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ARTICLE

THE EFFECT OF SCAFFOLD HYDROXYAPATITE DERIVED FROM *PORTUNUS PELAGICUS* SHELL ON THE EXPRESSION OF FIBROBLAST GROWTH FACTOR-2 (FGF-2) AND BONE MORPHOGENETIC PROTEINS-2 (BMP -2) IN THE EXTRACTION SOCKETS OF *CAVIA COBAYA*.

El efecto del scaffold de hidroxiapatita derivada de *Portunus pelagicus* sobre la expresión del factor de crecimiento de fibroblastos-2 (FGF-2) y las proteínas morfogenéticas óseas-2 (BMP-2) en los alvéolos dentarios de *Cavia cobaya*.

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ABSTRACT:

Objetive: To determine the expression of Fibroblast Growth Factor (FGF)-2 and Bone Morphogenetic Protein (BMP)-2 after application of scaffold hydroxyapatite from Rajungan crab shell (Portunus pelagicus) in the tooth extraction socket of Cavia cobaya.

Material and Methods: This study used a post-test only control group design with 28 Cavia cobaya separated into two groups, control and treatment group. The left mandibular incisor was extracted and socket preservation was conducted. A hydroxyapatite graft derived from crab shells was mixed with gelatin and eventually turned into a scaffold, which was afterwards put into the extraction socket. After 7 days and 14 days, each group was terminated and examined using immunohistochemical staining to observe the expression of FGF-2 and BMP-2. One-Way Anova and Tukey HSD were used to examine the research data.

Results: FGF-2 and BMP-2 expressions were observed higher in the group that received hydroxyapatite scaffold at the post-extraction socket than those in the group that did not receive hydroxyapatite scaffold.

Conclusion: The application of a hydroxyapatite scaffold from Rajungan crab shell (Portunus pelagicus) to the tooth extraction socket can increase FGF-2 and BMP-2 expression.

KEYWORDS:

Portunus pelagicus; hydroxyapatites; fibroblast growth factor 2; bone morphogenetic proteins; tissue scaffolds; tooth socket.

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RESUMEN:

Objetivo: Determinar la expresión del factor de crecimiento de fibroblastos (FGF)-2 y la proteína morfogenética ósea (BMP)-2 después de la aplicación de hidroxiapatita de andamio de caparazón de cangrejo Rajungan (Portunus pelagicus) en el alvéolo de extracción dental de Cavia cobaya.

Material y Métodos: Este estudio utilizó un diseño de grupo de control solo posterior a la prueba con 28 Cavia cobaya separados en dos grupos, grupo de control y grupo de tratamiento. Se extrajo el incisivo mandibular izquierdo y se realizó la preservación del alvéolo. Un injerto de hidroxiapatita derivado de caparazones de cangrejo se mezcló con gelatina y se convirtió en un andamio, que luego se colocó en el alvéolo de extracción. Después de 7 días y 14 días, se terminó cada grupo y se examinó mediante tinción inmunohistoquímica para observar la expresión de FGF-2 y BMP-2. Se utilizaron One-Way Anova y Tukey HSD para examinar los datos de la investigación.

Resultados: Las expresiones de FGF-2 y BMP-2 se observaron más altas en el grupo que recibió la estructura de hidroxiapatita en el alvéolo posterior a la extracción que en el grupo que no recibió la estructura de hidroxiapatita.

Conclusión: La aplicación de un andamio de hidroxiapatita de caparazón de cangrejo Rajungan (Portunus pelagicus) al alvéolo de extracción dental puede aumentar la expresión de FGF-2 y BMP-2.

PALABRAS CLAVE:

Portunus pelagicus; hidroxiapatitas; factor 2 de crecimiento de fibroblastos; proteínas morfogenéticas óseas; andamios del tejido; alveolo dental.

INTRODUCTION.

Patients may frequently experience unpleasant consequences resulting from tooth loss, after which the redundant alveolar bone atrophies due to a lack of physiological stimulation.[1] Alveolar ridge resorption reduces patient comfort with dentures while simultaneously increasing the number of complaints about denture stability which is influenced by the vertical height of the residual ridge.[2]

Resorption ridges will provide less stability than residual ridges with sufficient vertical height.[3] Following tooth extraction, efforts should be made to maintain the vertical dimension of the residual ridge. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is a bioceramic material with frequent biomedical applications due to its close resemblance to the primary mineral ingredient of bones and teeth. Numerous investigations have established that hydroxyapatite is biocompatible and possesses osteoconductive properties. It can prove an effective therapy in the prevention of post-extraction physiological bone loss, in both horizontal and vertical dimensions.[4]

Hydroxyapatite synthesized from natural raw materials or natural waste can be more beneficial because it contains many valuable ions, also found in biological hydroxyapatite.[5] One of the sources of natural hydroxyapatite is the shell waste of *Portunus Pelagicus*, known as the Rajungan crab in Indonesia. A previous study revealed that the Calcium hydroxyapatite content of Rajungan crab shells can be as high as 66.62%.[6]

Hydroxyapatite scaffolds from waste bone from different species have been studied, such as in a study by Sharifianjazi *et al.*,[7] which concluded that hydroxyapatite scaffolds from waste bone pigeon could be a promising and economically viable material for bone grafting. According to Raya *et al.*,[8] calcium hydroxyapatite from the Rajungan crab was effective in inhibiting demineralization of the tooth *in vitro*. Its effect as a scaffold after tooth extraction has not been studied.

In this study, administration of hydroxyapatite scaffold derived from Rajungan crab shell to the tooth extraction socket was expected to increase alveolar bone formation and reduce alveolar bone resorption, thereby enabling the bone replacement

process in the extraction socket to be completed. Fibroblast Growth Factor-2 (FGF-2) has been shown to activate the transcription factor Runx2 in osteoblasts by increasing the protein's stability and acetylation level via extracellular signal-regulated kinase mitogen-activated protein (ERK MAP kinase).[9] A subsequent study conducted by Ai., reported that Bone Morphogenetic Proteins-2 (BMP-2) functions as an osteoinductive growth factor since it recruits mesenchymal progenitor cells and induces their differentiation to bone-forming osteoblasts. FGF-2 and BMP-2 have also been found to work synergistically in the formation of bone and fibrous tissue.[11]

This study aimed to determine the expression of FGF-2 and BMP-2 in the post-extraction socket following application of hydroxyapatite scaffold from Rajungan crab shell in order to investigate bone forming ability and cellular activity.

MATERIALS AND METHODS.

This study was conducted between April and October 2020. The Federer formula was used to calculate the number of Cavia cobaya for this study, resulting in a final research population of 28. The subjects had to meet certain criteria: healthy, 3-3.5 months old, weighing 300-350 grams, being lesion-free, and having complete use of their five senses.

The Cavia cobaya were provided with standard pellets and water ad libitum during a one-week acclimatization period prior to being randomly assigned to one of two groups; control and treatment. The latter group was given a hydroxyapatite scaffold from Rajungan crab shell. Both groups were observed after 7 and 14 days.

Soft tissue was removed from Portunus pelagicus crab shell waste using distilled water. The crab shells were then immersed in a chlorine solution at a ratio of 30 ml of chlorine to 5 liters of water. Immersion in 3% H_2O_2 was continued for another 24 hours before the shells were dried at room temperature. Shell calcination was carried out in a furnace at 1000°C. The initial heating temperature of approximately 50°C was increased at a rate of 5°C/min. On reaching 1000°C, it was kept constant for around two hours before being rapidly reduced to 100°C; SEM-EDX was used to characterize hydroxyapatite compounds. The powder sifting process was conducted using a sifting machine to obtain crab shell hydroxyapatite powder measuring less than 150 µm.[6]

The hydroxyapatite scaffold was produced by adding 0.5 grams of gelatin to distilled water and stirring it for one hour at 40°C. The scaffold was created by stirring 1.5 grams of hydroxyapatite powder into the gelatin solution for six hours. The gel was then centrifuged for ten minutes. Following extraction of excess water, the residue was poured in a cylindrical, 5mm x 2mm (2mm high, 5 mm diameter) sized mold and, having cooled, freeze dried at -800C for 24 hours.[14]

Ketamine 20mg/300mg body weight was injected intramuscularly into the Cavia cobaya subjects for anesthetic/sedation and analgesic purposes. The region surrounding the left mandibular incisive tooth was cleansed of debris prior to the tooth being carefully extracted in a specified direction with a sterile needle holder. The socket was irrigated with sterile distilled water after removal and hydroxyapatite scaffold applied in accordance with the previously defined group. Simple suturing was subsequently performed using DS 12 3 / 8c, 12 mm, 6/10 met, 0.7 (Braun Aesculap) polyamide monofilament.[13]

After 7 and 14 days, the subjects in each group were sacrificed in order to observe FGF2 and BMP2 expression. The mandible was cut and removed with samples of the tooth subsequently being fixed in 10% formalin buffer for 24 hours at room temperature and decalcified with ethylenediaminetetraacetic acid (EDTA). Dehydration by means of graded alcohol concentration was then completed, followed by clearing in xylol and embedding in paraffin. Finally, 4-micron thick paraffin blocks were cut and placed on the object glass.

Staining of Fibroblast Growth Factor-2 (FGF-2) and Bone Morphogenetic Proteins-2 (BMP-2) was

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performed on the 7th and 14th-day observation groups using single staining technique.

The preparations were immersed in Fibroblast Growth Factor-2 (FGF-2) and Bone Morphogenetic Proteins-2 (BMP-2) primary antibodies for 30 minutes, then washed three times with PBS. At that point, the preparation was immersed in a secondary antibody, namely; anti-mouse monoclonal antibody (Thermo Scientific, USA), for 30 minutes, washed twice with PBS, and immersed in a chromogen substrate for five minutes. After rinsing with distilled water, the incision was placed in a haematoxylin mayer for six minutes, washed with running water and, finally, mounted and placed under a cover glass to enable it to be viewed with a light microscope.[14]

The area under observation was the apical third of the socket.Statistical analysis was performed using the Statistical Package for the Social Sciences Software (SPSS) edition 24.0 (SPSSTM, Chicago, Illinois, USA). One-way analysis of variance (ANOVA) was used to analyze the data followed by a Tukey HSD comparison multiple test at a confidence level of 95%.

RESULTS.

The statistical analysis revealed significant differences between the control and treatment groups ($p<0.05$) on both days 7 and 14 (Table 1). Compared to the other groups, group C7 had the lowest level of FGF-2. The control group, designated C7, was observed on day 7. On the other hand, Group HS14, which was treated with hydroxyapatite scaffolds made from crab shells and tested on the 14th day, had the highest level of FGF-2 (Figure 1). Brown staining was utilized to detect osteoblasts expressing FGF-2 (Figure 2).

Similarly, a statistically significant difference ($p<0.05$) was found between the observations in the BMP-2 treatment group and the control group (Table 1). The levels of BMP-2 in each group demonstrated a similar pattern to the levels of FGF-2. The BMP-2 levels in the C7 group were the lowest. In a related manner, after 14 days of treatment with a hydroxyapatite scaffold produced from crab shells, the HS14 group had the highest BMP-2 concentrations compared to the other groups in the experiment (Figure 1). Brown staining was utilized to detect osteoblasts expressing BMP-2 (Figure 3).

Figure 1. Mean number of osteoblast cells expressing FGF-2 and BMP-2 in each group.

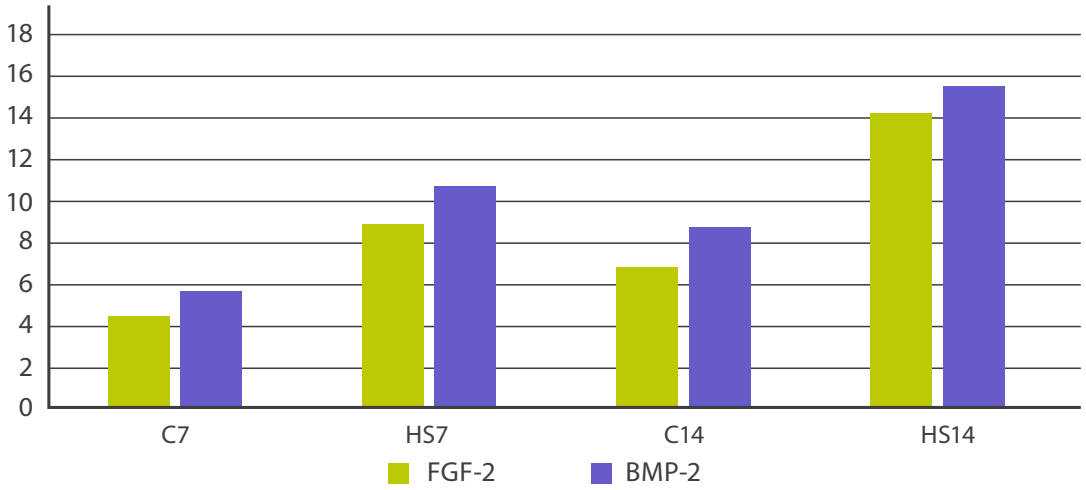
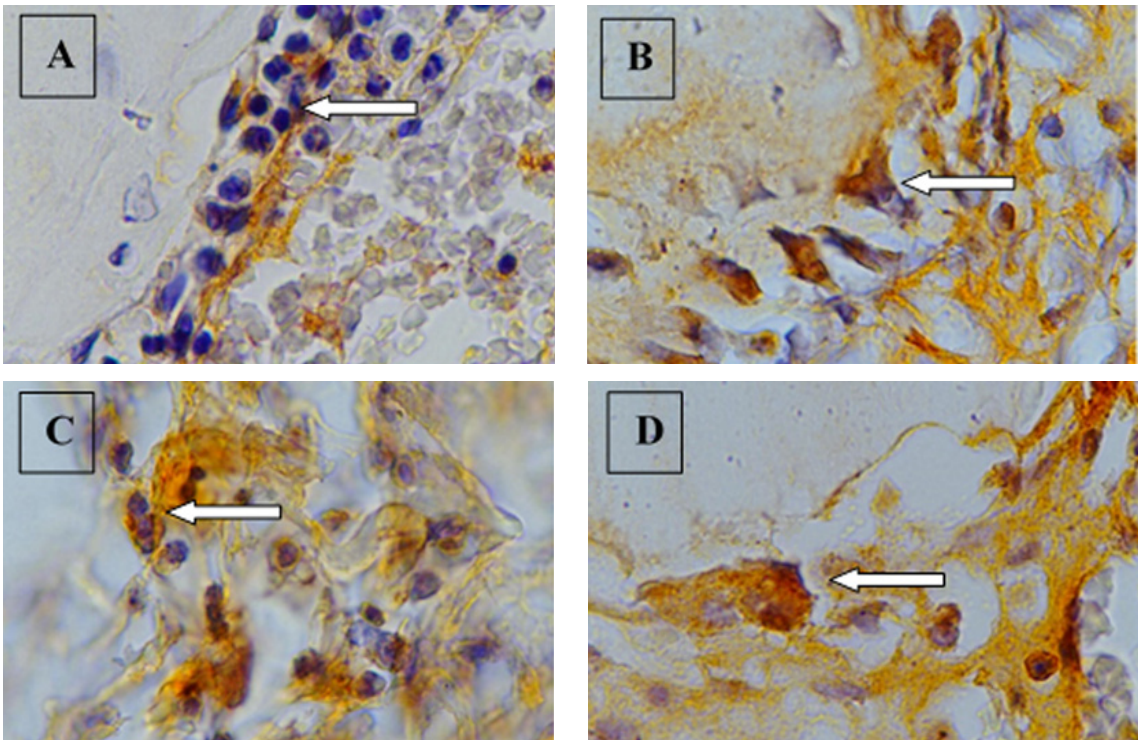
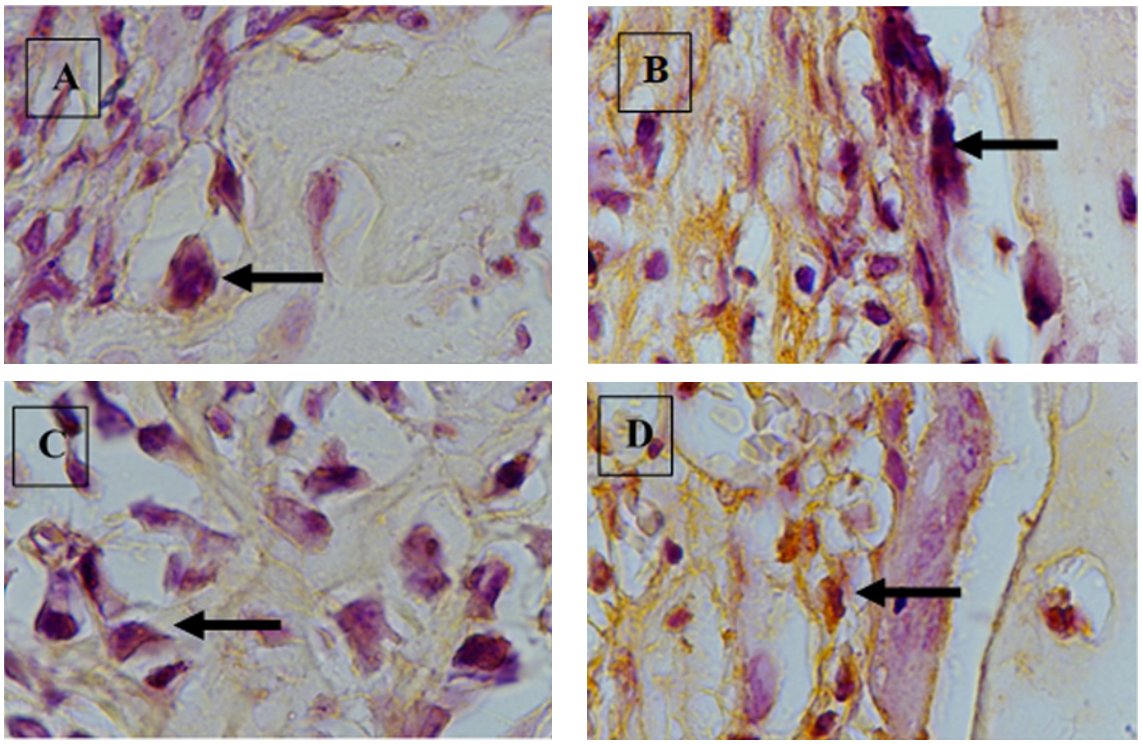


Figure 2. Expression of FGF-2 in osteoblasts located in the third apical after tooth extraction on day 7 (A, B) and day 14 (C, D) using a 1000x magnification light microscope is shown by the white arrows.



A and C: The untreated control group. **B and D:** Extraction followed by administration of Hydroxyapatite scaffold.

Figure 3. Expression of BMP-2 in osteoblasts located in the third apical after tooth extraction on day 7 (A, B) and day 14 (C, D) using a 1000x magnification light microscope is shown by the black arrows.



A and C: The untreated control group. **B and D:** Extraction followed by administration of Hydroxyapatite scaffold.

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Table 1. Tukey HSD comparison multiple test result between group.

	FGF-2					BMP-2			
Group	C7	HS7	C14	HS14	Group	C7	HS7	C14	HS14
C7		0.004*	0.190	0.000*	C7		0.002*	0.081	0.000*
HS7	0,004*		0.284	0.001*	HS7	0.002*		0.394	0.005*
C14	0,190	0.284		0.000*	C14	0.081	0.394		0.000*
HS14	0,000*	0.001*	0.000*		HS14	0.000*	0.005*	0.000*	

DISCUSSION.

The findings showed that the group receiving the crab shell hydroxyapatite scaffold in the extraction socket expressed significantly more FGF-2 and BMP-2 than the group that was not provided with the crab shell hydroxyapatite scaffold. There was a significant difference in the expression of FGF-2 and BMP-2 between the two groups ($p<0.05$). These findings demonstrate that placing a hydroxyapatite scaffold derived from the *Portunus pelagicus* shell into a tooth extraction socket increases the expression of FGF-2 and BMP-2 in *Cavia cobaya* alveolar bone. This significant difference is evident in the 7th and 14th-day groups.

The findings of this study are similar to those of research conducted by Ramadhani *et al.*,[15] as there is a significantly higher FGF-2 expression in the Wistar rats group to whose femoral bone defect hydroxyapatite had been applied, compared to the control group which was not administered with hydroxyapatite. In another study conducted applying a hydroxyapatite xenograft to the extraction sockets of guinea pigs induced a higher BMP-2 expression than in the untreated control group.[16] Both studies indicate that the administration of hydroxyapatite has a positive effect on the number of FGF-2 and BMP-2 expressions found. The increase in FGF-2 and BMP-2 expression identified in this study could have occurred due to the osteoinduction properties of the gelatin-hydroxyapatite scaffold of crab shells applied to the extraction sockets of the *Cavia cobaya*. Previous studies asserted that the osteoconductive nature of hydroxyapatite crab shell stimulated stem cells and osteoblasts to proliferate and differentiate in the formation of new bone or the process of bone regeneration.[17]

The content of hydroxyapatite crystals in the scaffold, which is homogeneously distributed throughout it, can result in an increase in mechanical properties and cellular activity on the surface of the scaffold.[18] Moreover, calcium phosphates such as HA are well known for their affinity for binding to various proteins, including BMPs, and their increase on a particular surface area may be required to accumulate sufficient amounts of BMPs to induce osteoinduction.[19] During the bone formation phases and acti-vation of osteogenesis, several growth factors are present in the early and intermediate stages, with some differences in each peak expression.

FGF-2 and BMP-2 will reach their peak on day 14, at which point the amount will decrease.[20] The results of this study support this finding since they indicated that the highest FGF-2 and BMP-2 expression in both the treatment and control groups was found on day 14.

This finding is also in line with the results of the research conducted by Huang et al., which revealed that the expression of mRNA expressed coding for FGF-2 and BMP-2 was at its highest point on day 14, during the differentiation phase.[21]

Hydroxyapatite can be synthesized from various resources such as eggshells, bones of various animals, shells, and plants. Research conducted to date shows that this natural resource can be a good source of biologically and thermally stable hydroxyapatite.[22] On this occasion, the researchers chose to use hydroxyapatite from natural sources, namely; from processed crab (*Portunus pelagicus*) shell. This action was intended to reduce crab shell waste that often produces an offensive odor and pollutes seawater. However, in reality, the amount of hydroxyapatite contained in crab shells is relatively high. Crab shell waste (*Portunus pelagicus*) has been used as raw material in the synthesis of calcium hydroxyapatite [$Ca_{10}(PO_4)6(OH)_2$] because of its high calcium content of 66.62%.[6]

Other research conducted by Wibisono *et al.*,[23] found that crab shells contain more calcium (93.78%) than fish scales (82.31%) making them suitable as raw material in the synthesis of hydroxyapatite. Also, crab shells demonstrate high compatibility. Research conducted by Kamadjaja *et al.*,[24] revealed grafts made from crab shells have strong biocompatibility in cell culture and have the optimum biocompatibility at a concentration of 25 ppm. In this study, gelatin was selected as the scaffold material because gelatin-based scaffold demonstrates excellent biocompatibility and biodegradability, while possessing a porous structure.[25]

Microporosity is also an essential characteristic of a scaffold in a bone graft. It will increase the specific surface area, providing more protein adsorption sites, where cells have more numerous opportunities to interact with osteogenic-related proteins, thereby facilitating cellular osteogenic function to form new bone tissue.[26]

Finally, further development of this research in the future remains a strong possibility because its findings show that natural ingredients, especially hydroxyapatite derived from crab shells, have a positive effect on the increased expression of growth factors, especially FGF- 2 and BMP-2 which play an essential role in helping post-extraction bone regeneration.

Furthermore, to identify the additional benefits of this material, a long-term study must be conducted until the bone regeneration process is complete in order to enable comparisons with other bone grafting materials to be made.

CONCLUSION.

The application of hydroxyapatite scaffold derived from Rajungan crab shell (*Portunus pelagicus*) in the tooth extraction socket affects the increased expression of FGF-2 and BMP-2 in the alveolar bone of *Cavia cobaya*. Furthermore, in order to identify the additional benefits of this material, a long-term study should be conducted until the bone regeneration process is complete to allow comparisons with other bone grafting materials to be undertaken.

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Conflict of interests:

The authors declare no conflict of interest.

Ethics approval:

Ethical approval for this study was granted by the Ethics Committee of the Faculty of Dentistry, Universitas Airlangga (528/HRECC.FODM/XII/2020).

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Authors' contributions:

Kridanto-Kamadjaja MJ: conceived and designed the analysis, contributed data, performed the analysis, edited the manuscript.

Salim S: conceived and designed the analysis, contributed data, performed the analysis, edited the manuscript.

Gemiudeas G: conceived and designed the analysis, collected the data, contributed data, performed the analysis, wrote the manuscript.

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