The Toxicity Assessment of The Nanohydroxyapatite, Epigalocathecine-3-Gallate, And Hydroxyprophil Methylcellulose Hydrogel

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ABSTRACT
The previous study showed that the hydroxyapatite, Epigalocathecine-3-Gallate (EGCG), and hydroxypropyl methylcellulose (HAp-EGCG-HPMC) material has the potential to be combined into a useful material in the field of dentistry, and one of them is as a pulp regeneration material. To determine the safety of the HAp-EGCG-HPMC formulation, the characterization of this new material should be performed using the cytotoxicity and proliferation test. It can show the number of living cells and it can indicate the vitality mechanism in certain proteins, also knowing the cell survival after exposure to HAp-EGCG-HPMC material. The method used to detect cell proliferation is commonly similar to the viability test. The cytotoxicity and proliferation were performed **Methods**: The HAp-EGCG-HPMC hydrogel was made by dissolving hydroxyapatite powder in distilled water to make a concentration of 1%, 2%, 4% with 10µmol/mL EGCG, and hydroxypropyl methylcellulose carriers were added. The cytotoxicity and proliferation in fibroblast cells of HA-EGCG-HPMC hydrogel was done using by CCK-8 dye and read by spectrophotometer with 450nm wavelength. **Results**: Hydroxyapatite, EGCG, and HPMC hydrogels were nontoxic to fibroblast cells 24 hours after mixing and it can induce proliferation of fibroblast cells. **Conclusion**: The hydrogel containing hydroxyapatite, EGCG, and HPMC is non-toxic and promotes the proliferation of fibroblast cells, making it suitable as a material for pulp regeneration.

INTRODUCTION

The latest technology has greatly improved, including nanotechnology in the dentistry field. Nanomaterials including nanohydroxyapatite should have strict rules for use in the medical field. (1) The Nanohydroxyapatite (HAp) is a material that is similar to bone and teeth, nanohydroxyapatite can be taken from eggshells and it can be crosslinked with epigallocatechin-3-gallate into the hydrogel. It also potential to be used as a pulp regeneration material. (2) To form a gel, several studies used hydroxypropyl methylcellulose as a thickening material. (3) Previous findings showed that HAp from chicken eggshell waste can induce human dental pulp cell proliferation. It is biocompatible, nontoxic, and can be an antioxidant. (4,5) Hydroxyapatite in several concentrations can induce human dental pulp cell (hDPSc) proliferation for 24 hours. (4) In the dentistry field, epigallocatechin-3-gallate (EGCG) has the potential to reduce inflammation. (6) Hydroxyl propyl methyl cellulose (HPMC) is an odorless and tasteless fibrous substance powder that is creamy with white color. HPMC is used as a polymer matrix which is a stable carrier material compared to other carrier materials and it is essential in the gelling form process.(7) In this study, several HAp concentrations were added with EGCG and HPMC to become hydrogel and the toxicity assessment was analyzed. Mineral Trioxide Aggregate (MTA) was used as a control group. An invention in medical science material, suggests that a new formulation material may have

an interaction with cells. The toxicity assessments commonly used are cytotoxicity and cell proliferation assay. A decrease in cell viability may show essential disturbance in physiological conditions. The cessation of the proliferation process may lead to a cell death process.(8) Many conventional standard method assays can applied in cytotoxicity analysis. One of them is the cell counting kit-8 (CCK-8) assay and it reads by spectrophotometer.(9)

RESEARCH METHOD

Formulation of HA-EGCG-HPMC hydrogel

HAp formulation refers to the previous hydrogel preparation by Elline et al (2) Hydroxyapatite powder (ProDB , PT.Aleesha Berkah Utama, Bekasi, Jawa Barat, Indonesia) is dissolved in deionized water and stirred with a magnetic stirrer at a speed of 350 rpm with a concentration of 1%, 2%, and 4%.(10) 10µg/mL EGCG (Sigma Aldrich, E4268, 80 %, USA).was added to the hydroxyapatite solution. Each sample was stirred until homogeneous at a temperature of 40℃ for 30 minutes and the carrier material 2% HPMC (Benecel, K100M, Ashland, Wilmington,USA) was added. Samples were divided into 8 groups, negative control and an MTA group as a positive control.

The cytotoxicity Test

The HA-EGCG-HPMC hydrogel was placed in a 96-well plate and incubated for 24 hours in a 5% CO_2 incubator. Then the test solution of various concentration mixtures was added in 3 replications, then the plate was incubated in a 5% CO_2 incubator for 24 hours at a temperature of 37°C. At the end of incubation, the medium in each well was removed and washed with phosphate-buffered saline (PBS) (Gibco, Thermofisher Scientific, USA), then 100 µl of 0.5% CCK-8 in PBS was added. The plate was incubated again for 1 hour at 37˚C. In the CCK-8 assay, the dye of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenol-yl)-5-(2,4disulfophenyl)-2H-tetrazolium, monosodium salt] was decreased by dehydrogenase in cells to make a water-soluble orange-colored product (formazan) and it correlates with living cells that act with CCK-8 to form orange formazan. Absorption is read with a spectrophotometer with a wavelength of 450 nm using a wellplate. The Cytotoxicity was assessed 24 hours after stain exposure using the CCK-8 toxicity assay. (9,11)

Cell Proliferation Test

The cells that had been planted in 10,000 cells/well using a 96-well plate in the culture medium were replaced with 200 µl of sample solution and then incubated for 24 hours. After 24 hours, the treatment was discarded and the wells were washed with DMEM (Gibco, Thermofisher) once. Then add 100 µl of CCK-8 solution (Sigma-Aldrich,USA) to each well and incubate for 90 minutes. Observations were made after 24 hours, 3, 6, 8, and 10 days. Measure the absorbance at a wavelength of 450 nm using a spectrophotometer. The percentage of cell growth can be calculated using the formula (12):

$$\% \text{ Viability} = \frac{\text{absorbance of test cell}}{\text{Absorbance of control cell}} \times 100\%$$

Statistical analysis

A normality test was carried out with Shapiro–Wilk on all data obtained. If the data was normal, a two-way analysis of variance (ANOVA) was used. Otherwise, the data was analyzed using Kruskal–Wallis. Data are described as the mean and standard deviation values at $P < 0.05$. Statistics were performed using SPSS 25 (SPSS Inc, Chicago, IL, USA).

RESULT AND DISCUSSION

Cytotoxicity Test

The value of the cytotoxicity test using the CCK-8 method is calculated in percent (%) and it was performed for 24 hours. It describes the percentage of the number of fibroblast cells that survive after treatment is presented in Figure 1.

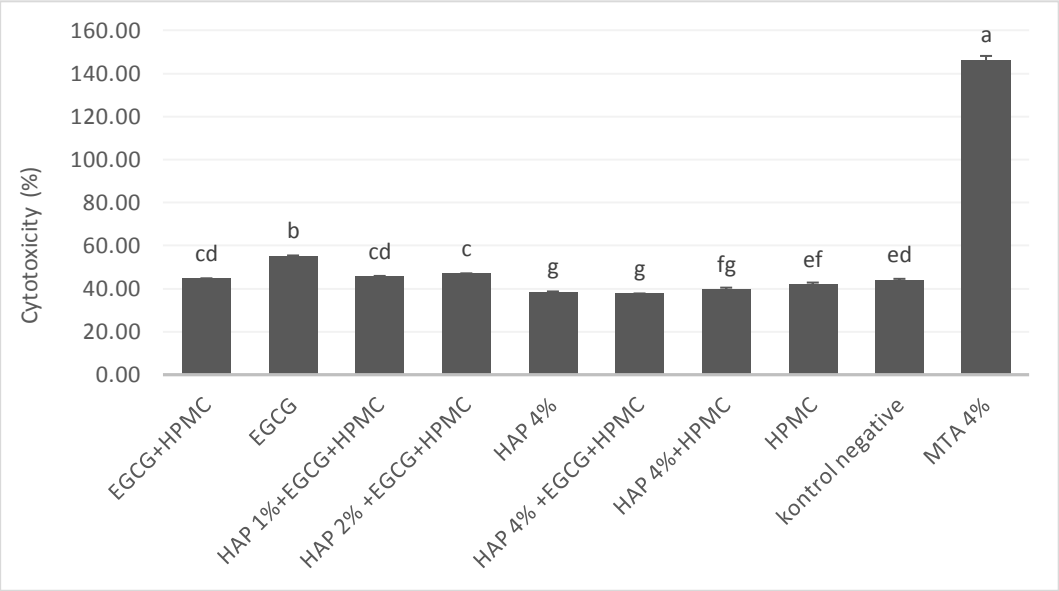

Figure 1. Cytotoxicity of several HA concentrations with -EGCG-HPMC to fibroblast cells

One-way ANOVA shows that there was a significant difference in all groups. The Post hoc test results showed that the negative control toxicity value of 43.96% was not significantly different ($p>0.05$) from HPMC, EGCG+HPMC, and HAp1%-EGCG-HPMC with a mean cytotoxicity value of 41.83%, 44.62%, and 45.53%. However, it was significantly different ($p<0.05$) from the HAP 4% EGCG-HPMC, HAP 4%, HAP 4%-HPMC, HAP 2%-EGCG-HPMC, EGCG, MTA 4% groups with mean cytotoxicity values respectively 37.71%, 38.31%, 39.53%, 46.85%, 55.01%, and 146%.

The positive control MTA 4% was significantly different ($p<0.05$) from the HAP 4%-EGCG-HPMC, HAP4%, HAP4%-EGCG, HPMC, negative control, EGCG-HPMC, HAP1%-EGCG-HPMC, HAP2% groups. EGCG-HPMC, and EGCG. Based on the results of the post-hoc test, it was found that the HAP-EGCG-HPMC hydrogel had low cytotoxicity when compared to the negative control and MTA.

Cell Proliferation

The results of the cytotoxicity test showed that the HAP-EGCG-HPMC mixture was not toxic to fibroblast cells, then continued with a cells proliferation test with percentage (%) data values which describes the percentage of proliferation of the number of living fibroblast cells after treatment on days 3, 6, 8, and 10. They are presented in Figure 2.

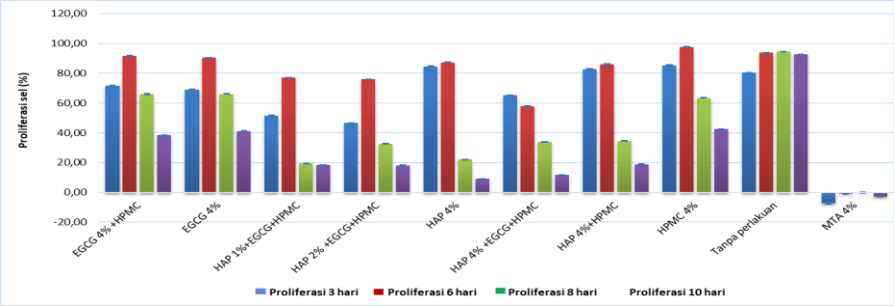

Figure 2. The percentage of the proliferation of the number of living fibroblast cells after treatment on days 3, 6, 8, and 10

The results of the two-way ANOVA test showed a p-value <0.05, so a Bonferroni post-hoc test was carried out. The results of the post-hoc test on proliferation based on treatment days proved that proliferation on day 6 was significantly more than on day 3 (p<0.001). In the contrary, the cell proliferation on day 3 was significantly more than on days 8 and 10 (p<0.001). Post-hoc test results based on days are presented in Table 1.

Tabel 1. The Post-hoc Proliferation Based on Days of Observation

Days		Days	Mean Difference	P Value
Day 3	-	Day 6	-12,7	0 ,001
	-	Day 8	19,8	0 ,001
	-	Day 10	34,2	0 ,001
Day 6	-	Day 8	32,5	0 ,001
	-	Day10	46,9	0 ,001
Day 8	-	Day 10	14,4	0 ,001

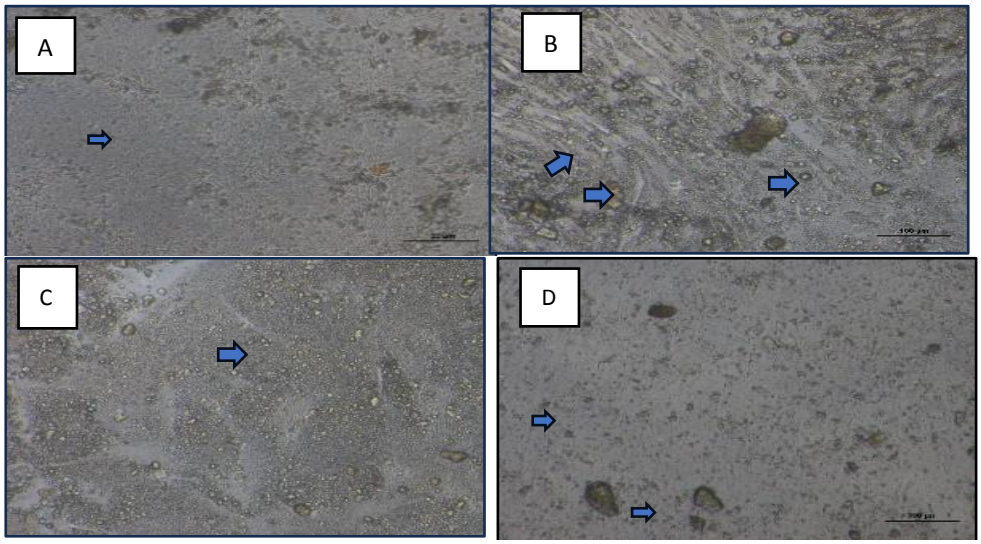

Figure 3. Group 2 (HAP 4%-EGCG-HPMC) proliferation test results days 3 (a), 6 (b), 8 (c), 10 days (d). The fibroblast cells are indicated by blue arrows

Based on days, there are significant differences between each group, so it is necessary to test proliferation based on treatment groups which can be seen in table 2. In the posthoc test of proliferation by group, it was proven that there was a significant difference in proliferation rate in most of the groups (p<0.001), except between the EGCG group and the

EGCG+HPMC group (p<0.05) Table 2. This study showed that the HAP-EGCG-HPMC material did not have a proliferation value that exceeded the untreated group, which shows that the mixture of materials did not have a proliferative effect.

Table 2. Post-hoc Test Based on Group

Groups		Groups	Mean Difference	P Value
NEG CONTROL	-	HAP 4% +EGCG+HPMC	48,311	0 .001
	-	HAP 2% +EGCG+HPMC	47,072	0 .001
	-	HAP 1%+EGCG+HPMC	48,902	0 .001
	-	HAP 4%	39,704	0 .001
	-	HPMC	18,226	0 .001
	-	EGCG	23,714	0 .001
	-	HAP 4%+HPMC	34,832	0 .001
	-	EGCG+HPMC	23,513	0 .001
	-	MTA 4%	93,354	0 .001
HAP 4% +EGCG+HPMC	-	HAP 2% +EGCG+HPMC	-1,238	0 .001
	-	HAP 1%+EGCG+HPMC	0,591	0 .001
	-	HAP 4%	-8,607	0 .001
	-	HPMC	-30,085	0 .001
	-	EGCG	-24,597	0 .001
	-	HAP 4%+HPMC	-13,479	0 .001
	-	EGCG +HPMC	-24,797	0 .001
	-	MTA 4%	45,043	0 .001
HAP 2% +EGCG+HPMC	-	HAP 1%+EGCG+HPMC	1,829	0 .001
	-	HAP 4%	-7,368	0 .001
	-	HPMC	-28,847	0 .001
	-	EGCG	-23,358	0 .001
	-	HAP 4%+HPMC	-12,241	0 .001
	-	EGCG +HPMC	-23,559	0 .001
	-	MTA 4%	46,281	0 .001
HAP 1%+EGCG+HPMC	-	HAP 4%	-9,198	0 .001
	-	HPMC	-30,676	0 .001
	-	EGCG	-25,188	0 .001
	-	HAP 4%+HPMC	-14,070	0 .001
	-	EGCG +HPMC	-25,388	0 .001
	-	MTA 4%	44,452	0 .001
HAP 4%	-	HPMC	-21,478	0 .001
	-	EGCG	-15,990	0 .001
	-	HAP 4%+HPMC	-4,873	0 .001
	-	EGCG +HPMC	-16,191	0 .001
	-	MTA 4%	53,650	0 .001
HPMC	-	EGCG	5,488	0 .001
	-	HAP 4%+HPMC	16,606	0 .001
	-	EGCG +HPMC	5,288	0 .001
	-	MTA 4%	75,128	0 .001
EGCG	-	HAP 4%+HPMC	11,118	0 .001

	-	EGCG +HPMC	-0,201	0.543 *
	-	MTA 4%	69,640	0.001
HAP 4%+HPMC	-	EGCG +HPMC	-11,318	0.001
	-	MTA 4%	58,522	0.001
EGCG+HPMC	-	MTA 4%	69,841	0.001

*p>0,05

Discussion

Pulp regeneration aims to establish recovery of inflamed pulp. Nowadays, pulp regeneration relates to the tissue engineering concept that combines stem cells, bioactive material, and growth factors.(13) In this study, a formulation of HAp-EGCG-HPMC material with different concentrations (1%,2%,4%) of HAp was used to make a hydrogel form. A cytotoxicity test was carried out using the Cell Counting Kit-8 (CCK-8) method to evaluate fibroblast cell metabolism against new substances. The data were read by a spectrophotometer with 450 nm wavelength. Based on dye labels, conventional colorimetric assays such as CCK-8 assay were commonly used for the cytotoxic analysis of new material because of their excellent sensitivity and ease of operation. The highly water-soluble tetrazolium salt used made the CCK-8 assay give more sensitive detection than other tetrazolium.(9,14)

The results of this study showed that the HPMC, EGCG and the EGCG+HPMC toxicity values were significantly higher compared to the 4% MTA group ($p<0,05$). However, there was no significant difference between other sample groups when compared with MTA 4% ($p>0.05$).

According to Escobar et al., the cytotoxicity of MTA material on fibroblast cells was not toxic at 24 hours of exposure to the material, but the number of fibroblast cells decreased on the 7th day after the material was exposed to MTA.(15) It almost in line with Noites et al., have also carried out a cytotoxicity test on the Bio MTA material on fibroblast cells of human gingiva using Methyl tetrazolium (MTT) and Sulforhodamine B (SRB) media and the results showed a decrease in fibroblast cell viability at a concentration of 100 mg/ml after 24 and 72 hours.(16)

This study also proved that HAp-EGCG-HPMC was biocompatible material to fibroblast cell. It similar to Utama et al., that proved hydroxyapatite from chicken eggshells is not toxic to human dental pulp cells. (4) Maria et al. also obtained results that nano-hydroxyapatite from chicken egg shells and the addition of sucrose are safe to human stem cells by staining resazurin assay. (17) Rohmadi et al., have carried out a systematic review of various articles on the cytotoxicity of hydroxyapatite eggshell and found to have cell viability of 70% with the conclusion that the eggshell hydroxyapatite material is not toxic. (18)

This study also showed that EGCG has higher cytotoxicity than MTA. According to a previous study, EGCG itself is toxic when used without other crosslinking material and in high dosage. (19) Kucera et al. analyzed the effect of EGCG on the primary culture of rat hepatocytes for 24 hours. It causes cellular damage and lowers the hepatocyte functions. The EGCG made the form of reactive oxygen species (ROS) in the biphasic phase. However, lower doses of EGCG can decrease ROS production. (20) According to Li et al., 10 µg/mL EGCG had no effect in hDPSc proliferation, but it decreased inflammation, so it can preserve cell proliferation ability. (10)

According to several studies, HPMC is a nontoxic agent.(21) However, in this study, HPMC has a higher cytotoxicity value but it also can induce cell proliferation. So the HPMC in this formulation can be concluded as non toxic material. HPMC can cross-link with calcium ions

for stable network composition. Gel form can be a carriers according to the mineralization process. Good mineralization can not be achieved if HPMC lack of Ca or P ions. (22) So in this research, the HAp and HPMC can fulfill the mineralization criteria, such as the existence of Ca and P ions from HAp, and HPMC as the stabilizer of network structure.

The toxicity assessment of novel bioactive material can be difficult to determine. Many studies only focus on single parameters in a single material. Here we present the result of two parameters used in determining the biocompatibility. We also analyzed the toxicity HAp,EGCG, and HPMC material in single compound. Our findings confirm that HAp-EGCG-HPMC has a potential in fibroblast cell regeneration. As a limitation, we had not determined several other characteristics needed as supporting tissue regeneration material, such as setting time, viscosity, injectability, and others. Future research also needs to be carried out in animal models.

CONCLUSION

The Nanohydroxyapatite, Epigallocatechin-3-Gallate, and Hydroxypropyl Methylcellulose Hydrogel are non-toxic. Although the proliferation assay for HAp-EGCG-HPMC has not yet shown significant cell proliferation, it has the potential to preserve cell proliferation ability.

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