Volume 56, Issue 1, March 2023

p-ISSN: 1978-3728
e-ISSN: 2442-9740

Dental Journal

Published quarterly per year

Majalah Kedokteran Gigi

Interdisciplinary management of Class III malocclusion with cleft lip and palate • Management of bimaxillary protrusion with missing molar using T-loop and couple force • Molecular docking study of *Zingiber officinale* Roscoe compounds as a mumps virus nucleoprotein inhibitor

Accredited No. 158/E/KPT/2021

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Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2023 March; 56(1): 53–57

Original article

Physical characterization and analysis of tissue inflammatory response of the combination of hydroxyapatite gypsum puger and tapioca starch as a scaffold material

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ABSTRACT

Background: *Cases of bone damage in the oral cavity are high, up to 70% of which consist of cases of fracture, tooth extraction, tumor, and mandibular resection. The high number of cases of bone damage will cause the need for bone graft material to increase. The bone graft material that we have developed is a combination of hydroxyapatite gypsum puger (HAGP) and tapioca starch (TS) scaffold.* ***Purpose:*** *This study analyzes the physical characterization and tissue inflammatory response of the combination of HAGP+TS as a scaffold for bone graft material.* ***Methods:*** *Eighteen Wistar rats were used. HAGP+TS were installed into the molar 1 socket for 7 and 14 days. First, HAGP was evaluated using XRF and SEM before setting up the in vivo experiment. A blood sample was drawn and then tested for TNF-αlevels using ELISA.* ***Results:*** *The XRF revealed that the main constituents of hydroxyapatite were Ca and P. Next, SEM characterization on the HAGP+TS showed an average pore size of 112.42 μm^2, which is beneficial for cell activity to grow as new bone tissue. In addition, TNF-αon days 7 and 14 on the HAGP+TS scaffold did not elicit an inflammatory response.* ***Conclusion:*** *The combination of HAGP+TS contains a high amount of Ca and also has excellent interconnectivity between pores. It also does not trigger an inflammatory response in the tissue; therefore, it is a good candidate as an alternative bone graft material.*

Keywords: *bone graft; characterization; hydroxyapatite gypsum puger scaffold; tapioca starch; inflammation*
Article history: *Received 17 April 2022, Revised 19 August 2022, Accepted 15 September 2022*

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INTRODUCTION

The occurrence of bone destruction related to oral health is prevalent. There are several factors causing bone destruction, such as fracture, tooth extraction, periodontitis, tumor, mandibular resection, alveolar cleft, and cleft palate.[1,2] The high number of bone destruction cases is causing a rise in the demand for bone replacement materials, bone implants, and bone grafts.[3]

In prosthodontics, the technological advancement of bone grafting techniques can help cases of bone destruction and implant placement. The ideal bone graft material should be biocompatible, osteoconductive (i.e., providing a framework or scaffold for the new bone to grow), and osteoinductive (i.e., growth-stimulating materials).[4] Materials that can be used as bone substitutes or bone grafts include autograft, allograft, xenograft, alloplastic, or synthetic bone substitute (bioceramic).[5]

A bioceramic material that a previous study has recently developed is hydroxyapatite gypsum puger (HAGP). The gypsum puger has been synthesized into a hydroxyapatite scaffold, and thus it becomes an alternative graft material at a more affordable price and is easy to obtain.[6,7] However, it still poses some weaknesses, such as low biomechanical properties, low porosity, and brittleness.[8] Therefore, to improve its material properties, it needs to be enhanced by combining it with biopolymer materials. One of the natural biopolymer materials is cassava.

Cassava is an abundant raw material at a relatively affordable price, and it is easy to obtain. Cassava is a tuber plant with the Latin name *Manihot utilissima* from the Euphorbiaceae family. Cassava is a polysaccharide

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containing starch with amylopectin and amylose content. Cassava can be processed to produce tapioca starch (TS) with many benefits. Tapioca starch is beneficial for health since it is a source of carbohydrates, high in calories and proteins, and contains B complex vitamins, minerals, fibers, and vitamin K, which are beneficial for building bone mass.[9] HAGP is a new development in bone graft material that has improved mechanical properties and porosity when combined with cassava starch; therefore, the HAGP+TS scaffold material is firm and has good mechanical characteristics.

In the case of fractured bones, tissue engineering procedures are needed to accelerate the healing process and formation of new bone. One of the components of tissue engineering is a scaffold. A scaffold is a component for inducing cell growth.[10] The scaffold material that will be used as a bone graft must have characterization in the form of an elemental composition test/x-ray fluorescence (XRF), a morphological test using scanning electron microscopy (SEM), and an inflammation test to determine the tissue response to the incoming scaffold/foreign object. Inflammation is a reaction to a foreign agent entering the body. The tissue damage is caused by the invasion of microorganisms, harmful chemicals, and physical factors. Inflammatory signs are usually seen as redness, heat, swelling, pain, and impaired function.

TNF-αis a cytokine that plays an important role in the inflammatory response.[11] Inflammation is an initial response from the body tissues if there is an injury. A bone graft applies foreign matter to the tissues of the human body. Therefore, it may cause injury, such as inflammation. The purpose of this study is to analyze the elemental composition and morphological characterization of the combination of HAGP+TS scaffold as a candidate new bone graft material. Next, we also examine the tissue inflammatory response of the combination to get an overview of the biocompatibility of the new bone graft material.

MATERIALS AND METHODS

The HAGP sample was made using the following procedure. Weigh 0.5 g of gypsum, 0.5 g of diammonium hydrogen phosphate (DHP), and 500 ml of distilled water. Mix them in a beaker and then put the beaker on a magnetic hotplate for 15 minutes. Then, put the beaker in an oven at 100°C for 30 minutes. Wash the solution using distilled water, and at the same time, filter it using filter paper several times until the pH is neutral. Then, dry the powder in a microwave at 50°C for 5 hours to create hydroxyapatite powder.

The process of making tapioca starch generally comprises peeling, washing, grating, extracting, settling, and milling. First, weigh and wash 500 g of cassava, and then grate it. Add 1 l of water and filter it. Next, let the contents rest for 12 hours to settle the starch at the bottom. Then dry, mash, and sieve the starch.

The composite HAGP+TS scaffolds were made through the following procedure. Weigh 250 mg hydroxyapatite and 300 mg solid gelatin. Add 10 ml distilled water and heat to 40°C. Then add 250 mg of HAGP and mix until homogeneous. Then add 250 mg TS and 10 ml distilled water and mix using an ultrasonic homogenizer for 6 minutes. Put the mixture into cylindrical molds with a diameter of 5 mm and a height of 5 mm. Then freeze and dry with a sublimation/freeze-drying system. Sterilize using gamma irradiation. Next, characterize them to see the concentration of scaffold content using XRF and SEM.

This study was an *in vivo* laboratory experiment using a posttest-only control group design. The independent variable was the HAGP+TS scaffold, and the dependent variable was tumor necrosis factor alpha (TNF-α) levels. Eighteen Wistar rats with the following inclusion criteria : 12-to-14-week-old male Wistar rats with a body weight of around 200–250 g were used. The rats were kept with the same feed, the rats' drinks (distilled water), and the rats' caring method. In addition, also the evaluation time, the skin area for injection, the time and level of material given, and the application method were controlled. Wistar rats were divided into 6 sampling groups, with 3 samples (n=3) in each group. Inflammatory responses were evaluated by enzyme-linked immunosorbent assay (ELISA) analysis method,

The prepared HAGP+TS scaffold was ready to be applied to the rats with the following steps. First, anesthetize the Wistar rats intra-muscularly using 100 mg/ml ketamine and 20 mg/ml xylazine base and xylazine ratio with a dose of 0.08–0.2 ml/kg body weight. Once the rats were anesthetized, the first molar on the left mandible was extracted using a needle holder. Then, put HAGP+TS scaffold material into the extraction socket. Sew it using a 75 cm sewing thread (Dr. Stella Silk Braided USP 3/0). Then, wait for 7 and 14 days. Collect the rats' blood for ELISA analysis by using 5 ml of ether on a cotton swab. Put the cotton swab in a closed glass box. Put the rats into the glass box singly for 5 minutes. Dissect the thoracic area of the rats and collect 2 ml of blood from the heart. Determine TNF-αlevels using ELISA.

The data obtained were subjected to statistical analysis with a Statistical Package for the Social Sciences (SPSS) software, version 22 (IBM, USA). All scale variables were analyzed for normality and homogeneity tests. The data were normally distributed; therefore, parametric tests using one-way analysis of variance (ANOVA) were conducted, followed by least significant difference (LSD), with $p < 0.05$ indicated as statistical significance.

RESULTS

The results of the characterization analysis using XRF, the HAGP scaffold, and the HAGP+TS scaffold produced elemental percentages that can be seen in Table 1. The

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results of SEM characterization used to determine the morphology (including the shape, pore diameter, and pore area) of the HAGP+TS composite scaffold are shown in Figure 1.

The SEM test results of the HAGP scaffold and HAGP+TS scaffold in Figure 1 show an irregular pore edge shape and inhomogeneous interconnectivity with different sizes among samples. The results of the diameter and the pore area data for the HAGP scaffold and the HAGP+TS scaffold are presented in Tables 2 and 3.

The diameter and pore size of the HAGP+TS and HAGP groups were analyzed using the Shapiro-Wilk normality test. The analysis showed $p > 0.05$, which indicated that the data is normally distributed. A homogeneity test was also conducted using the Levene test. The results showed that the significancy of pore diameter $p = 0.13$ and pore area $p = 0.06$. It indicated that the variance of the data is homogeneous between groups. Moreover, an ANOVA test was also conducted. The results of pore diameter showed $p = 0.03$, and pore area showed $p = 0.02$. It showed the difference in pore diameter and pore area between groups.

The tissue inflammatory response analysis was conducted to analyze the TNF- αlevels after the application of the HAGP+TS group and HAGP group. Negative controls on days 7 and 14 were analyzed using the Shapiro-Wilk normality test. The results showed $p > 0.05$, which indicated that the data was normally distributed. A homogeneity test using the Levene test was also conducted. The results showed $p = 0.22$, which indicated that the data variance was homogeneous between groups. Furthermore, an ANOVA test was conducted. The results showed $p = 0.75$, indicating no difference in TNF- αlevels between groups. The results of TNF- αlevels are presented in Table 4.

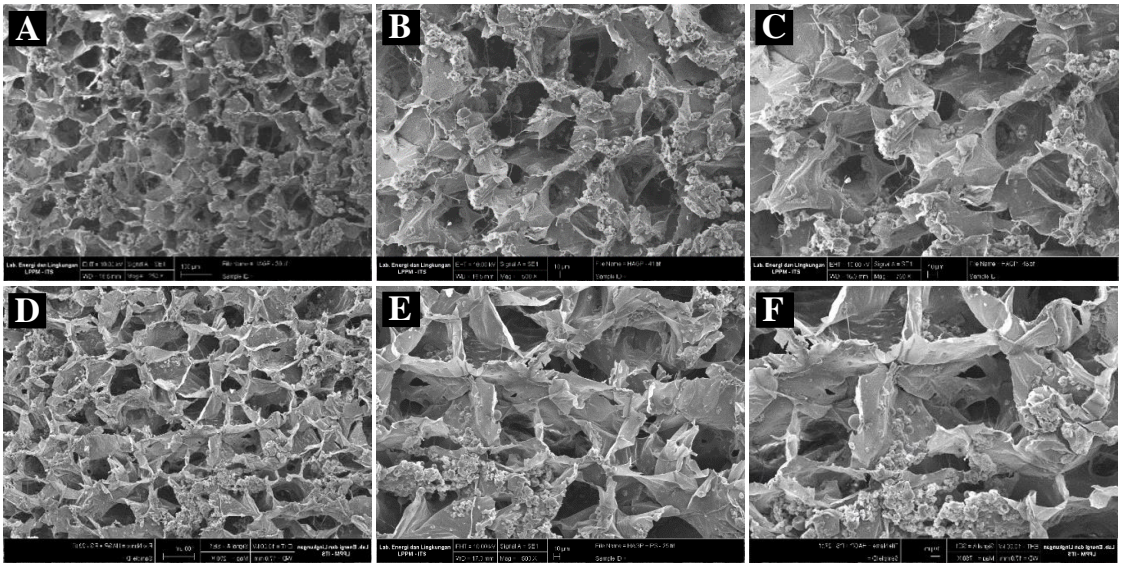

Figure 1. SEM analysis of HAGP scaffold and HAGP+TS scaffold. A. HAGP scaffold 250x magnification, B. HAGP scaffold 500x magnification, C. HAGP scaffold 750x magnification, D. HAGP+TS scaffold 250x magnification, E. HAGP+TS scaffold 500x magnification, F. HAGP+TS scaffold 750x magnification.

Table 1. The results of XRF test on HAGP and HAGP+TS scaffold

Compound	P	Ca	Cr	Fe	Ni	Cu	Yb	S
HAGP scaffold	34%	49.5%	5.8%	5.0%	3.4%	1.9%	0.9%	-
HAGP+TS scaffold	13.5%	82.9%	-	0.39%	-	0.081%	0.46%	2.70%

Table 2. Pore diameter of HAGP scaffold and HAGP+TS scaffold

Group	Mean	SD	Minimum	Maximum	*p*
HAGP scaffold	143.97 μm	36.15	105.20 μm	217.90 μm	0.03
HAGP+TS scaffold	112.42 μm	18.80	85.33 μm	142.90 μm	

Table 3. Pore area of HAGP scaffold and HAGP+TS scaffold

Group	Mean	SD	Minimum	Maximum	*p*
HAGP scaffold	16155.08 μm^2	6615.77	8687.62 μm^2	27272.10 μm^2	0.02
HAGP+TS scaffold	10168.69 μm^2	3321.66	5715.74 μm^2	16030.01 μm^2	

DOI: 10.20473/j.djmkg.v56.i1.p53–57

Table 4. The results of TNF- αlevels

Scaffold	N	TNF- α		
		Mean	SD	*p*
K (-) 7	3	0.10	0.02702	0.75
K (-) 14	3	0.12	0.01493	
HAGP 7	3	0.11	0.00902	
HAGP 14	3	0.12	0.00987	
HAGP+TS 7	3	0.12	0.00513	
HAGP+TS 14	3	0.12	0.02178	

DISCUSSION

The results of the XRF test showed Ca and P elements, which were the main elements of hydroxyapatite with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ contained in the HAGP+TS scaffold. The addition of TS to the process of HAGP scaffold-making resulted in a greater amount of Ca compared to the HAGP scaffold without TS. A greater amount of Ca can improve the material properties of the scaffold. The presence of element P is related to the deposition of phosphate from hydroxyapatite.[12] There was a hydrothermal reaction in the process of making the scaffold to obtain single crystals. Hydroxyapatite crystals are the same size as bone hydroxyapatite crystals.[13,14]

The scaffolds used in this research were made of HAGP powder mixed with TS, while the scaffolds for the control group were processed using a freeze-drying system. Microstructural characterization of the HAGP scaffold using SEM obtained a three-dimensional interconnected pore structure with an average diameter of 143.97 µm, and the HAGP+TS scaffold showed an average diameter of 112.42 µm. For the pore area (this pore is a hole formed on the surface of the HAGP scaffold), the HAGP scaffold showed 16155.08 μm^2, and HAGP+TS showed 10168.69 μm^2. The HAGP+TS scaffold showed better interconnectivity between each pore than the HAGP scaffold. It can be assumed that the addition of TS to gelatin and HAGP caused interactions with TS particles; thus, separating gelatin particles from one another, which caused the formation of interconnectivity and more even pore distribution.[15]

The HAGP+TS scaffold had a smaller pore size than the HAGP scaffold. This was due to the constant freezing temperature and the addition of TS that helped decrease the pore size.[16] TS can lower pore size by forming hydrogen bonds that increase the strength of the scaffold through an interlocking mechanism.[16] The small pore size exists because of the presence of hydroxyapatite (HA), causing the pore size and pore wall to decrease after freeze-drying because of the increasing number of ice crystals and the closing distance among ice crystals.[17] The addition of cassava starch polymer allows bond formation between the cassava starch polymer and HAGP. The concentration of cassava starch polymer influences the pore size. By triggering hydrogen bonds to form, the pores shrink.. It is conducive to cell activity so that it enters and grows in it, which is useful for bone remodeling and bone tissue engineering.[17,18] The minimum scaffold pore size was between 75–100 µm. However, if the pore size diameter is > 300 µm, it will be better for bone formation since it is easier to facilitate and vascularize the tissue.[19] Scaffold pores have a retentive shape for cell attachment, being a place for cell proliferation and differentiation into osteoblast cells.[20] In this study, all sample groups produced pore sizes with the potential to assist bone growth. This is ideal as it meets the requirements for bone graft material, i.e., being osteoconductive, that is, providing a framework or scaffold to grow.[4]

In this study, TNF- αlevels were observed on days 7 and 14. The results of the TNF- αlevel of the negative control group showed that the HAGP scaffold and HAGP+TS scaffold were almost the same. In the HAGP+TS group, the levels were higher but statistically within the range of the HAGP+TS group. For the HAGP and negative control groups, the results showed p = 0.75, which indicated no significant difference, and thus did not cause an inflammatory response. Inflammation is an important mechanism needed by the body to defend itself from dangers such as tissue damage and invasion of microorganisms, antigens, and foreign materials that disrupt the balance of the tissue. As a foreign object that enters the rats' body through the tissue, the scaffold can activate macrophages and other cells to produce and release various cytokines, including TNF- α[21]

TNF- αlevels on days 7 to 14 were associated with the recruitment of osteoclast precursors and differentiation into mature osteoclasts in the defect. It corresponds to other research findings that defects treated with HA in the first week are associated with TNF- αexpression from the surface osteoblasts markers.[22] On day 7, the inflammation peaks after the application of the material to the tissues, while day 14 has a decrease in the inflammatory response and begins to form tissue regeneration. TNF- αlevels report levels of cytokines, which play an important role in the inflammatory response.[21]

In this study, there was no significant difference between the control and treatment groups concerning the inflammation test. Thus, the HAGP+TS scaffold material is safe for the body (biocompatible) and an ideal candidate for bone graft material.[4] HAGP+TS scaffold is a type of alloplastic bone graft material or synthetic bone replacement material (bioceramic), which can be referred to as a regenerative material. Based on a systematic review study on graft materials related to alveolar regeneration, there were no significant differences between regenerative materials and iliac crest grafts in the meta-analysis. It indicates that this regenerative material still meets the requirements for treating bone destruction.[3]

The conclusions of this study are that the combination of HAGP+TS scaffold contains high amounts of Ca and P by XRF test, has excellent inter-pore interconnectivity by SEM test, and does not trigger an inflammatory response in tissue by ELISA test; therefore, this material is a good

DOI: 10.20473/j.djmkg.v56.i1.p53–57

candidate for alternative bone graft material. However, further research is needed using other biomarkers before it can be applied clinically.

ACKNOWLEDGEMENTS

The authors would like to thank all those who have participated in this research and in particular the Bioscience laboratory of Dental and Oral Hospital, Universitas Jember which has allowed and facilitated this research.

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DOI: 10.20473/j.djmkg.v56.i1.p53–57