The Characterization Of Hydroxyapatite, Eppigalocathecine-3-Gallate, Hydroxypropyl Methylcellulose As Bioactive Material For Pulp Capping

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ABSTRACT

Background: *According to a previous study, hydroxyapatite (HA) has the potential to be used in dentistry, but it still needs further research, especially as a biomaterial for pulp capping. The pulp capping treatment can maintain pulp vitality and induce dentin reparative formation. Calcium hydroxide is the gold standard of pulp capping material, but it can cause tunnel defects. Several studies made a crosslink with epigallocatechin-3-gallate (EGCG) and hydroxypropyl methylcellulose (HPMC) to improve the effect of hydroxyapatite as a biomaterial. The HA-EGCG-HPMC combination is expected to have the potential as a bioactive material. In this study, HA-EGCG-HPMC was characterized physicochemically by several criteria, such as the gelation time, pH, and antibacterial effect against Aggregatibacter actinomycetemcomitans. This study aims to determine the physicochemical characteristics of the HA-EGCG-HPMC material as a pulp capping material*

Method: *This research is an in vitro laboratory experimental study design. Hydroxyapatite powder was dissolved with distilled water at concentrations of 4%, 2%, and 1%, with 10µmol/mL EGCG and hydroxypropyl methylcellulose as the carrier agent. Characterization of the samples measured are gelation time, pH, and antibacterial effect by microdilution method.*

Result: *The HA-EGCG-HPMC combination has an ideal gelation time of about 23-31 minutes, and its pH is within the range of 7.28 - 7.33. However, the HA-EGCG-HPMC does not yet have an antibacterial effect against Aggregatibacter actinomycetemcomitans.*

Conclusion: *The hydroxyapatite, EGCG, and hydroxypropyl methylcellulose can be used as a bioactive material, such as pulp capping material seen from physicochemical characterization that can be developed further*

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INTRODUCTION

Dental caries is the most prevalent disease worldwide, with 2.3 billion people suffering from caries in permanent teeth.[1] The results of Hasil Riset Kesehatan Dasar (RISKESDAS) or Basic Health Research in 2018, the most significant dental problems in Indonesia are cavities and toothache at 57.6%, and 76.2% of children aged 12 years have caries so needs to be a concern. Untreated caries can cause dental pulp necrosis, which causes pulp vitality to be lost and teeth to become brittle.[2]

The pulp capping treatment is used when the pulp is exposed due to caries, trauma, or iatrogenic causes, such as during caries cleaning or tooth preparation. Direct or indirect pulp capping is usually used in deep cavities with unexposed pulp.[3] The commonly used reconstructed pulp cap therapy materials are calcium hydroxide Ca(OH)2 or MTA. Ca(OH)2 has a high pH of up to 12.5, which is an antibacterial material that provides hemostasis and stimulates mineralized tissue and the formation of dentin bridges.[3,4] According to William, under The International Standard (ISO) 9917-1 guidance, all water-based dental cement takes 1 hour to become rigid when stirred, so it can be used for applications.[5]

The limitations of Ca(OH)2 are that the high pH can irritate the dental pulp and can cause inflammation or necrosis of the exposed pulp surface, poor seal strength and no adhesion quality to the tissue, poor physical properties, and dissolves over time, causing tunnel defects in the reparative dentin to form under the calcium hydroxide, facilitating pulp recontamination.[6]

Mineral trioxide aggregate (MTA) is often used as a recycled pulp cap treatment because it has the advantages of good mechanical properties, good seal formation, antibacterial, biocompatible, and high pH.[7] However, MTA limitation leads to pulp obliteration, which makes it difficult to treat a root canal.[8] There are limitations to the materials commonly used in pulp cap treatment, so other materials that function optimally but can reduce the shortcomings of the previous materials are needed.

Hydroxyapatite (HA) has been used extensively in dentistry, including in the field of vital pulp therapy.[9] HA may form dentinal bridges in pulp capping treatments.[10,11] The advantage of HA from chicken eggshells is the level of hydroxyapatite (0.0950g/g).[12] *Epigallocathecine-3-gallate (EGCG) is 50-60% of the content of catechins, which are polyphenolic chemical compounds from tea leaves.*[13] EGCG has antioxidant, antitumor, antibacterial, and anti-inflammatory effects and is good at maintaining cell viability.[14] EGCG is proven to increase the mechanical properties of materials.[15] 10µg/mL EGCG has anti-inflammatory effects without affecting the proliferation and differentiation of pulp cells so that it can be used for pulp capping.[13]

HA and EGCG are to be applied as pulp capping materials, and a suspending agent using a 2% hydroxypropyl methylcellulose (HPMC) polymer is required to form a phase from liquid to gel.[16] The mixture of HA, EGCG, and HPMC carrier agents is expected to support and accelerate the formation of reparative dentin without the occurrence of severe inflammation.

Lactobacillus, Actinomyces, and Olsenella dominate bacteria in deep carious lesions. Actinomyces is the second most common bacterium in deep carious lesions, and it can induce leukocytes to apoptosis and lysis.[17] So, it is expected that the mixture of HA and EGCG can be used as a therapeutic material for recycled pulp caps and can kill actinomyces bacteria.

MATERIALS AND METHODS

1. Formulation of HA-EGCG-HPMC hydrogel

   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%.[1] 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.[18]

2. Gelation time

   Gelation time was performed with an inverted tube method test. Gelation time was determined by changes in sHAe from liquid to gel (sol-gel phase). Three replications were done to measure the average value.

3. pH

   The pH was measured using a pHmeter, and it was calibrated first with an acid solution with 4.0 pH and 10,0 base pH. The pH value of HA-EGCG-HPMC was determined after the formulation was mixed at 1,20,40, and 60 minutes with four repetitions each.[4]

4. Antibacterial Test

   a. The Microdilution Test Preparation

      0,1 ml samples of 4% HA-EGCG-HPMC, 2%HA-EGCG-HPMC, and 1%HA-EGCG-HPMC, positive control (BioMTA+, PPH Cerkamed, Stawola Wola) were prepared into a 96-well plate with four repetitions. Each well was inoculated with 20 µL of the *Aggregatibacter actinomycetemcomitans* microbial suspension with the turbidity of the test colony suspension standardized to 0.5 McFarland (approximately 1.5x108 CFU/mL). Samples were incubated for 24 hours at a temperature of 37℃.[5]

   b. The Bacterial Suspension Test Preparation

      The *Aggregatibacter actinomycetemcomitans* test colony suspension was made by taking one colony phase from the solid agar culture medium (24 hours of bacteria cultured on the agar medium) into a reaction tube containing 5 mL of physiological NaCl. The tube was then vibrated, and the turbidity of the test colony suspension was standardized to 0.5 McFarland (approximately 1.5x108 CFU/mL). The suspension must be used as an inoculum within 15 minutes.

5. Statistical Analysis

   All data obtained were subjected to a Shapiro–Wilk normality test. If the data were normal, a one-way analysis of variance (ANOVA) was used. Otherwise, the data were analyzed using Kruskal–Wallis. The Wilcoxon statistical test measured the pH because the data was not normally distributed.

RESULTS

The gelation time test results obtained with 4% HA+EGCG+HPMC, 2% HA+EGCG+HPMC, and 1% HA+EGCG+HPMC are presented with the mean and standard deviation (Sd) in Table 1.

Table 1. Descriptive gelation time data

Group name	N	Mean ± Sd (minutes)
HA 4%	9	23,78 ± 0,88
HA 2%	9	27,67 ± 0,50
HA 1%	9	30,00 ± 0,71

Based on Table 1., the average gelation time of the HA-EGCG-HPMC mixture at a concentration of 4% obtained a gelation time of 23.78 minutes, 2% HA-EGCG-HPMC obtained a gelation time of 27.67 minutes, and 1% HA-EGCG-HPMC obtained a gelation time of 30 minutes, then continued with a non-parametric statistical test, namely the Kruskal Wallis test (p = 0.000). The results of the Kruskal Wallis test followed by the Mann Whitney test showed that the 4% HA-EGCG-HPMC group had a significant difference with 2% HA-EGCG-HPMC (p=0.045) and 1% (p=0.000), for 1% HA-EGCG-HPMC showed a significant difference with the 2% HA-EGCG-HPMC group with a value of p=0.045. Concentrations of 4%, 2%, and 1% showed gelation times below 60 minutes to qualify as pulp cap treatment materials (table 2.).

Table 2. Post-hoc Mann Whitney test of gelation time

Sample 1(HA-EGCG-HPMC)	Sample 2 (HA-EGCG-HPMC)	p
HAP 4%	HAP 2%	*0,045
HAP 4%	HAP 1%	*0,000
HAP 2%	HAP 1%	*0,045
*P<0,05		

The pH test was conducted to determine the basicity contained in the sample. The pH test was carried out using a sample tested using a pH-meter calculated at the time after the material was stirred until homogeneous and measured periodically at 1 minute, 20 minutes, 40 minutes, and 60 minutes, presented with the mean and standard deviation in Table 3.

Table 3. Mean and standard deviation of pH test

Time	Group Name	N	Mean ± Sd
1 minute	HA 4%	9	7,32 ± 0,01
	HA 2%	9	7,30 ± 0,02
	HA 1%	9	7,39 ± 0,01
20 minutes	HA 4%	9	7,32 ± 0,09
	HA 2%	9	7,29 ± 0,01
	HA 1%	9	7,31 ± 0,01
40 minutes	HA 4%	9	7,31 ± 0,01
	HA 2%	9	7,30 ± 0,01
	HA 1%	9	7,29 ± 0,01
60 minutes	HA 4%	9	7,31 ± 0,01
	HA 2%	9	7,30 ± 0,01
	HA 1%	9	7,29 ± 0,01

The test results were continued with Wilcoxon non-parametric (p <0.05). The results of further tests using Post-hoc Mann Whitney obtained 1minute group (HA 2%) and 1 minute (HA 1%), 20 minutes group (HA 2%) and 20 minutes (HA 4%) (p=0.04), 40 minutes group (HA 2%) and 1 minute (HA 1%), 20 minutes group (HA 2%) and 1 minute (HA 1%), 60 minutes group (HA 1%) and 1 minute (HA 1%), 40 minutes group (HA 1%) and 1 minute (HA 1%) (p=0, 00), group 40 min (HA 1%) and 20 min (HA 4%), group 40 min (HA 1%) and 1 min (HA 4%) (p=0.005), group 60 min (HA 2%) and 1 min (HA 1%) (p=0.02), group 60 minutes (HA 1%) and 60 minutes (HA 4%) (p=0.03), group 60 minutes (HA 1%) and 1 minute (HA 4%) (p=0.01), and group 60 minutes (HA 1%) and 20 minutes (HA 4%) (p=0.01) significantly different. Based on the results of the material test, it was found that the mixture at concentrations of 4%, 2%, and 1% showed an average value of neutral pH with a range of 7.29-7.31. These results show that the tested HA-EGCG-HPMC material is as expected, which is close to the normal pulp pH of 7.3.

The antibacterial test was conducted in 5 test groups, namely: negative control group (distilled water), 4%HA-EGCG-HPMC, 2%HA 2%-EGCG-HPMC, 1%HA- EGCG-HPMC, and positive control group (4%MTA) samples were mixed with a solution that already contained *Aggregatibacter actinomycetemcomitans* bacteria followed by incubation of samples for 24 hours at 37 ℃, then the results were observed by turbidimetric method, and all samples were turbid in color indicating that all samples had bacterial growth, then the samples were applied to MHA agar media to confirm the samples had bacterial growth. The calculation of the number of colonies formed on agar media is presented in Figure 1.

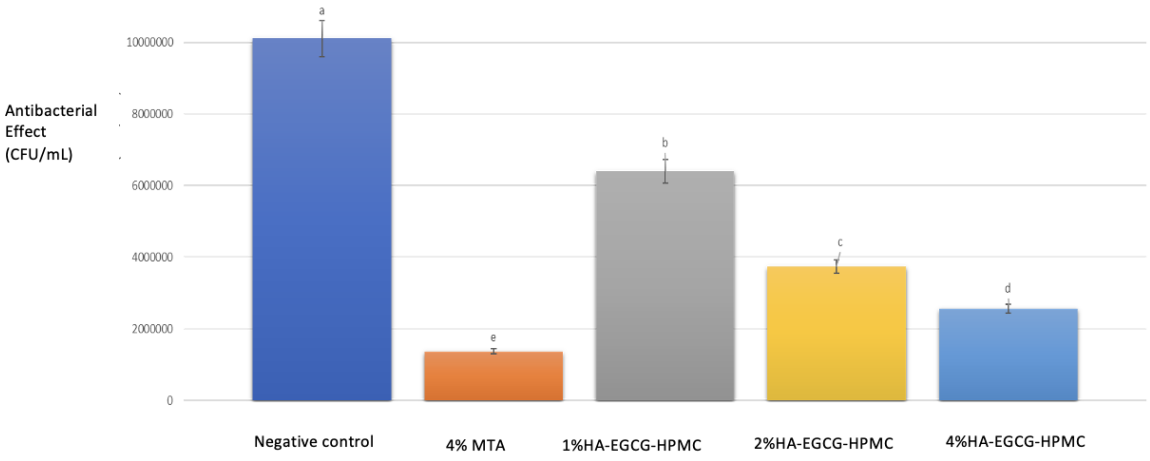

Figure 1. Picture of the number of colonies formed in each sample treatment

The ANOVA test results showed significant differences between the test groups (p<0.01). Further tests were conducted to see the differences between groups. The results of the post-hoc test showed that the number of colonies in the negative group was significantly different from 4%HA-EGCG-HPMC, 2%HA -EGCG-HPMC, 1%HA-EGCG-HPMC, and 4%MTA (p<0.01) with mean colony counts of 1x108 ± 55x104, 2.5x107 ± 28x104, 3.7x107 ± 14x104, 6.4x107 ± 87x103, and 1.3x107 ± 16x104 CFU/mL, respectively. The number of colonies of 4% HA-EGCG-HPMC significantly differed from 1% HA -EGCG-HPMC(p<0.001), which showed that 4% HA-EGCG-HPMC was lower than 1% HA. The

number of colonies of 2%HA-EGCG-HPMC was slightly lower than that of 1%HA -EGCG-HPMC ($p<0.001$) and 4%MTA ($p<0.001$), 1%HA-EGCG-HPMC group was higher than 4% MTA group ($p<0.001$) (Figure 2).

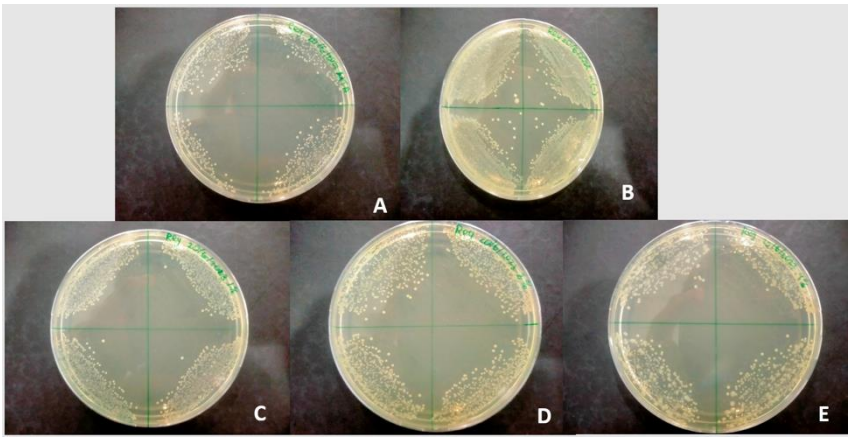

Figure 2A-E. Sample test results against the bacteria *A. actinomycetemcomitans* (A. MTA, B. Negative control, C. 1%HA-EGCG-HPMC, D. 2%HA-EGCG-HPMC, E. 4%HA-EGCG-HPMC)

In Figure 2, the density of *A.actinomycetemcomtans* in 4% MTA is the least formed when compared to the control (-), but there is no significant difference in the 4% MTA group with 4% HA-EGCG-HPMC. There was a significant difference in the number of colonies in the 4% HA-EGCG-HPMC group with 1% HA-EGCG-HPMC. Based on the post-hoc test, there is a density of *A. actinomycetemcomitans* in each test sample of the HA-EGCG-HPMC mixture, so it can be concluded that the HA-EGCG-HPMC mixture does not have an antibacterial effect on *A. actinomycetemcomitans* bacteria.

DISCUSSION

The purpose of performing a reconstructed pulp capping is to increase the healing ability of the pulp and promote the formation of a mineralized tissue layer by placing the biomaterial directly on the exposed pulp roof.[19] In this study, a mixture of HA-EGCG-HPMC materials was tested to find alternative materials for pulp capping treatment. First, a characterization test of the hydrogel suspension material with hydroxyapatite, EGCG, and HPMC was carried out, and it was seen that the gelation time was sufficient so that it could be used for clinical applications, whereas slow gelation time made it difficult to applicate in a certain area.[20] The hydrogel suspension mixture with a concentration of 4% HA-EGCG-HPMC had a gelation time an average of 24-26 minutes, at a concentration of 2% the gelation material at an average time of 27-30 minutes, and at a concentration of 1% the gelation material averaged 29-31 minutes so that the hypothesis was accepted because it had sufficient gelation time for clinical applications, which was under 60 minutes.[21]

In this study, the level of basicity of the HA-EGCG-HPMC material mixture was tested using a pH meter, and samples with three concentration mixtures were shown in Table 5.2. The results show that the material after adding the gelation agent and after the gelation material shows a neutral pH (Table 2), so it is to the pH conditions in the pulp chamber, namely 7.3-7.8.[22] According to Elline et al,

pH is essential in determining the morphology and structure of a formulation. The pH can influence the ion balance. The increase or decrease of OH^- ion, will affect the Ca^{2+}, $PO4^{3-}$, and $(HPO4)^{2-}$ ions[18]

The results of the antibacterial effect test of the HA-EGCG-HPMC mixture using the dilution method of 4%, 2%, and 1% concentrations of mixed materials mixed with *Aggregatibacter actinomycetemcomitans* bacterial suspensions in 96-well plates and incubated at 37˚C for 24 hours, the results showed that all samples contained bacterial growth. Confirmation of bacterial growth and development is achieved by scratching on agar discs to see the growth of the number of bacterial colonies present (Figure 2). Thus, the HA-EGCG-HPMC mixture has no antibacterial effect on *Aggregatibacter actinomycetemcomitans* bacteria. According to a previous study, EGCG would break the bacterial plasma membrane in a certain concentration. For example, the *Streptococcus mutans* plasma membrane will destroyed with 0,2 mg/ml after EGCG treatment.[23] So, in this study may be the concentration of EGCG has not fulfilled the minimum criteria as a treatment

CONCLUSIONS

Based on the test results, the HA-EGCG-HPMC mixture at a concentration of 4% has a gelation time of 23.78 minutes. It is concluded that this material has a qualification to be used in clinical conditions. It also has a degree of acidity that resembles the vital pulp at 7.30 after gelation. In the antibacterial test results, the 4% HA-EGCG-HPMC mixture had a lower antibacterial effect when compared to 4% MTA. Further research is needed to develop a mixture of HA-EGCG-HPMC materials to increase the ability of antibacterial effects that can support the potential of the material to become a pulp capping material

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