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Original Article

Evaluation and comparison of synthesised hydroxyapatite in bone regeneration: As an *in vivo* study

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الملخص

أهداف البحث: يعاني العديد من المرضى من عيوب في العظام غير قابلة للإصلاح وما يتبعها من مشاكل جمالية ونفسية عقب كسور العظام أثناء الحوادث. تهدف الدراسة إلى مقارنة وتقييم هيدروكسيباتيت الاصطناعي مع الطعم المغاير وهيدروكسيباتيت التجاري في إصلاح العظام وإعادة بنائها.

طرق البحث: في هذه الدراسة، تم تصنيع وتحديد هيدروكسيباتيت الاصطناعي وإجراء اختبارات السمية الخلوية (تحليل البلازما المقترنة بالحث، وتحليل الكثافة والمسامية، ومقايسة فحص المجهر الإلكتروني). وتم اختبار هيدروكسيباتيت الاصطناعي، والطبقة الخارجية، والهيدروكسيباتيت التجاري في دراسة الحيوانات. أخيرا، تم تقييم تجديد العظام عن طريق صبغة الهيماتوكسيلين والأيوسين.

النتائج: تم قياس نسبة الكالسيوم/الفسفور للعينات الطينية والعينات التجارية التي كانت أقل من هيدروكسيباتيت الاصطناعي. وكانت كمية مسامية السطح في العينة المركبة أكثر من العينات التجارية وعينات الطعوم المغايرة. بالإضافة إلى ذلك، كانت كثافة هيدروكسيباتيت المُصنّع أقل من عينات الطعوم المغايرة والعينات التجارية. في مجموعة الطعوم المغايرة ومجموعة هيدروكسيباتيت التجارية، لوحظت كمية قليلة من التعظم من هوامش العظام الطبيعية في أربعة أسابيع. في المجموعة الاصطناعية، لوحظ عدم نضج تكوين العظام في أربعة أسابيع. كان معدل التعظم وتسلل الخلايا في عينات طعم أجنبي وعينات هيدروكسيباتيت التجارية أعلى عند ٨ أسابيع مقارنة مع ٤ أسابيع وكان هذا المعدل أقل من مجموعة هيدروكسيباتيت الاصطناعي. كان لمجموعة هيدروكسيباتيت المُصنّعة تحجر أكثر من الطعوم المغايرة، هيدروكسيباتيت تجاري ومجموعات تحكم في ١٢ أسبوعا.

الاستنتاجات: خلصت هذه الدراسة إلى أن هيدروكسيباتيت الاصطناعي كان له تأثير أفضل على تجديد العظام ويمكن استخدامه في هندسة أنسجة العظام.

الكلمات المفتاحية: هيدروكسيباتيت؛ الطعوم المغايرة؛ إصلاح؛ هندسة أنسجة العظام

Abstract

Objectives: Many patients suffer from non-repaired bone defects and subsequent aesthetic and psychological problems following bone fractures from accidents. The main goal of the study was to compare and evaluate synthetic hydroxyapatite with xenograft and commercial hydroxyapatite for bone repair and reconstruction.

Methods: In this study, synthetic hydroxyapatite was fabricated and verified. Cytotoxicity tests (i.e., induction coupled plasma [ICP], density and porosity analysis, scanning electron microscope [SEM] analysis, and thiazolyl blue tetrazolium blue [MTT] assay) were performed. Synthetic, xenograft, and commercial hydroxyapatite were tested in the animal study. Finally, bone regeneration was assessed using haematoxylin and eosin (H&E) staining.

Results: The Ca/P ratio was measured for xenograft and commercial samples, and values were lower than those for the synthesised hydroxyapatite. The amount of surface porosity in the synthesised sample was greater than in the commercial and xenograft samples. Additionally, the density of the synthesised hydroxyapatite was lower than that of the xenograft and commercial samples. A small amount of ossification from natural bone margins was observed at 4 weeks in the xenograft and commercial hydroxyapatite group. In the synthetic group, immature

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bone formation was observed at 4 weeks. The rate of ossification and cell infiltration in the xenograft and commercial hydroxyapatite samples was higher at 8 weeks than at 4 weeks, and this rate was lower than in the synthesised hydroxyapatite group. The synthesised hydroxyapatite group exhibited greater ossification than the xenograft and commercial hydroxyapatite, and control groups at 12 weeks.

Conclusion: This study showed that synthesised hydroxyapatite had better effects on bone regeneration and could be used in bone tissue engineering.

Keywords: Bone; Bone tissue engineering; Hydroxyapatite; Repair; Xenograft

Introduction

Currently, because of the high rate of accidents and fractures that lead to bone defects, the limited possibility of proper bone grafting, and the concomitant functional and psychological problems from these defects, researchers are attempting to discover new solutions for the repair of damaged bones.[1–3] Although bone fractures heal with orthopaedic and supportive treatments because of their high healing power, in many cases, the broken bone cannot heal, and patients may suffer problems, such as amputation and psychological problems.[4–6] Tissue engineering plays a very important role in science and technology, and consequently, has attracted many researchers who have conducted research worldwide, particularly in specific scientific and research communities. The purpose of tissue engineering is to restore and activate lost capabilities in dysfunctional parts of the body, which, if possible, repair damaged tissue with the implantation of new tissue. Tissue regeneration in humans could imitate that of other animals (e.g., planarians).[7,8]

The question is what factors are needed for tissue growth, such as bone and skin. This requires cells, cell–matrix interactions, cell–cell interactions, the extracellular matrix, and growth factors from a biological viewpoint.[9,10] Therefore, to regenerate and preserve tissue, a temporary replacement for cell proliferation and intercellular matrix formation is needed until new tissue is formed. The sample must also be able to form a suitable environment for the angiogenesis of new tissue. Therefore, a three-dimensional sample with suitable properties is a serious necessity in tissue engineering. Many natural and synthetic materials have been designed, and the construction of engineered samples and the reconstruction of many tissues have been studied. With sufficient control of sample ratios, desirable conditions for cell survival and proliferation, followed by tissue formation, can be achieved.[11–13]

Composite materials play a significant role in the construction and development of absorbable and bioactive samples.[14] Development of composite materials, especially in tissue engineering of cartilage and bone, using a combination of a degradable polymer phase, inorganic bioactive stage-like hydroxyapatite (HA), and bioactive glass or tricalcium phosphate for a significant function in sample construction has been considered.[15] The most popular type of calcium phosphate is HA. It forms the basic inorganic component in human bone tissue. Calcium and phosphorus separate from the surface of the material after implantation, and they are absorbed by body tissues. Next, they develop into new tissues.[16] HA is also commonly used as an artificial element for bone grafts. Consequently, it has inherent bioactive characteristics, which support osteoconduction when it is used for bone regeneration. Additionally, HA has high biocompatibility in regards to its composition, which resembles the apatite found in normal bone.[17] Porous samples of HA with high interconnectivity and porosity are often surveyed and used in fillers for skeletal deficiencies and tissue engineering of bone.[18] The main goal of this study was to compare and evaluate synthesised HA with xenograft and commercial HA in bone regeneration.

Method and Materials

Materials

Calcium chloride phosphoric acid and liquid ammonia were purchased from Merk, Germany. Tween 80 and ammonium hydrogen phosphate were purchased from Sigma–Aldrich, Germany. Ammonium hydrogen phosphate (from Reidel-de Haën), DMSO (dimethyl sulphoxide), PBS (phosphate buffer saline powder), FBS (foetal bovine serum), DMEM (Dulbecco's Modified Eagle's Medium), and MTT (thiazolyl blue tetrazolium blue) were purchased from Sigma–Aldrich, Germany. No further purification was conducted for any of the chemicals.

Synthesis of powder

This method was conducted as previously described,[19] with several minor modifications. Rapid stirring of the aqueous solution of calcium chloride (1.0 M) was conducted at room temperature 37 °C. 0.6 M phosphoric acid solution was added slowly to the solution of rapidly stirred calcium chloride. Tween 80 (5%) aqueous solution was added to the phosphate and calcium solution mixture, which was rapidly stirred. Next, we added the proper volume of ammonia solution (25% v/v) dropwise to the solution to hold the mixture pH at 11. The reaction was performed for 5 h (at 50 °C). Then, we placed the reaction mixture at room temperature to rest for another 20 h for completion of the reaction. Suspension centrifuging was conducted at 10,000 rpmg (for 15 min) using a table-top centrifuge. Moist powder was obtained from the sample of synthesised powder. The transfer of the solution to the oven (ATRA 50) was performed, and it remained in the oven for 4 h at 105 °C followed by an additional 4 h at 130 °C.

Centrifugation of the wet powder was conducted at 10,000 rpm for 5 min. After adding ethanol to the pellet, sonication of the suspension was accomplished for 20 min using an ultrasonicator and then dried in an oven at 100 °C for 3 h. Before sample analysis, calcination of the dried powder was performed for 3 h at 1000 °C.

Characterisation tests

Induction coupled plasma (ICP)

Chemical analysis of P and Ca contents were conducted using the quantitative ethylenediamine tetraacetic acid (EDTA) titration technique, ICP with an Agilent 7800 ICP-MS instrument, respectively, for calculating the Ca/P molar ratio of the precipitated powder. Sputtering (EMITECH K450X, England) was performed to thin-layer gold coat the powder sample,[20] and then a scanning electron microscope (SEM, Tescan Vega3) with an acceleration voltage of 20 kV was used to observe the microstructure of the powder sample.

SEM, density, and porosity analyses

An SEM (Amsterdam, Netherlands) was used to view and examine samples, including synthesised, xenograft, and commercial HA. The operation of the SEM was at 20 kV, and its working distance was 10 mm. Porosity and density were measured using the ASTM B311 standard. The density and porosity of the samples were determined by the Archimedes method according to ASTM.[21]

In vitro and in vivo analysis

MTT assay of xenograft, commercial, and synthesised HA samples

Analysis of the cytotoxicity of the samples mentioned above (synthesised, xenograft, and commercial HA) was performed by MTT cytotoxicity analysis (ISO 10993−5). Next, we incubated the HEK293 cells in 96-well sheets for a full day (24 h). This work was performed before the same amount of samples were added. The cultured cells without sample treatment were used as the control group. Discarding and replacing the medium were accomplished with MTT solution (5 mg/ml) after 24 and 48 h, respectively. Then, MTT solution was removed, and 0.1 ml isopropanol was added after 3 h to dissolve the formazan product. A microplate reader was used to monitor their absorbance at 570 nm. Finally, the findings were presented as a percentage (control = 100%). Assays were conducted in triplicate.

Animal study

In this study, 15 New Zealand white rabbits (male, 2.5−3 kg, 12 months old) were used and divided randomly into three groups (n = 5). They were maintained in different cages. The Animal Research's Ethical Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran approved this project (IR.BMSU.REC.1398.400). Each rabbit was kept in an isolated cage (total = 12 plastic cages) before and after surgery. The environmental conditions were standard (e.g., temperature and humidity) and on a 12/12 h light/dark cycle with food and water supplied ad libitum.

Implantation and surgical procedure

Each rabbit was intramuscularly injected with acepromazine maleate (0.5 mg/kg). Then, they received ketamine (5−8 mg/kg) accompanied by chlorbutol and atropine (0.05 mg/kg) intravenously after 15 min. The back of the orbit was shaved and rinsed with povidone iodine. Next, a 5 cm longitudinal incision was made in the parietal bone using a sharp scalpel. Four critical zone defects in the bone were created using a low-speed handpiece. Irrigation was conducted with a constant flow of saline using a trephine (8 mm diameter). In the next stage, four defects were made in parietal bones at both left and right sides over the top of the orbits.[22] Filling the first was done with synthesised HA. The second and third defects were filled with xenograft and commercial HA, respectively. The fourth remained depleted as a control group. Incisions were sutured, and we then applied topical tetracycline ointment to the surgical area. Afterward, each rabbit was intramuscularly injected with 0.1 mg/kg amoxicillin.

Radiographic analysis

For each rabbit, anteroposterior (frontal) digital radiographs were prepared from the calvaria at 4, 8, and 12 weeks. The exposure settings were 7 mA and 60 kV (Helsinki, Finland). The differences in radiopacity of images were evaluated to detect the formation of new bone in the bone defects of each group, including xenograft, commercial, and synthesised HA, and the control. Using Mimic software, we compared the radiographs based on differences in radiopacity in both the peripheral and central regions of the defects (Materialise NV, Leuven, Belgium).

H&E analysis

In weeks 4, 8, and 12, five rabbits were randomly sacrificed with an anaesthetic overdose after the operation, and then radiographic, histologic, and histomorphometric evaluations were performed. These assessments were analysed by removal of the rabbit calvaria using a bur (disc-shaped). The samples were sent to a laboratory for analyses. First, histological samples were immersed in 10% formalin for fixation. Next, samples were immersed in 10% nitric acid for a week (7 d) to decalcify them. Then, samples were embedded in paraffin, sectioned into 5 μm pieces, and used for H&E staining.[23−30] The cases were evaluated using a light microscope at × 100 magnification (Wetzlar, Germany) for the formation of new bone. Further, the amount of graft material and connective tissue were measured.

Histomorphometric analysis

After implantation, rabbits were sacrificed in the groups at 4, 8, and 12 weeks. Then, statistical analyses of the quantity of new bone, connective tissue, and remaining graft were performed in histological sections. Next, sections were

randomly selected from each group, including the xenograft, commercial, and synthesised HA, and the control. Then, a light microscope (Wetzlar, Germany) was used to observe each section at × 100 magnification. Image J software (Maryland, USA) was used for all histomorphometric analyses. New bone formation, connective tissue, and the remaining graft were expressed as a percentage.

Data analyses

The cases were assessed according to the percentage of newly formed bone, the remaining graft material, and connective tissue during histomorphometric analysis. SPSS, Inc. (SPSS version 20, IL, USA) software was used, and the Kruskal–Wallis test (non-parametric) was performed for all histomorphometric information. Differences were considered significant if the p-value was <0.05.

Results

Characterisation analysis

Table 1 shows the measurement results for the elemental mixture of P and Ca and the molar ratio of Ca/P. The results of synthesised, xenograft, and commercial HA samples showed that the bulk Ca/P molar proportion was 1.67, 1.64, and 1.43, respectively. The expected stoichiometric proportion for the pure HA phase was 1.667. In the xenograft powder and commercial samples, carbonate apatite was present locally, in which the molar Ca/P proportion could be increased to higher than 3.33.[31] Impurities, such as CaO was also present, which could be another reason for the increased Ca/P molar ratio. For these reasons, the ratio mentioned above was less in these two groups than in pure HA. SEM was used to analyse the prepared powder microstructure. Figure 1 shows synthesised powder SEM micrographs under two different magnifications.

Table 1: Ca and P content in the synthesised, xenograft, and commercial hydroxyapatite samples and the Ca/P molar proportion. Significance of differences ($p < 0.05$) was calculated.

Sample	Measured content [wt%] of Ca Element	Ca/P molar ratio
Synthesised	37.6	1.67
Xenograft	40.07	1.64
Commercial	37.03	1.43

Table 2: Density and superficial porosity content in the synthesised, xenograft, and commercial hydroxyapatite samples. Significance of differences ($p < 0.05$) was calculated.

Sample	Superficial Porosity (%)	Density (gr/cm^3)
Synthesised	88.5	2.55
Xenograft	82.4	2.92
Commercial	61.1	3.12

The morphologies of particles revealed small particles and gross agglomerates, including fine grains that were cold-welded to each other. Table 2 shows the results of density and porosity measurements. The amount of surface porosity in the synthesised sample was greater than that of the commercial and xenograft samples, and the density of the synthetic HA sample was lower than that of the xenograft and commercial HA samples. The MTT test revealed that all of the samples lacked any significant cytotoxicity (Figure 2).

Macroscopic and radiographic analysis

Macroscopic observations and histopathological examinations were performed to evaluate ossification. Macroscopic observations of the specimens at 4, 8, and 12 weeks indicated that the fibrosis scar covered the entire area of the defect in the cranial specimens. In the defect area of all specimens, there was no depression, inflammation, or infection. No tissue movement was observed in the repaired defects by xenograft, synthesised, or commercial HA, which were completely integrated with the peripheral tissue. The bound between the peripheral bone and the sample was difficult to distinguish. Macroscopic observations were performed in the control group that indicated the defect area was covered by a thin layer of connective tissue (Figure 3).

Figure 3 shows the radiological evaluation wherein the repaired defects in cranial specimens with the synthesised HA samples were better than the xenograft and commercial synthesis HA, and control group. Additionally, at week 12, the defects repaired with synthesised HA samples showed greater radiodensity and non-uniformity in the entire defect than in the other three groups. An increase in opacity indicates an increase in bone formation in the defect area.

Microscopic analysis

The observation of the prepared sections by H&E staining 4 weeks after the surgery in the control group showed that

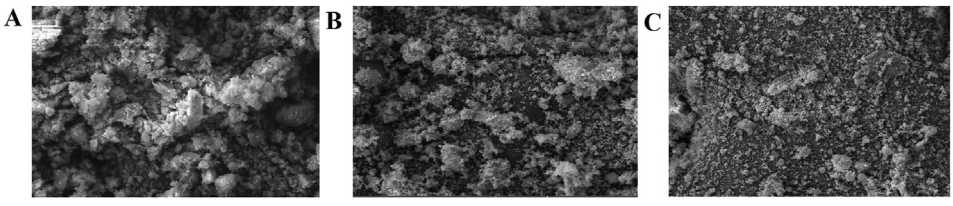

Figure 1: SEM of the (A): synthesised hydroxyapatite powder, (B): xenograft hydroxyapatite, and (C): commercial hydroxyapatite sample. (MAG: 1.00 kx, HV: 20.00 kv).

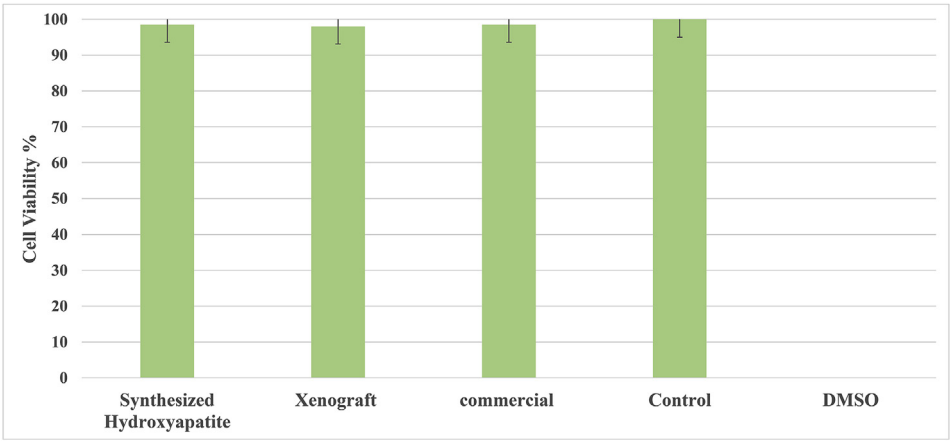

Figure 2: MTT test of samples. There was no indication of a noticeable difference among these groups. Control: Positive control, DMSO: Negative control.

the borders between the two ends of the bone were covered by narrow fibrous tissue and no observable ossification. After 4 weeks, the xenograft and commercial synthesis HA groups showed a small amount of ossification on the edges of normal bone, and the samples were covered with connective tissue. However, a significant infiltration of cells was not evident. In the synthesised sample, 4 weeks after surgery, the formation of immature bone was observed near the two edges of the sample. The formation of new bone was from the margin to the centre. Additionally, the presence of blood vessels and cell penetration into the sample were also observed. The rest was surrounded by connective tissue.

In the control sample, 8 weeks after surgery, the border between two edges of the bone was covered by narrow fibrous tissue, and immature bone formation from the borders was seen at 8 weeks and was not significant. At week 8 after surgery, the synthesised, xenograft, and commercial HA groups, cell penetration and bone formation were greater than at 4 weeks, and defects were covered with connective tissue. In the synthesised HA sample group,

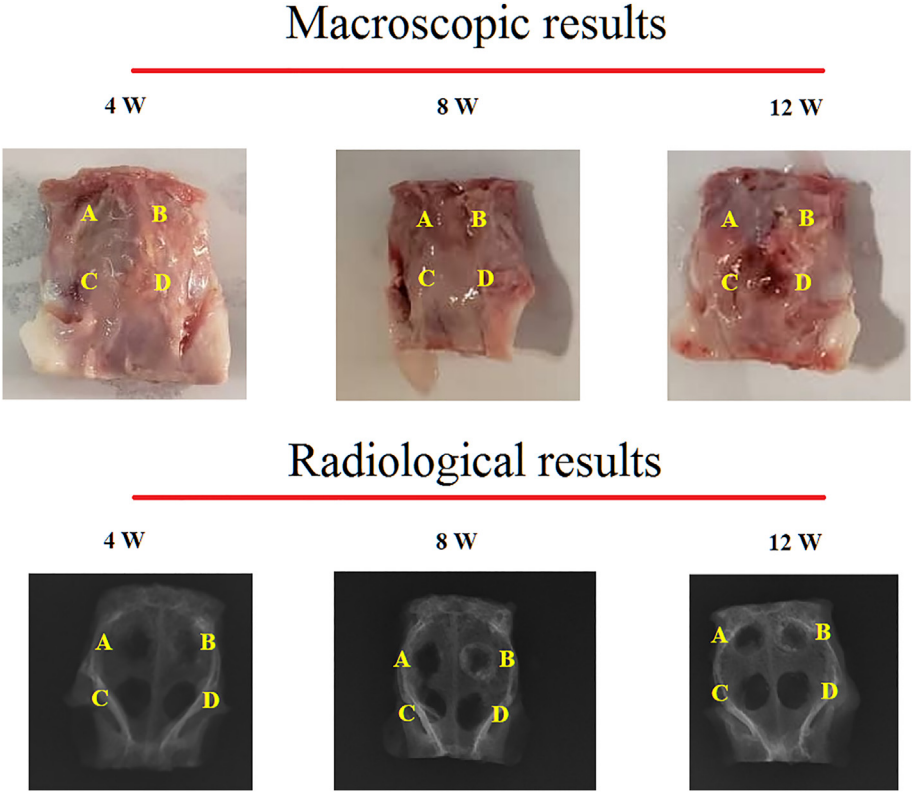

Figure 3: Macroscopic and radiological evaluations of defect repair by commercial synthesis (A), synthesised (B), and xenograft hydroxyapatite (C), and the control (D) group after 4, 8, and 12 weeks.

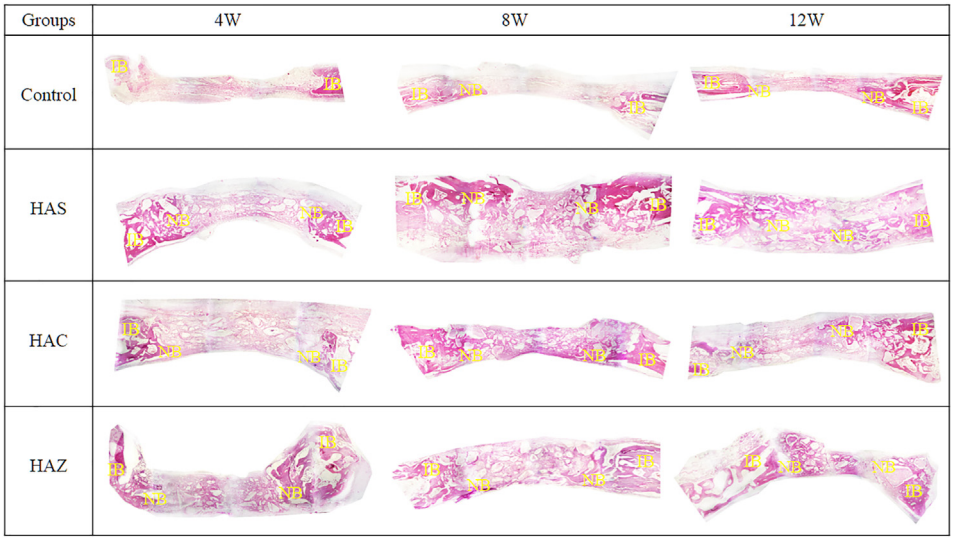

Figure 4: Microscopic evaluations of defect repair by synthesised, commercial (HAC), and xenograft hydroxyapatite (HAZ) samples, and the control group after 4, 8, and 12 weeks.

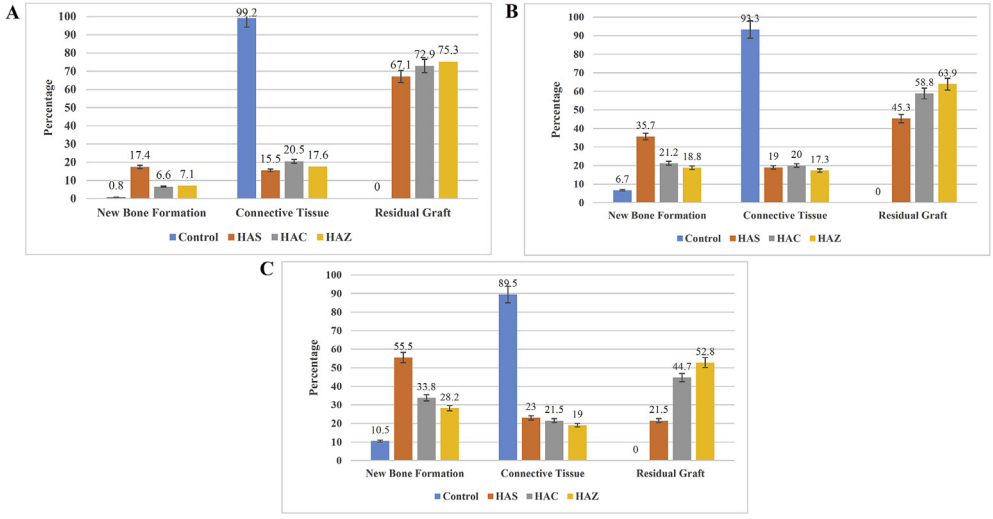

Figure 5: Results of histomorphometric examinations of repaired defects by synthesised, commercial (HAC), and xenograft hydroxyapatite (HAZ) samples, and the control group at 4 (A), 8 (B), and 12 (C) weeks. (New Bone Formation: the amount of newly formed bone, Residual Graft: the amount of the residual sample, Connective Tissue: the amount of connective tissue).

ossification was seen in the 8th week near the two ends of the sample, and its rate was higher than at 4 weeks. Additionally, the presence of cell penetration and blood vessels into the sample was observed, and new bone formation occurred from the margin to the centre. The remnants of the sample were surrounded by connective tissue that could be seen within the distance of the defect. In general, the rate of ossification at 8 weeks from the margin to the centre in the group of synthesised HA samples was higher than in that of the xenograft and commercial synthesis HA, and the control group. However, the rate of new bone formation in the commercial and synthetic HA samples was higher than that in the xenograft HA samples. However, the xenograft HA samples were higher than that in the control samples.

At 12 weeks, in the control sample, bone formation from the margin to the central area was low, and the filling of the cavity with connective tissue occurred. In the xenograft and commercial synthesis HA groups, at 12 weeks after surgery, the penetration of new bone in the centre was much lower than that of the synthesised HA sample but more than that in the same group at 8 weeks. This amount was most visible at week 12. The remainder of the samples was surrounded by connective tissue, which could be seen at a distance from the defect. New bone formation and cell infiltration in the synthesised HA group were significant at 12 weeks, and their rates were much higher than at 4 and 8 weeks. Additionally, its rate of ossification was much greater than in the xenograft and commercial synthesis HA groups. Furthermore, the rate of new bone formation in the commercial synthetic HA sample was higher than that of the xenograft HA sample. Additionally, that of the xenograft samples was higher than that of the control group. Histopathological

examination of skull specimens by H&E staining is shown in Figure 4.

Histomorphometric analysis

Figure 5 shows the histomorphometric results for the samples. The rate of new bone formation in the repaired samples in the synthesised synthetic, commercial synthetic, xenograft, and control group at week 4 was 17.4, 6.6, 7.1, and 0.8%, respectively.

The amount of new bone was greater in the repaired defects in the synthesised HA samples during weeks 8 and 12 (35.7 and 55.5%, respectively), which were significantly different than that of the repair process of other groups at the same time (P-value < 0.05). The amount in remaining samples in the synthesised HA group gradually decreased over time and was significantly different from that of other groups at weeks 4, 8, and 12.

Discussion

Bone injuries are one of the most common causes of morbidity and disability in elderly patients, and they can lead to decreases in general health and quality of life. Developing technology, lifestyle changes, immobility, sitting and standing in inappropriate positions, and sudden accidents cause weakness of muscles, especially around the spine. Fractures and serious injuries in this area can cause side effects on physical and mental health.[20,32–34] Bone grafts, which are used to rapidly restore the complete function of the affected area, are the most common treatment for autograft bone replacement.[1,32,35]

In this study, samples of the synthetic powder prepared by a chemical deposition method, xenograft powder prepared by combustion synthesis method, and commercial samples were compared. Experiments were performed to analyse chemical composition using the ICP method, conduct microstructural analysis using the SEM method, and evaluate the density and porosity of the samples using the Archimedes method. As mentioned before, the isometric Ca/P ratio for pure HA was 1667. Among the samples, only the Ca/P ratio of synthesised HA was close to this number, indicating that it approached pure HA. Microscopic images of the specimens showed that the specimens were lumpy and porous. Among all samples, density analysis revealed that the synthesised sample had the lowest density and highest porosity percentage. All results indicated that the synthesised sample contained pure HA with the highest percentage of porosity, and these properties accelerate ossification and bone cell growth during bone implantation. In this study, three sample groups, including synthesised, commercial, and xenograft HA, and the control group (unfilled) were placed in the cavities created (8 mm) in rabbit skulls and examined at 4, 8, and 12 weeks. The rabbits were humanely sacrificed, and their skulls were removed and sent to the laboratory for photography and histopathological tests. Macroscopic observations of samples at 4, 8, and 12 weeks showed that in the skull specimen, fibrous scars covered the entire area of the defect, and no inflammation, infection, or depression was observed in the defect area of any specimen. No tissue movement was observed in the defects repaired by xenograft, synthesised, and commercial synthesis HA groups in the repaired defects. Radiological evaluation showed that the repaired defects in the skull samples with synthesised HA were far more advanced than the xenograft and commercial synthesis HA, and control groups. Additionally, at week 12, the repaired defects in synthesised HA samples showed greater radiodensity in the entire defect area than did the other three groups. An increase in opacity indicated an increase in bone formation in the defect area. Calcium phosphate bioceramics are important for repairing bone owing to their structural resemblance to the bone mineral phase, such as HA.[36–38] Despite advances in the use of natural polymers and ceramics for bone replacement, no optimal approach has yet been reported.[39]

Recent studies have mostly been based on the use of composites with a combination of natural polymers such as fibrin, chitosan, and collagen, which are reinforced by ceramics, such as calcium phosphates or HA.[40,41] Xu et al. (2020) showed that the short fibres that contained BMP-2 were coated on HA samples using porous samples. They concluded that fibre coating is an efficacious method to improve both mechanical and osteogenic characteristics of HA samples.[18] Chi et al. (2020) produced the composite 3D-HA/BMSC-ECM (three dimensional-printed HA/bone marrow mesenchymal stem cell-extracellular matrix) samples using an inventive linking technique. The composite samples revealed good bone repair and osteogenic ability *in vivo* that greater than 3D-HA samples alone.[16] Veremeev et al. (2020) evaluated the rate of bone repair using different bovine biomaterials, including purified bone collagen [COLL], demineralised bone matrix [DBM], and refined HA) in comparison with accessible commercial bone autografts and bone minerals. They suggested that DBM and HA powder from bovine bones were both efficient in the repair of bone defects and showed acceptable potential to be utilized in clinical surveys.[42]

In this study, the xenograft and commercial synthesis HA groups showed a small quantity of ossification at the edges of natural bone at 4 weeks. However, samples were covered with connective tissue, whereas bone and significant cell infiltration were not evident. In the synthesised sample at 4 weeks after the sample placement in the skull cavity, the formation of immature bone was observed on the edges near the two ends of the sample, in the vicinity of healthy bone. The formation of new bone occurred from the margin to the centre, and the presence of blood vessels and cell penetration into the sample was also observed. At week 8 post-surgery, the synthesised, xenograft, and commercial synthesis HA groups, had cell penetration and bone formation were greater than at 4 weeks. The remainder of the sample was covered with connective tissue. In the synthesised HA sample groups, ossification was observed at the 8th week near the two ends of the sample, and the rate was greater than at 4 weeks in this group. The presence of cell infiltration and blood vessels in the sample was also observed. The rate of ossification at 8 weeks from the margin to the centre in the synthesised HA group was higher than that of the xenograft HA, commercial synthesis HA, and control groups. In the xenograft and commercial synthesis HA groups at 12 weeks after sample placement in the skull cavity, the penetration of bone blades to the centre was much lower than that of the synthesised HA sample but greater than that at 8 weeks for

the same group. The amount had greater visibility at week 12. Other parts of samples were surrounded by connective tissue, which could be observed at a distance from the defect. New bone formation and cell infiltration in the synthesised HA group were significant at 12 weeks, and rates were much higher than at 4 and 8 weeks.

Conclusion

In this study, three samples, including synthesised, commercial, and xenograft HA, were investigated, and the synthesised type of HA exhibited significantly greater results in macroscopic and microscopic tests. We concluded that synthesised HA could be used as a suitable candidate in clinical treatments for bone regeneration (e.g., orthopaedics and dentistry).

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The experimental protocol was approved by the Animal Research part of Baqiyatallah University of Medical Sciences, Tehran, Iran (IR.BMSU.REC.1398.400), 2019.

Authors' contributions

The authors attest that this study was conducted by the authors whose names are on this article. AR, HH, AR, SK, and KK were involved in the investigation (methodology), design of the study, data collection, and writing of the original draft. AR, HH, SK, and KK were involved in writing the review section of this article and critically reviewed the data. All authors have critically reviewed and accepted the final draft of this article and are responsible for the content and similarity index of the article.

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