time and not being easy to manipulate as a direct pulp capping material.[9]

Other alternative materials for vital pulp treatment are formulations of hydroxyapatite and theobromine. Hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$ is a biomineral material of hard tissue in bones and teeth containing phosphate and calcium.[10] Hydroxyapatite is synthesized from calcium-rich materials such as mollusk shells, chicken eggshells, and cow and goat bones.[11] In vital pulp therapy, HA forms reparative dentin,[12] without tunnel defects with the inflammatory response caused more effectively reduced compared to $Ca(OH)_2$.[13] Theobromine (TB) $C_7H_8N_4O_2$ is an alkaloid compound of the methylxanthine group contained in the cocoa plant. Theobromine is often used in dental health care, including vital pulp therapy.

HA-TB comes from a natural composition, so it has high biocompatibility and can increase tissue regeneration. Apart from that, hydroxyapatite can support bone remineralization such as pulp capping,[12] theobromine has antibacterial properties and stimulates the formation of HA crystals,[14] this formulation of HA-TB can potentially become a pulp capping material by combining the remineralization ability of HA and the antibacterial properties and structural strengthening of theobromine. This formulation can provide better results in pulp and dentin regeneration, so it can potentially be an alternative material for vital pulp therapy treatments.

Research on HA-TB formulation is still rare because it is a new material, so toxicity and cell proliferation testing is needed to ensure the safety of the material if it enters the oral cavity and to determine whether the formulation of the material can induce cell proliferation.

This study discusses the toxicity and proliferation tests of HA formulations derived from chicken eggshells and theobromine from cocoa on hDPSC cells with ratios of 1:1, 1:2 and 2:1 for both test materials, to see whether hydroxyapatite or thebromine is more dominant in the results of the toxicity and proliferation tests, so that it has the potential as an alternative vital pulp treatment.

MATERIALS AND METHODS

This is a laboratory experimental study with a posttest-only control group design divided into three groups with HA/TB ratios of 1:1, HA/TB 1:2, HA/TB 2:1, and a control group with $Ca(OH)_2$ (n=6). The samples of this study were hDPSC cells that were first cultured at the ProSTEM Laboratory.

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Culture Cell of hDPSC Procedure

hDPSC cells were cultured in 50 mm cell culture dishes (5-10 ml of complete medium) and incubated for 2-7 days until confluence. The cells were then harvested by discarding the old medium and washing with fetal bovine serum, adding trypsin to release the cells, and then incubating in a CO_2 5% incubator at 37°C for 7 minutes. Next, PBS (1:1) was added to wash the cells, then the cells were taken and collected in a tube. Furthermore, centrifugation was carried out at 300g for 5 minutes to separate the cells from the supernatant. After that, the supernatant was discarded, and 1 ml of cell medium was added to stabilize the cell condition. The number of cells was counted using an automated cell counter, then 5000 cells were put into each well of a 96-well plate. Furthermore, the cells were incubated for 3-4 days.

HA-TB Formulation Procedure

The Hydroxyapatite and theobromine were dissolved in 100 mL of deionized water with ratios of 1:1, 1:2, and 2:1.

The formulation was made by mixing hydroxyapatite and theobromine that had been prepared in a dish and then dissolved with 0.5 mL of Dulbecco Modified Eagle Medium (DMEM). The HA-TB formulation that had been dissolved was transferred to a 96-well microplate and incubated for 24 hours.

Toxicity Test and Proliferation Cell Test

Toxicity test was conducted by MTT method, where 100 µL of MTT solution was added to each well of 96-well microplate using micropipette. The microplate was then incubated at 37°C for 24 hours. After incubation, the MTT reaction was stopped by adding 100 µL of solubilizer to each well. Absorbance was measured using an Elisa reader at 450 nm wavelength, and cell viability at various concentrations was calculated using viability formula.

Cell proliferation measurements were performed by calculating cell viability after 48 hours using the MTT method. The hDPSC cells were grown on 96-well microplates at a density of $5x10^3$ cells per well and incubated for 24 hours. Then, the culture medium was replaced, and test solutions with various concentrations were added and then incubated for 24 hours. Next, 100 µL of culture medium and 100 µL of MTT solution were added to each well and incubated for 4-6 hours in a CO_2 5% incubator at 37°C. Absorbance was measured using a microplate reader at a wavelength of 450 nm.

RESULTS

Table 1 and Figure 1 show the results of the toxicity test. Table 1 shows the mean, standard deviation, and significance of the effects of the various treatment groups on human dental pulp stem cells (hDPSC). Statistical analysis was performed using *One-Way* ANOVA. There were significant differences between the test groups ($p < 0.05$).

Table 1. Mean value, standard deviation, and significance of the effect between groups of test materials on hDPSC cells

Group Test	Average ± SD	P
HA/TB 1:1	0,141517±0,0036108	
HA/TB 1:2	0,139700±0,0024706	<0,001
HA/TB 2:1	0,147833± 0,0033916	
Control (+)	0,151733± 0,0044107	

**One-way* ANOVA (p<0,05)

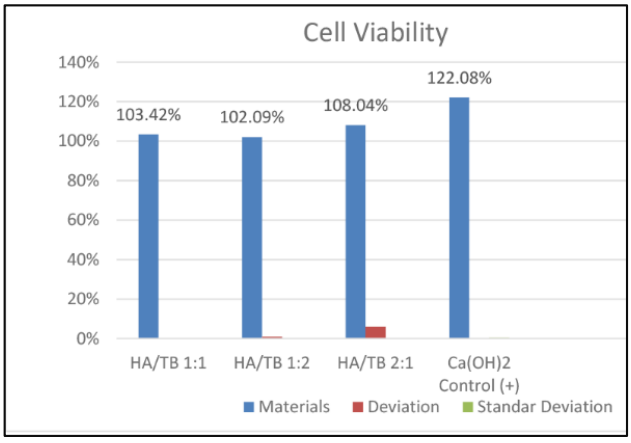

Figure 1. The human dental pulp stem cell viability chart (%).

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Figure 1 shows the results of the toxicity test calculation in percentages with values above 100 percent, indicating that the test material is not toxic. The highest value was the control (+) 122.08, HA/TB 2:1 108.04%, HA/TB 1:1 103.42%, and the lowest being HA/TB 1:2 102.09%.

Table 2. The significance value between groups

	HA/TB 1:1	HA/TB 1:2	HA/TB 2:1	Control (+)
HA/TB 1:1		0,978	0,155	0,004
HA/TB 1:2			0,033	<0,001*
HA/TB 2:1				0,639
Control (+)				

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

Table 2 shows the significance value between groups. There is no significant difference on HA/TB 1:1 and HA/TB 2:1, but on HA/TB 2:1, there is a significant difference from HA/TB 1:2.

Furthermore, proliferation tests were carried out to determine whether HA/TB formulations with various ratios could induce cell proliferation; the test results are in table 3 and figure 2.

Table 3. Mean, standard deviation, and significance of the test materials against hDPSC cells

Group Test	Average ± SD	P
HA/TB 1:1	0,141517±0,0036108	
HA/TB 1:2	0,139700± 0,0024706	<0,001
HA/TB 2:1	0,147833± 0,0033916	
Control (+)	0,151733± 0,0044107	

**One-way* ANOVA (p<0,05)

In Table 3, the results obtained from the One-way ANOVA test show significant differences within the tested groups (p<0.05).

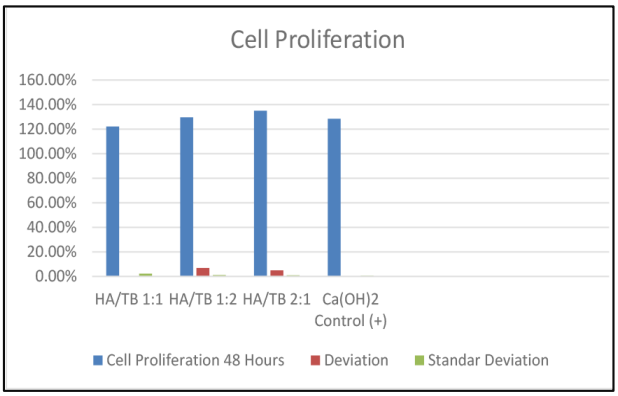

Figure 2. The Human dental pulp stem cell viability chart (%) showing hydroxyapatite formulation on hDPSC cell proliferation

Figure 2 Based on the test results, HA/TB 1:2 and 2:1 had a higher proliferation value than the positive control, but HA/TB 1:1 had a lower proliferation value than the positive control. It can be concluded that HA-TB formulation can induce proliferation but not significantly.

Table 4. Significance value between proliferation test groups

	HA/TB 1:1	HA/TB 1:2	HA/TB 2:1	Control (+)
HA/TB 1:1		0,561	0,074	0,731
HA/TB 1:2			0,838	1,000
HA/TB 2:1				0,685
Control (+)				

*Tukey test (p<0,05)

Based on the cell proliferation test, there is no significant difference between HA/TB 1:2, HA/TB 2:1, and HA/TB 1:1 test materials (Table 4). Compared with the positive control, there was a significant difference in HA/TB 1:1. It can be concluded that HA-TB formulation can induce cell proliferation but not significantly.

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DISCUSSIONS

Untreated dental caries can lead to the opening of the pulp chamber,[15] which leads to inflammation of the pulp tissue and loss of tooth vitality.[2] To prevent this, vital pulp treatments such as pulpotomy and pulpcapping can be performed to maintain pulp vitality.[3] Common materials used in these treatments are $Ca(OH)_2$ and MTA, although $Ca(OH)_2$ has some limitations,[4] so alternative materials are needed.

This aims to evaluate whether hydroxyapatite and theobromine formulations can be an alternative treatment for vital pulp therapy in human dental pulp stem cells (hDPSC) through toxicity and cell proliferation tests. The study was conducted with an hDPSC cell culture and cultured in vitro.[16] Primary cell culture was chosen because it has a lower risk of genetic mutation and ease of use. The hDPSC cells have an important role in pulp tissue regeneration and the ability to repair damage to dental pulp.[17]

Cytotoxicity and proliferation tests were conducted to evaluate the safety of the material and the increase in cell number. In this study, the test was conducted with the MTT method using several HA/TB treatment groups with a ratio of 1:1, 1:2, 2:1, and positive control Ca(OH)2. The cytotoxicity test was conducted for 24 hours, while the proliferation test was conducted for 48 hours, with 6 repetitions each.

The MTT method was used in this study because the time required is relatively short and can be measured accurately. The working principle of the MTT method is a colorimetric measurement by determining the functional state of mitochondria and indicating cell viability with a mechanism in the form of tetrazolium salts that are soluble in water by producing a yellow solution, which will be reduced in cells that have metabolic activity.[18] The results of the MTT test can be measured by absorbance of light at 450 nm wavelength. In living cells, it will react with MTT to form purple formazan crystals, while in dead cells, no color change occurs.

Based on the ISO 10993-5 standard, in measuring the cell viability value, if the value results in a test material above 90%, the material is biocompatible. In this study, all samples tested showed cell viability above 90%, indicating that the test material is biocompatible.[19] These results also indicate a relationship between cell viability and the amount of HA exposure. HA with a large concentration will increase the viability value. No significant difference was found between the viability values at HA/TB 1:1, 1:2, 2:1 ($P > 0.05$), indicating that the test samples will not produce different effects in clinical application.

HA/TB may have potential as an alternative material for vital pulp treatment, as it is non-toxic but not significant in inducing cell proliferation, so further studies with other characterizations are needed.

In conducting a study on test materials, the pH value is important to note because it can play a role in maintaining pulp vitality and regeneration. In previous studies, HA had a lower pH value than $Ca(OH)_2$. The high pH value of calcium hydroxide can cause tunnel defects that create the potential for bacterial infection and result in necrosis in the superficial part of the pulp layer.[7] In this study, $Ca(OH)_2$ can support cell viability because its high calcium concentration may induce cell proliferation.[20] Besides pH, the amount of hydroxyapatite used in the HA-TB formulation also has an effect, as from the results of this study the ratio of HA/TB 1:1 with the positive control the results obtained were higher values for the positive control compared to the results of the value of HA/TB 1:1, so that the amount of hydroxyapatite used if it was greater than theobromine, this could be effective in inducing cell proliferation. In previous studies, cell proliferation tests with 24 hours and 48 hours were shown to induce cell proliferation.[22] In this study, there are limitations, namely that the study only used 3 concentrations of HA / TB ratio and only used 1 time parameter in the research observation, namely 48 hours in the cell proliferation test, so that it has not shown

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the actual conditions in the cell life cycle, which are measured using several time parameters and several characterizations of materials that have potential as vital pulp therapy treatment materials. Further research needs to be done to identify other characteristics of the HAP and theobromine as alternative materials in vital pulp treatment.

CONCLUSIONS

Based on the results of the study, the formulation of HAP Theobromine to hDPSC with a ratio of 1:1, 1:2, 2:1, is non-toxic because it has a viability value above 100, but is not significant in inducing cell proliferation. Nevertheless, HAP and Theobromine have potential as alternative materials in vital pulp treatment.

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