Introduction

Periodontal tissue is an important part that maintains teeth function and stability. Bone defects due to periodontal disease or periodontal treatment procedures can cause problems related to aesthetic function, the healing process, and even the stability of the jawbone resulting in tooth loss [1]. The application of several materials to the area around these bony defects aids bone regeneration and eliminates the defects or limits their size [2].

Bone grafting is a surgical procedure that replaces missing bone with material from the patient’s own body or from synthetic, artificial, or natural substitutes. A bone graft can help regenerate bone through three methods: Osteoinductive, osteoconductive, and osteogenesis [3], [4], [5]. Osteoconduction is a bone graft material serving as a scaffold for new bone growth. Osteoinduction involves stimulating osteoprogenitor cells to differentiate into osteoblasts which then form new bone. Osteogenesis is the formation of new bone by the cells (Mesenchymal stem cells and progenitor cells) contained within the graft material. Bone morphogenetic proteins (BMPs) are osteoinduction cytokines that stimulate osteogenesis, that release substances, and that stimulate bone formation [6]. BMP2 stimulated the vascular expression of the osteoblast-specific transcription factor core-binding factor alpha 1 and of the downstream osteoblast programmed leading to increased mineralization [7].

Osteoprotegerin (OPG) is a natural inhibitor to inhibit receptor activator of nuclear factor kappa B ligand (RANKL) binding with receptor activator of nuclear factor kappa B (RANK) so that osteoclasts activity decrease. The biological effects of OPG on bone cells include resistance in the late stages of osteoclast differentiation, suppressing the activation of mature osteoclasts, inducing apoptosis, thereby decreasing the process of bone resorption and causing an increase in the amount of OPG so the osteogenesis process developed [8].
Graft material derived from the body itself is still the best choice in restoring bone defects [9] but is still limited in-process and source, so it needs a replacement bone graft material that can help bone regeneration.

Some marine biota structures can be used to develop tooth structures (bones, dentin, pulp, or periodontal ligaments) that are damaged or lost due to disease [10]. Pinctada maxima are pearl shells that have been cultivated in the Pangkep Islands of South Sulawesi [11]. Pearl shells are one of the marine biotas that have a long history of medical research. Nacre Pinctada maxima contain inorganic and organic materials that have a bone-like basic structure. Nacre was able to facilitate osteoblast proliferation, accelerate extracellular matrix production, and mineralization [12], [13].

Nacre, the inner layer of pearl shell of Pinctada maxima oysters often called the “mother of pearls” has shown promised results as a resorbable biomimetic graft material. Nacre often exhibits natural osteoconductive turnover with strong effects in osteoprogenitors, osteoblasts, and osteoclasts during bone tissue formation and morphogenesis. In addition, nacre also exhibits biocompatible and biodegradable properties in bone tissue [14]. Hence, the purpose of this study is to describe the effectivity of Pinctada maxima pearl shells as bone graft material using an animal model.

Materials and Methods

Pearl shell preparation

Pinctada maxima pearl shells were obtained from the Pangkep Islands of South Sulawesi, Indonesia. It was cleaned by brushing under running water, and then dried in the sun. After that, Pinctada maxima pearl shells were cut into smaller sizes and furnace for 2 h. The sample results were mashed with a metallic mortar and pastel and filter with a test sieve (100 mesh) to obtain Pinctada maxima pearl shell powder.

Hydroxyapatite (HAP) powder preparation

The synthesis of HAP from Pinctada maxima shell powder was carried out using the precipitation method. Pinctada maxima shells are synthesized using H$_3$PO$_4$ compounds with a temperature of 100°C. The solution is left for 24 h to obtain HAP deposits. These deposits are calcined at 800°C. The synthesis results in the form of HAP Pinctada maxima (HPM) powder. The chemical and characterization of HPM were assessed by X-ray fluorescence (XRF), X-ray diffraction (XRD), and Fourier transform infra-red (FTIR) analysis.

Animal model

This research proposal was reviewed by the Komisi Etik Penelitian Kesehatan Faculty of Dentistry, Dental and Oral Hospital of Hasanuddin University, Makassar, Indonesia (process no. 0043/2021) and the design of this study has been approved. This study used 30 healthy male guinea pigs weighing 300–350 g that were adapted for 7 days and given food in the form of pellets. Guinea pigs receive an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) which were mixed and injected with a dose of 1 mg/kg BB in Dexter’s femur bone. After being shaved and asepsis with 10% iodine povidone, a horizontal incision with a 2 cm long scalpel blade on soft tissue (skin and muscles) is lifted with a periosteal elevator where the defect will be made. After the bone tissue was exposed, a defect was made with a round bone bur (3 mm in diameter and 3 mm depth). Then, randomly, guinea pigs were divided into three treatment groups: Negative control (NC) group which was treated with placebo, positive control (PC) group which were treated with HAP of BATAN, and HPM group. After the surgical procedure, the suture was performed with absorbable suture (Vicryl 5.0) on the muscles and silk suture on the skin, with interrupted suture technique and given antibiotics as prophylaxis on the surface of suture area. For medication post-surgical, the guinea pig was given dexamethasone 0.6 mg/kg and ampicillin 10 mg/kg. Euthanasia was performed with an ether chamber at 14–21 days which were five animals are sacrificed in each group at a time.

The femur was taken through appropriate surgical techniques and the bone defects are separated. Pieces of the bone block are stored in a 10% formalin solution for 24 h. It is then included in a routine laboratory procedure.

Analysis of immunohistochemical (IHC)

IHC analysis was performed with Sony A7 digital camera connected to Nikon Eclipse E100 LED binocular microscope with magnification 40×,100×, and 1000× in 20 times per field of view to guarantee representation and reduce error results. Coded slides are closed code numbers and given new numbers randomly, so the examiner does not know what slides sample of the group was examined. Examination and calculation of expression are observed by looking at the presence of brown color in the cytoplasm of osteoblast cells. The test results are then analyzed in a statistical test.
Statistical analysis

The data were then analyzed with the help of IBM SPSS statistics data analysis program version 21. Analysis of primary data of OPG and BMP$_2$ was obtained to conduct the normality data using the Shapiro–Wilk’s test, and then the data have tested the homogeneity with Levene’s test. To analyze, the differences between the research groups conducted with one-way analysis of variance (ANOVA) and followed by Tukey HSD test with significance value $p < 0.05$.

Results

Characteristics of materials with XRF, XRD, and FTIR analysis

The mineral composition of HPM was analyzed with XRF and found that the calcium of the total sample was 69.46%. HAP content in HPM can be predicted in qualitative terms using data of FTIR analysis. It provides useful information about the composition of samples and the location of peaks, their intensities, width, and shape in the required wave-number range. From this analysis was known functional groups, namely, $\text{OH}^-$, $\text{PO}_4^{3-}$, and $\text{CO}_3^{2-}$. From the data of the analysis are known hydroxy group ($\text{OH}^-$) at wave-numbers 3641.73 cm$^{-1}$, phosphate groups ($\text{PO}_4^{3-}$) 563.59 cm$^{-1}$, 609.53 cm$^{-1}$, and 1035.81, and groups calcium carbonate ($\text{CO}_3^{2-}$) 1425.44 cm$^{-1}$ and 1512.24 cm$^{-1}$ (Figure 1).

IHC analysis

Through IHC analysis, OPG and BMP$_2$ expression was seen in all experimental groups. OPG and BMP$_2$ expression calculations were performed on days 14–21. The data were then analyzed with IBM SPSS statistics data analysis program version 21. Before the statistical test, first conducted a test of the normality data using Shapiro–Wilk’s test. The results of the Shapiro–Wilk’s test data for OPG and BMP$_2$ variables of NC, PC, HPM on days 14–21 showed a significance value of $p > 0.05$, so it concluded that the data were a normal distribution. Then, homogeneity test using Levene’s test obtained a significance value of OPG and BMP$_2$ expression $p > 0.05$, it can be concluded that the variant of NC, PC, and HPM on days 14–21 between groups was the same or homogeneous.

Parametric statistical tests use the one-way ANOVA test to determine the effect of graft material on OPG and BMP$_2$ expression in bone formation of male guinea pig femur defects and proceed to the Tukey HSD test to see the difference between each variable with a significance level of 5%.

OPG analysis

Figure 3 shows the results of IHC painting using anti-OPG monoclonal antibodies (MoAb) on 14–21 days with 30 slides of the guinea-pig bone tissue defect. Each tissue sample is made of slice preparations with a thickness of 4 um. To obtain the color intensity and area of OPG as quantitative values, it is observed by looking at the presence of brown color in the cytoplasm of osteoblast cells. There was a significant increase expression of brown colors on days.
of 4 um. To obtain the color intensity and area of BMP\textsubscript{2} expression as quantitative values, it is observed by looking at the presence of brown color in the cytoplasm of osteoblast cells. BMP\textsubscript{2} expression was seen on days 14–21. Predominant brown colors show the BMP\textsubscript{2} expression of guinea pig femur bone. There was a significantly increased expression of brown colors on days 14–21 in all experimental groups.

Table 1 shows observation of IHC expression of OPG in the male guinea pig femur defect on the HPM group against the NC group and PC group after 14 days observation showed the expression of OPG in HPM group 10.40 ± 2.408 in the defect area, which was significantly greater than the NC group (p < 0.05) and after 21 days observation, the expressions of OPG in HPM group were 11.20 ± 2.168 in the defect area, which was significantly greater than the NC group (p < 0.05). However, in the HPM and PC, there were no significant differences. OPG expression on days 14–21 shows that the entire NC group and HPM group descriptively increase, except for the PC group, which has decreased (Figure 4).

Table 1: OPG expression on guinea pig femur defect (mean ± SD)

| Days   | n   | Experimental Groups |
|--------|-----|---------------------|
|        |     | NC                  | PC                  | HPM                  |
| 14 h   | 5   | (4.00 ± 1.581)      | (11.40 ± 1.817)     | (10.40 ± 2.408)      |
| 21 h   | 5   | (6.80 ± 0.837)      | (10.60 ± 1.517)     | (11.20 ± 2.168)      |

OPG: Osteoprotegerin, HPM: Hydroxyapatite pinctada maxima, NC: Negative control, PC: Positive control.

**BMP2 analysis**

Figure 5 shows the results of IHC painting using anti-BMP\textsubscript{2} MoAb on 14–21 days with 30 slides of the guinea-pig bone tissue defect. Each tissue sample is made of slice preparations with a thickness of 4 um. To obtain the color intensity and area of BMP\textsubscript{2} expression (black arrow). (a and b) represent the negative control group, (c and d) represent the positive control group, and (e and f) represent the hydroxyapatite pinctada maxima group.

Table 2 shows observation of IHC expression of BMP\textsubscript{2} in the male guinea pig femur defect on the HPM group against the NC group and PC group after 14 days observation showed the expression of BMP\textsubscript{2} in HPM group 11.00 ± 2.000 in the defect area, which was significantly greater than the NC group (p < 0.05) and after 21 days observation, the expressions of BMP\textsubscript{2} in HPM group were 13.40 ± 2.074 in the defect area, which was significantly greater than the NC group (p < 0.05). However, there were no significant differences in the HPM and PC data.

Table 2: BMP2 expression on guinea pig femur defect (mean ± SD)

| Days   | n   | Experimental Group |
|--------|-----|-------------------|
|        |     | NC                | PC                | HPM                |
| 14 h   | 5   | (3.20 ± 1.304)    | (11.80 ± 2.280)   | (11.00 ± 2.000)    |
| 21 h   | 5   | (7.60 ± 2.608)    | (12.40 ± 2.302)   | (13.40 ± 2.074)    |

BMP2: Bone morphogenetic protein, HPM: Hydroxyapatite pinctada maxima, NC: Negative control, PC: Positive control.

Based on Figure 6, IHC observations of BMP\textsubscript{2} expression on days 14–21 show that entire experimental groups descriptively increase.

**Discussion**

Bones can regenerate from bone grafts. An ideal bone substitute still searches since autograft and allograft are limited in application [15], [16]. The use of Pinctada maxima shells as the main source of bone graft material has been widely researched before, but the differences in species and geographical origin can affect the component variation of shells [17].

The HAP and calcium phosphate content of nacre make it potential as alternative bone substitute.
composite material [18]. In this study, researchers used Pinctada maxima pearl shells that were processed into HAP powder. There have been many studies that process Pinctada maxima shell powder into HAP [19], [20]. It is common knowledge for geologists and agronomists who say that calcium carbonate can be converted into HAP using liquid phosphate at room temperature [21]. This study uses precipitation techniques to process the Pinctada maxima pearl shells powder into HAP powder [22], [23], [24].

Nacre and human bone are both mineralized structures, although their composition and organization differ [25]. The mineral phase of bone is composed of calcium phosphate in the HAP crystal form, whereas nacre is composed of calcium carbonate in an aragonite crystal form [19], [21], [26]. Researchers processed Pinctada shell powder into HAP powder to obtain materials that resemble human bones. After the HPM powder is produced, XRF analysis is done to find out the mineral content of HPM powder, which in this study obtained a calcium content of 69.46%. The amount of calcium in HPM decreased when compared to the results of other studies that calculated the amount of calcium from pearl shell powder which reached up to 95% of aragonite calcium carbonate crystals. This can be due to the release of calcium ions from shell powder when reacted with phosphate ions in the buffered solution. A few calcium ions are released and thus the nacre surface is activated at the sites where the calcium ions are released. The free calcium ions bind the phosphate ion in the buffered solution and precipitate it again at the active sites on the nacre surface in the form of HAP [17].

The characterization of HAP using FTIR analysis shows the peak of PO$_4^{3-}$ which is sharp. This indicates that much amount content of PO$_4^{3-}$ in HPM powder. This is in accordance with the theory that the sharper the peak of PO$_4^{3-}$ the growth of crystalline area better, which means the HAP, obtained the better [27]. PO$_4^{3-}$ and OH that appear with sharper peaks indicate higher absorbance intensity. The higher the absorbance intensity, the more the content of PO4- and OH- compound. Both groups show the synthesis of HAP where PO$_4^{3-}$ and OH form the formula Ca$_{10}$(PO$_4$)$_6$(OH)$_2$.

The FTIR results showed the presence of CO$_3^{2-}$ which is characterized by the presence of C-O bonds at the peak of 1512.24 cm$^{-1}$. The presence of the group is from the reaction of HAP with CO2 which is contained in the atmosphere at the time of synthesis and heat treatment. It is seen that the intensity of this peak decreased in FTIR analysis indicated by the peak at ~1425.44 cm$^{-1}$. The decrease in intensity of the peak indicates a decrease in the concentration of the CO$_3^{2-}$ group caused by an increase in the temperature of heat treatment. The presence of CO$_3^{2-}$ cannot be said to be bad because indeed in the human bones themselves have CO$_3^{2-}$ which is a substitution of PO$_4^{3-}$ which naturally follows the equation Ca$_{10}$(CO3)$\times$(PO4)$_6$-(2/3) × (OH)$_2$ [22], [23].

![Figure 6: Bone morphogenetic protein 2 expression between experimental groups at different periods. (*) indicates significant differences between groups at the same period](image)

Characteristics of HAP Crystalline (HAP) using XRD Analysis. The degree of crystallinity can be determined by the ratio of the area of the crystal fraction to the total fraction area (crystal + amorphous). The degree value of crystalline or the percentage of different crystalline can be due to differences in type, bone minerals, and genus factors, in addition, the differences can also be caused by the density of the shell itself [28]. HAP is the main inorganic component of bone-hard tissue that accounts for 60–70% of the mineral phase in human bones [29]. A study reported that the percentage of crystallinity affects the regularity of atoms structure in the material, where the higher the percentage of crystalline, the more orderly the arrangement of atoms structure in the material. It can be seen from the graph of results in the form of higher intensity and narrower the width of half the peak. The degree of crystalline HPM is 64.43%; this value is quite good because it meets the mineral phase value of human bones and teeth so that HPM has the potential to be used as bone graft material.

Bone cells biological effects of OPG include suppressing mature osteoclast activation, resistance osteoclast differentiation at the final terminal stages, promoting apoptosis, so the bone resorption process decreases [30]. Many cells’ types have secreted OPG; most factors that induce RANKL expression by osteoblasts also regulate OPG expression [31]. The discovery OPG/RANK/RANKL system has been most important in biology. This signaling is important for skeletal homeostasis, and inhibition of bone resorption occurs when there is a disruption of the system and it will lead to increase resorption [32]. An immunohistochemistry examination of this study showed increased OPG expression in both the NC group and the HPM group, but NC group showed fewer than HPM group. This suggests that the HPM component affects OPG formation faster than physiological healing or NC group. The presence of OPG expression affects the production of osteoclast and inhibits bone resorption in the bone remodeling cycle.
The presence of BMP₂ will accelerate bone healing, increase mineralization, remodeling, and biomechanical stiffness [33]. BMP₂ expression increases at the beginning of the latent phase and helps the cell’s differentiation process. Studies using mammalian bone marrow cells observed that WSM induces rapid mineralization and that fibroblasts displayed an early increase in alkaline phosphatase and BMP₂ activity [34], [35], [36], [37], [38]. Research using Pinctada maxima material in the form of chips in mammalian bone marrow cells found that nacre promotes the differentiating of bone cells [39].

In this study, BMP₂ expression was increased in all experimental groups, with the highest values appearance in the HPM group. This suggests that HPM materials affect the formation of BMP₂ expression than existing physiological healing and HAP component. These results indicated the osteogenic potential of HPM and it is possible that the greater the mineral availability, the better this organic-mineral interface formed. The chemical and characteristics of HPM could suggest the formation of bone and it is conducted by the IHC result which can be seen from the OPG and BMP2 expression. Further studies need to see the osteogenesis mechanism of this material through the progenitor cells that will optimize the possible use of this material as a bone graft in dentistry.

Conclusion

HAP powder of Pinctada maxima contains calcium and high crystallinity that meet the mineral phase value of human bones so it has good potential as a promising bone graft material.

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