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Original Article

EFFECTIVENESS OF CHITOSAN AND HYDROXYAPATITE FROM CRAB (*PORTUNUS PELAGICUS*) SHELLS AS BONE GRAFT ON BMP-2 EXPRESSION IN SOCKET PRESERVATION

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ABSTRACT

Objective: Socket preservation involves inserting graft material into the tooth socket to prevent alveolar bone resorption and sustain bone volume. Crab shells contain high protein, chitin, and calcium carbonate, making them a potential novel material for bone grafts. This study aims to evaluate the efficacy of chitosan and hydroxyapatite (HA) derived from small crab (Portunus pelagicus) shell waste on Bone Morphogenetic Protein (BMP)-2 expression during socket preservation procedures in experimental animals.

Methods: Chitosan derived from crab shells is produced through demineralization, deproteinization, and deacetylation processes. HA is derived from diminutive crab shell through a reaction with calcium and phosphate precursors. A total of 36 male *cavia cobaya guinea pigs* underwent mandibular incisor extraction, divided into four groups: the chitosan powder from blue crab shells group, the chitosan gel and HA from blue crab shells group, the positive control given commercially available HA bone graft, and the negative control group given a placebo gel. On days 7, 14, and 21, sacrifices were performed to collect the mandibular jaw tissue of the *cavia cobaya guinea pigs,* and immunohistochemical examinations were conducted to determine BMP-2 expression. Data analysis was conducted using the Shapiro-Wilk test, ANOVA, and post hoc LSD test.

Results: On days 7, 14, and 21, there was an increase in BMP-2 expression.

Conclusion: Chitosan and hydroxyapatite derived from the shells of *Portunus pelagicus* have been demonstrated to effectively enhance BMP-2 expression.

Keywords: BMP-2, Chitosan, Hydroxyapatite, *Portunus pelagicus*, Bone graft

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INTRODUCTION

Tooth extraction is the most recent treatment alternative applied to severe tooth decay, causing bone defects that lead to resorption of the alveolar bone and surrounding soft tissue [1]. After tooth extraction, the alveolar socket experiences a reduction in the dimensions of the ridge during the healing process [2]. Young-Kyun Kim *et al.* revealed in their research that, within one year post-extraction, there was an average reduction of 50% in ridge width. The average amount of alveolar bone loss ranges from 5 to 7 mm, with two-thirds of this loss occurring within the first three months, exhibiting a consistent pattern across all regions of the oral cavity [3]. Several studies indicate that two-thirds of hard and soft tissue experiences resorption within three months post-tooth extraction. Most bone resorption transpires within the initial six months following tooth extraction, with a potential annual increase in the resorption rate averaging 0.5-1%. It is estimated that the loss of alveolar bone width can reach up to 50% within the first 12 mo post-extraction. Alveolar bone resorption following tooth extraction can be mitigated through a technique known as socket preservation [1].

Socket preservation is a procedure involving the insertion of graft material into the tooth socket, designed to prevent alveolar bone resorption, maintain bone volume, and uphold bone structure for optimal function and aesthetics [4]. Bone grafts facilitate bone regeneration across three mechanisms: osteoinductive, osteoconductive, and osteogenesis [2]. Various types of bone graft materials can be used in the management of socket preservation. Chitosan and HA are among the materials that can be utilized as bone grafts. HA is an inorganic mineral constituting 70% of bone composition. HA sourced from bovine bones has been extensively researched due to its structural and morphological similarity to human bones. HA contains biocompatibility, bioactivity, and osteoconductivity, making it frequently utilized for hard tissue regeneration; however, it has drawbacks, including fragility and the relative difficulty of absorption by the body. To enhance its characteristics, biocompatible HA has been the subject of numerous studies utilizing chitosan and HA to address its inadequate mechanical properties and to augment the bioactivity and adhesion of the biomaterial to bone [5, 6].

Chitosan is a natural polysaccharide obtained from N-deacetylated chitin, consisting of a semicrystalline structure of glucosamine and N-acetylglucosamine units. Chitosan is mainly sourced from chitin, which originates from the cell walls of fungi, as well as the shell of crustaceans and insects [7, 8]. Chitin can be transformed into chitosan via a deacetylation process utilizing a strong base [4]. Chitosan is biocompatible and exhibits significant osteointegration and osteoconductive properties. It promotes osteoblast formation and inhibits osteoclast activity, making it an appropriate alternative for bone regeneration following alveolar bone damage post-tooth extraction. Research by Shavandi *et al.* (2016) demonstrated that a combination of chitosan and HA, with a scaffold pore size of 90-220 µm and porosity of 70-80%, exhibits physical and biological properties such as osteoconduction, osteoinduction, and osteogenesis, making it a promising biomaterial for bone tissue regeneration as a bone graft. Research by Kamadjaja *et al.* demonstrated that HA content in small crab shells at concentrations of 100 ppm, 50 ppm, and 25 ppm was categorized as a bioactive material with favorable biocompatibility [9].

Crabb shell are a leading export commodity from the fisheries of South Sulawesi and have become a popular food with significant economic value [10]. Crab shell waste contains significant chemical compounds, including protein (15.60%-23.90%), calcium carbonate (53.70%-78.40%), and chitin (18.70%-32.20%) (Focher *et al.*, 1992). The substantial calcium carbonate content in crab shells indicates that crab shell waste can serve as a fundamental material for producing chitosan and HA, which are applicable as bone substitutes [11]. This study used small crab shells due to the extraction of two materials: chitosan and HA.

BMP-2 is a polypeptide growth factor composed of 396 amino acids that promote the differentiation of undifferentiated mesenchymal cells into cartilage and bone tissue. It acts as a substrate for bone

development in tissue engineering and promotes osteoblast differentiation, which is crucial for the production of new bone matrix. The presence of BMP-2 signifies the osteoconduction process in bone regeneration [12].

This research examined the effectiveness of chitosan and HA from small crab (*Portunus pelagius*) shell waste on BMP-2 expression in socket preservation procedures on experimental animals.

MATERIALS AND METHODS

Preparation of chitosan gel from crab shells [13]

The chitosan gel was prepared from crab shells by washing them and drying them in sunlight for 48 h. Once dry, the shells are ground into a powder and sifted with a 100-mesh size to produce crab shell powder.

The demineralization procedure involved the addition of 1.5 M HCl to the raw material powder at a ratio of 1:15 (w/v) of sample to solvent. The mixture was heated to a temperature range of 70–120 °C for 4 h while being stirred, followed by filtration. The material was rinsed with distilled water to eliminate residual HCl. The final filtrate was analyzed using an AgNO3 solution; the absence of a white precipitate indicated the complete removal of Cl ions. Subsequently, the solid was dried in an oven at 70 °C for 24 h to yield a mineral-free powder, which was then cooled in a desiccator.

Deproteinization was performed by adding a 3.5% NaOH solution at a 1:10 (w/v) ratio to samples that had undergone demineralization. The mixture was heated to a temperature of 65-70 °C for two hours while stirring. The solid was subsequently filtered and chilled to yield chitin, which is then washed with distilled water till achieving a neutral pH. The filtrate recovered was analyzed with a biuret reagent; a blue coloration indicates the loss of protein content. The sample also exhibited chitin by the Van Wes slink color response. Chitin was treated with a 1% I2-KI solution, yielding a brown coloration. Introducing 1M H_2SO_4 results in a color shift to violet, signifying a positive indication of the presence of chitin.

The deacetylation process was modified using different alkalis, at a concentration of 60% and a chitin-to-solvent ratio of 1:20 (w/v). The amalgamation was agitated and subjected to a temperature of 120 °C for 4 h. The solution was separated and filtrated utilizing Whatman filter paper, followed by titration with 7 N HCl to reprecipitate the chitosan. Subsequently, it was centrifuged at 2,000 rpm for 5 min to isolate the chitosan, after which the precipitate was separated. The solid was dehydrated at 80 °C for 24 h.

Chitosan gel was prepared by mixing KH_2PO_3 and $CaCl_2$ with an acetic acid solution in distilled water. Deacetylated chitosan was then added. Separately, Na_2CO_3 was dissolved in a 0.1 M acetic acid solution. To produce the appropriate chitosan/Na_2CO_3 mixture, 1 ml of Na_2CO_3 solution was used.

Making crab shell powder

The crab shells were washed with water, dried in the sun, then ground and sifted using a 100-mesh sieve. 8g of crab shell powder was weighed and calcined in a furnace at 1000 °C for 5 h. Next, it was transferred to a desiccator.

Preparation of crab shell hydroxyapatite [14, 15]

The crab shells were washed with water, dried in the sun, and then ground and sifted using a 100-mesh sieve. 500 g of crab shell powder was weighed and calcined in a furnace at 1000 °C for 5 h, and then it was transferred to a desiccator.

The fabrication of HA involved the reaction of calcium and phosphate precursors at a Ca/P molar ratio of 1.67, employing 100 ml of 0.5 M Ca(OH)2 solution. Subsequently, 100 ml of 0.3 M phosphoric acid was introduced into the beaker while agitating. Subsequently, 2 M NaOH was used to achieve a pH of 10. The solution was maintained at room temperature for 24 h to facilitate the growth of HA crystals. The precipitate was filtered and subsequently washed with distilled water. Subsequently, it was rinsed with alcohol and dried at a temperature of 110 °C. The HA was crushed, sieved, and subsequently subjected to a furnace at 900 °C for 2 h. The crystals were let to cool in the furnace for 24 h before being moved to a desiccator.

Treatment of experimental animals

This experiment received approval from the Faculty of Dentistry Ethical Committee. All animal treatments were executed strictly according to applicable rules and laws, with a concerted attempt to mitigate animal suffering. Thirty-six male *Cavia cobaya guinea pigs* tails used in this study, weighing 300-500 g, were kept in groups (1-4 *cavia cobaya guinea pigs* per cage) and adapted for one week before treatment to condition the animals in a healthy condition. Environmental and animal health monitoring was carried out every day.

Cavia Cobaya guinea pigs received intramuscular injection of 0.2 cc of ketamine (20 mg/300 mg). Following shaving and antisepsis with 10% povidone-iodine, the mandibular right incisor was extracted, and the socket was then rinsed with a saline solution. The socket preservation method was executed in accordance with each treatment group. Group 1 contained crab shell chitosan powder at a concentration of 2%; Group 2 sockets were filled with a mixture of crab shell chitosan gel (2% concentration) and crab shell HA in a 1:1 ratio; Group 3 sockets were filled with commercially available HA bone graft (positive control); Group 4 sockets were filled with placebo gel (negative control). Following the surgical procedure, silk sutures were utilized for closure.

On the 7, 14, and 21 d, cavia cobaya guinea pigs (n=3) from each group were sacrificed. The mandibular jaw of Cavia cobaya guinea pigs was excised, thereafter preserved in a 10% buffered formalin solution for 24 h, and then dispatched to the Anatomical Pathology Laboratory to prepare immunohistochemical slides.

Immunohistochemical test

Paraffin blocks containing bone tissue were sectioned at a thickness of 4 µm using a microtome and then deparaffinized with xylene. Rehydration was performed with decreasing concentrations of ethanol, followed by rinsing with Phosphate Buffer Saline (PBS) for three sets of five-minute intervals each. The tissue slides were then incubated in DAKO® Antigen Retrieval Buffer in a microwave at 94 °C for 20 min, followed by cooling at room temperature for 20 min. The slides were then washed with PBS for 3 x 5 min and incubated in Peroxidase Block (Novocastra®) for 20 min.

Furthermore, the slides were washed again with PBS for three sets of five-minute intervals and incubated in Protein Block for 20 min. Afterward, the slides were washed again with PBS three sets of five-minute intervals and incubated overnight (12-18 h). The following day, the preparations were rinsed with PBS for three sets of five-minute intervals each. Then, they were treated with post-primary and post-protein solutions for 45 min, followed by incubation with secondary antibody (Novolink® Horse Radish Peroxidase (HRP)) for 60 min at room temperature. After incubation, the slides were washed with PBS for PBS three sets of five-minute intervals minutes and counterstained with hematoxylin (Novocastra). Subsequently, they were dehydrated using increasing concentrations of ethanol, clarification using xylene, and mounting. The last step slides were dried, applying Entellan and covered with a glass slide. The prepared slide was then observed under a microscope for analysis.

Image analysis

Immunohistochemical analysis was performed to determine the level of BMP 2. The analysis was carried out by observing the presence of brown color in the cytoplasm of osteoblast cells. The quantitative assessment was carried out following the protocol described by Soini *et al.* (1998), with modifications based on the specifications of Pizem and Cor (2003), and further adapted for the evaluation of osteoblast cells expressing BMP-2. Observations were performed across 20 microscopic fields at 1000× magnification using a light microscope. Immunohistochemical staining was classified as negative when no brown-stained nuclear granules were detected, whereas the presence of brown-pigmented granules within the cell nuclei was interpreted as a positive outcome.

Statistical analysis

Numerical information is expressed using mean–standard deviation.

The normality of the data was assessed using the Shapiro-Wilk test, while the homogeneity of the data was evaluated using Levene's test. Data that is normally distributed will then be tested using ANOVA, while data that is not normally distributed will be tested using Kruskal-Wallis. The type of data used is primary data. Data processing uses IBM SPSS Statistics V.24.

Research ethics

This research has obtained approval from the research ethics committee of the Faculty of Dentistry, Hasanuddin University, with the number: 0073/PL.09/KEPK FKG-RSGM UNHAS/2023.

RESULTS

Characteristics of *Portunus Pelagicus* graft materials

Fourier Transform Infra-Red (FTIR) was a test used to qualitatively analyze functional groups in chemical compounds such as chitosan. Fig. 1 compares sample chitosan's infrared spectrum and standard chitosan's infrared spectrum. The functional groups that appear in chitosan from crab shells (*Portunus pelagius*) and standard chitosan show that the crab shell chitosan samples show chitosan functional groups.

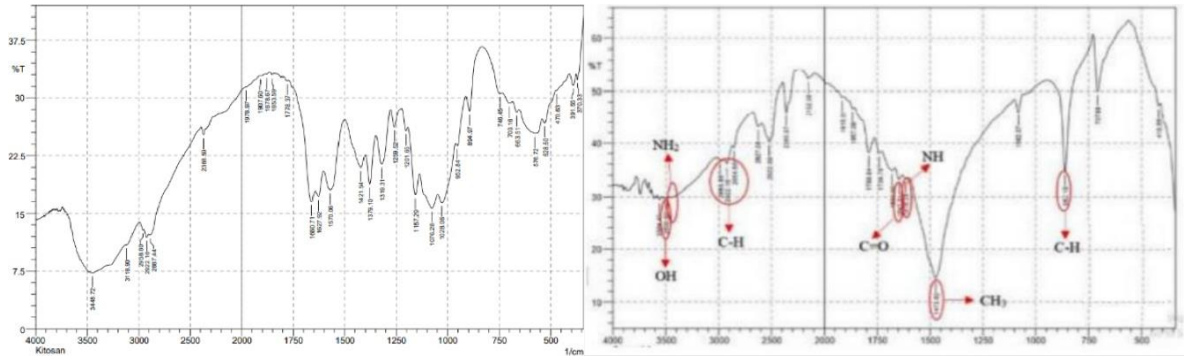
X-ray diffraction (XRD) provides specific existence information regarding the sample's phases. The results of the XRD analysis, shown in fig. 2, showed that the sample was a single phase without any other compounds formed, namely the hydroxyapatite phase. The highest peak in the hydroxyapatite sample is at an angle of 2Θ= 2=37.26.

BMP-2 expression examination and statistical tests

The Shapiro-Wilk test for data normality yielded a significant value (p>0.05), indicating that the data was normally distributed, therefore proceeding with the parametric test. Levene's homogeneity test yielded a p-value of 0.992 (p>0.05), indicating that the data in this investigation was homogenous. A comparison of BMP2 expression based on observation time can be seen in table 1. On the 7th d, the statistical test results using the one-way ANOVA test showed a significant difference among the four treatment groups with a p-value of 0.008 (p<0.05). On the 14th d, the statistical test results using the one-way ANOVA test showed a significant difference among the four treatment groups with a p-value of 0.005 (p<0.05). On the 21st d, the statistical test results using the one-way ANOVA test showed a significant difference among the four treatment groups with a p-value of 0.004 (p<0.05).

A comparison of BMP2 expression based on observation time can be seen in graphic 1. On d 7, 14 to 21, BMP-2 expression increased in the chitosan and hydroxyapatite gel treatment groups, followed by the chitosan powder group, which showed the same value on 14 and 21, the positive control group experienced an increase in BMP-2 expression and for the negative control group on d 7, 14, and 21 showed an increase in BMP-2 expression values.

The results of BMP-2 immunohistochemical examination between the chitosan powder, gel (chitosan+hydroxyapatite), positive control (hydroxyapatite), and negative control (placebo gel) treatment groups on d 7, 14, and 21 can be seen in fig. **3** and fig. **4**. The image of BMP-2 is visible in red (black arrow) on bone preparations. The gel treatment group (chitosan+hydroxyapatite) exhibited the highest number of cells expressing BMP-2, followed by the chitosan powder treatment group, then the positive control group (hydroxyapatite) and the negative control (placebo gel).



A **B**

Fig. 1: A. Infrared spectrum of *Portunus pelagicus* chitosan. B. Infrared spectrum of standard chitosan

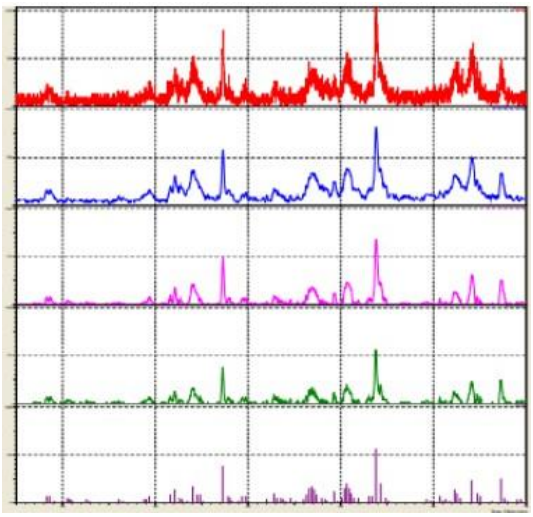
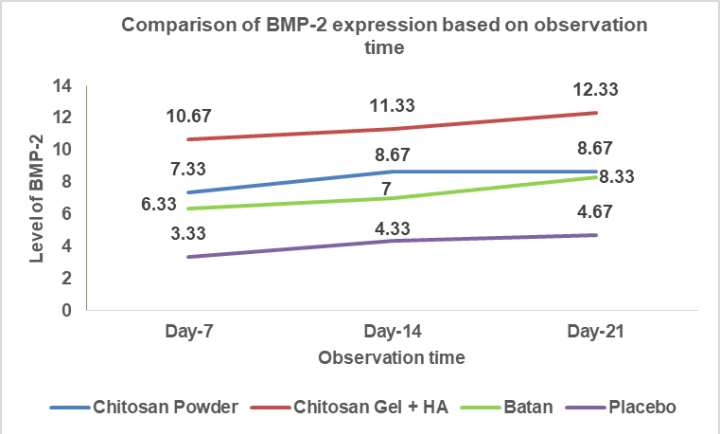


Fig. 2: Degree of X-ray diffraction crystallization of hydroxyapatite from crab (*Portunus pelagicus*) shells

Table 1: Comparison of average BMP-2 expression based on treatment groups on d 7, 14, and 21

Group	Day 7	Day 14	Day 21
	Mean	Mean	Mean
	SD	SD	SD
Chitosan powder	7.33	8.67	8.67
	2.082	1.528	1.528
Chitosan gel+HA	10.67	11.33	12.33
	2.082	1.528	2.082
Control positif	6.33	7.00	8.33
	1.528	2.000	1.528
Placebo	3.33	4.33	4.67
	1.528	1.528	1.528
p value	0.008*	0.005*	0.004*

One way Anova; *significant (p<0.05), SD: Standard Deviation, HA: Hydroxyapatite



Graphic 1: Comparison of BMP-2 expression based on observation time

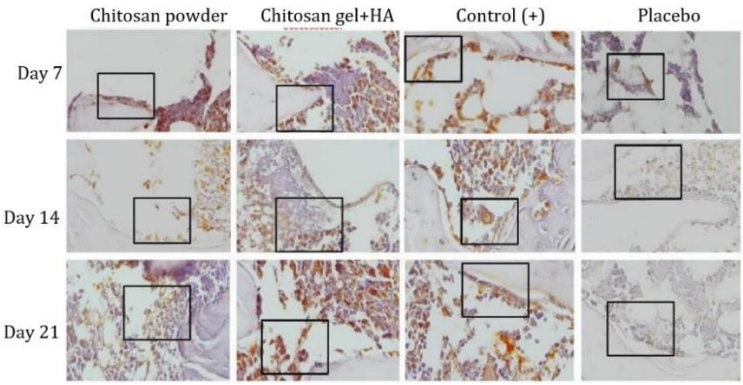


Fig. 3: Expression of BMP-2 on d 7, 14, and 21 under a 400x magnification light microscope. BMP-2 level marked in brown (box)

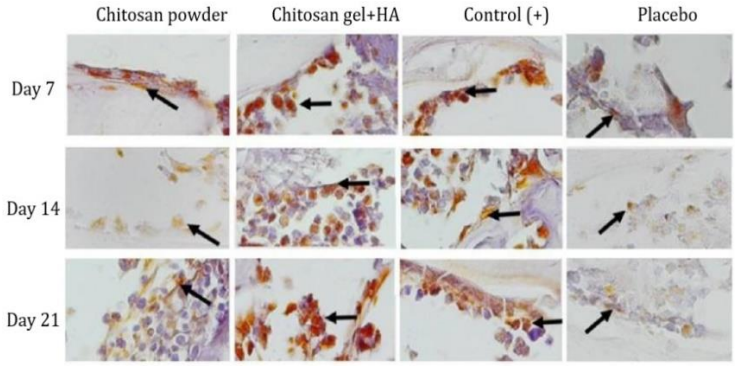


Fig. 4: Expression of BMP-2 on d 7, 14, and 21 under a 1000x magnification light microscope. BMP-2 level marked in brown (arrowheads)

DISCUSSION

This research was conducted to examine the effectiveness of chitosan powder, chitosan gel, and hydroxyapatite from blue crab shells (*Portunus pelagicus*) as bone grafts in increasing BMP-2 expression during socket preservation procedures. Socket preservation is a procedure on the tooth socket by inserting a graft material aimed at preventing alveolar bone resorption, maintaining bone volume and structure so that it can function optimally and have good aesthetics. The graft materials that can be used in socket preservation must have osteoconductive, osteoinductive, and osteogenic properties [16].

The researchers observed the expression of BMP-2 on the dental sockets of Cavia Cobaya guinea pig on d 7, 14, and 21 after the application of chitosan powder and a combination of chitosan gel from blue crab shells with HA This is based on research by Khoswanto [17], which states that BMP-2 expression in the dental socket was observed from day 7 post-application of 10% A. cordifolia gel.

In table 4, the comparison of BMP-2 expression in each treatment group on d 7, 14, and 21 is shown. The highest increase in BMP-2 expression was observed in the treatment with Chitosan Gel+HA, followed by the chitosan powder treatment group, the control positive treatment group, and the lowest in the placebo group, indicating a significant difference among the four treatment groups ($p<0.05$). This indicates that the application of chitosan powder, chitosan gel, and HA from crab shells can enhance BMP-2 expression, which plays a significant role in tissue formation and maturation. The activity of BMP-2 is necessary for the initiation of bone healing [17]. According to research by Chevrier *et al.* [18], chitosan was shown to enhance vascularization and induce the formation of granulation tissue on the 7th post-operative day.

Supported by research by Gani *et al.* [19], it was found that the addition of a combination of chitosan gel and HA to Wistar rat femur defects can reduce IL-1, a pro-inflammatory cytokine, and thereby preventing inflammation. These results are consistent with *in vitro* research by Matsunaga *et al.* [20], which stated that the application of chitosan can increase the expression of BMP-2 mRNA expression. BMP-2 is the prototype subgroup of BMPs that the differentiation of multipotent mesenchymal progenitor cell lines into the osteogenic lineage. BMPs stimulate activator protein-1 (AP-1) to increase the expression of alkaline phosphatase (ALP) and initiate the mineralization process.

Transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs) are critical members of the TGF-β superfamily that regulate lineage commitment, differentiation, and function of osteoblasts, osteoclasts, and chondrocytes. These pathways orchestrate skeletal development, postnatal remodeling, and long-term bone homeostasis. While BMPs function primarily as osteogenic, chondrogenic, and osteoclastogenic inducers at all stages of cellular differentiation, TGF-β signaling exhibits stage-specific and context-dependent effects. In articular cartilage, BMPs and TGF-β exert opposing influences: BMPs enhance anabolic extracellular matrix synthesis, whereas TGF-β contributes to catabolic regulation. Moreover, TGF-β is essential for the preservation of osteocyte viability and maintenance of the lacuno-canalicular network, which coordinates mechanosensation and skeletal remodeling. A finely tuned equilibrium between bone formation by osteoblasts, resorption by osteoclasts, and signaling by osteocytes is indispensable for skeletal integrity. Disruption of this balance results in impaired bone remodeling and contributes to metabolic bone disorders such as osteopenia, osteoporosis, and osteosclerosis. The integration of BMP and TGF-β signaling into these processes underscores their fundamental role in maintaining skeletal homeostasis [21].

HA material can be obtained from natural biological sources, one of which is the waste shells of blue crabs (*Portunus pelagicus*), a natural raw material that is abundant in South Sulawesi. Hydroxyapatite has bioactive and osteoconductive properties that are beneficial in promoting rapid and biologically strong bone formation within bone tissue. This is in line with the research by Kamadjaja *et al.* that the application of hydroxyapatite derived from the shells of blue crabs (Portunus pelagicus) in socket preservation procedures in Wistar rats was proven to increase TGF-β1. BMPs are a group of signaling molecules belonging to TGF-β protein superfamily. Analysis of the expression of members of the TGF-β superfamily shows that BMP-2 expression occurs earliest which will continue to increase until it reaches its peak on day 21. The presence of BMP-2 will accelerate bone healing, increase mineralization, remodeling, and biomechanical stiffness [22].

Chitosan is a compound obtained from the deacetylation process of chitin, which is commonly found in the shells of arthropods such as the crustacean family, including crabs, blue crabs, shrimp, and lobsters. Some functions of chitosan can be found in biomedical applications, such as biocompatible, biodegradable [23].

The utilization of the biopolymer chitosan can be processed into various forms such as solutions, gels, pastes, mixtures, sponges, membranes, tablets, and microbeads. In this study, chitosan is used in powder and gel forms. The powder, when placed into the socket after tooth extraction, has a good binding ability with blood, stabilizing the condition in the socket. This also shows that chitosan in gel form has good mucoadhesive properties, capable of absorbing exudate, maintaining wound moisture, and being easily applied to the wound. In gel form, chitosan protects the wound area and has a cooling effect, thereby reducing pain [19, 24] Sularsih [25] chitosan gel preparation, which is quite thick, has a viscosity that facilitates the application of chitosan in the healing of tooth extraction wounds in Rattus norvegicus rats. Although both preparations are effective in the bone regeneration process, the drawback of the powder preparation is its application in the tooth extraction socket compared to the gel preparation. The parameters that affect the properties of chitosan are molecular weight (MW) and degree of deacetylation (DD). Chitosan with a high molecular weight has high viscosity, while chitosan with a low molecular weight has low viscosity, because the higher the molecular weight, the larger the particle size, resulting in a slower dissolution process [26].

In this study, the highest BMP-2 expression was observed in the group treated with chitosan and hydroxyapatite gel. This is because chitosan has osteointegrative and osteoconductive properties, so when combined with hydroxyapatite, which also has bioactive and osteoconductive properties, both can enhance the bone regeneration process.

The research results are not optimal due limitations such as a smal sample size, a short research period, and a lack of mechanical stability testing, necessitating further research on these two materials to achieve maximum bone regeneration effects.

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AUTHORS CONTRIBUTIONS

DS (Conceptualization, Data curation, Formal Analysis, Methodology, Validation, Supervision, Writing – review and editing), SO (Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Supervision, Writing – review and editing), AG (Data curation, Supervision, Validation, Writing – review and editing), AI (Data curation, Validation, Writing – review and editing), USS (Resources, Software, Visualization, Writing – original draft).

CONFLICT OF INTERESTS

Declare that there is no conflict of interest regarding the publications of this paper.

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