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Workflow-Friendly Bone Paste: Hydroxyapatite and Decellularized Bone Powder in Starch for Efficient Socket Preparation

Maryam Sadeghi
Isfahan Payam Noor University
Tahereh Talaie-Khozani
Shiraz University of Medical Science
Farzaneh Bozorg-Ghalati
Shiraz University of Medical Sciences
Shahram Ahmadi
Payam Noor University,Department of Science, Tehran, Iran
Maryam Asadi
reihaneasadi@yahoo.com

Shiraz University of Medical Science

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Maryam Sadeghi, Department of Biochemistry, Faculty of Basic Sciences, Isfahan Payam Noor University, Isfahan, Iran

Email: mmsadeghi290@gmail.com, +989391140927

Tahereh Talaie-Khozani, Morphometry and Stereology Research Center, Shiraz University of Medical Science, Shiraz, Iran, talaeitkh@gmail.com, +989171177285
Farzaneh Bozorg-Ghalati, Clinical Biochemistry Department, Medical School, Shiraz University of Medical Sciences, Shiraz, Fars, Iran, fbozorgghalati@yahoo.com, +989177011191
Shahram Ahmadi, Assistant Prof., Department of Biology, Payam Noor University (PNU), Tehran, Iran, sh.ahmadi@pnu.ac.ir, +989177429656
Correspondence: Maryam Asadi, Department of Molecular Medicine, School of Advanced Medical Sciences and Technologies, Shiraz, Iran . Department of Human Genetics, Iranian Academic Center for Education, Culture and Research (ACECR), Fars Branch, Shiraz, Iran.

Email: reihaneasadi@yahoo.com

Running title: Fabrication of bone-like composite

Abstract

Purpose: Successful implantation requires a suitable bone substrate, which can be provided by the bone extracellular matrix (ECM). This research aims to develop a cost-effective and user-friendly bone-like composite for filling dental sockets after tooth extraction, which is intended to reduce the waiting period prior to dental implants.
Methods : Bovine pelvic bone was defatted and decellularized to serve as a source of bone ECM. Mineral hydroxyapatite (HA) was also extracted from bovine bone. To confirm successful decellularization and ECM retention, DNA quantification, H&E, Masson's trichrome, PAS, and colloidal iron staining were performed. X-ray diffraction (XRD) verified HA extraction. To fabricate the bone substitute, decellularized bone powder (organic part) was combined with HA (inorganic part) and mixed in crosslinking starch. Biocompatibility was assessed using both indirect and direct MTT assays. The degradation rate of the composite material was also evaluated.

Results: Microscopic images and DNA quantification revealed cell removal from decellularized tissue, while preserving the main part of ECM, including collagen, neutral sugars, and glycosaminoglycans. X-ray diffraction also confirmed that the extracted inorganic material was HA. Analysis of scanning electron micrographs by ImageJ showed that the average particle size of HA was 3.28 ± 2.56 µm, the median pore size was 75.5 nm ± 6.24, and the porosity was 76.8% ± 2.99. Moreover, cell viability tests showed that the fabricated moldable bone substitute was non-toxic.

Conclusion: This study demonstrates a cost-effective, user-friendly bone-like composite, combining decellularized bone ECM with hydroxyapatite and starch, that supports cell viability and can potentially be used for post-extraction socket augmentation.

Key words: Tissue Engineering, Bone, Hydroxyapatite, Extracellular Matrix

1. introduction

The loss of permanent teeth is one of the most significant causes of alveolar bone loss in humans and has numerous side effects on a patient's quality of life (1). Evidence shows that most people start losing their teeth in their late twenties, and nearly 23% of people after age 60 have complete tooth loss (2).

When a tooth is lost due to aging, injury, or disease, the individual may experience complications such as rapid alveolar bone loss, impaired speech, or changes in chewing patterns (3). One method for replacing lost teeth is the use of dental implants (4). The placement of implants requires suitable infrastructure (5), and the assessment of alveolar

bone quantity and quality is crucial to ensure the success rate of dental implant treatment (6).

Although dental implant success depends on various factors, one of the essential factors is the complete osseointegration of the bone and the implant (7). Unfortunately, in many individuals who need dental implants, the jawbone may have deteriorated due to various reasons, including aging, the presence of cysts or trauma, bone or skeletal deficiencies, periodontal disease, craniofacial surgery, infections, congenital abnormalities, oral cancer, and more. In such cases, insufficient alveolar bone density and height may lead to failed tooth implantation (8, 9). In such situations, the dentist must improve the jawbone infrastructure before performing the implant. Filling the tooth socket can be done using several methods. Autologous bone grafts are considered the gold standard for preparing the alveolar bone infrastructure (9). The use of decellularized scaffolds from human or animal sources (10) has been suggested as bone substitutes.

Applying engineering principles and biological sciences is a novel approach for repairing natural body tissues (11). In tissue engineering, a suitable biomimetic scaffold is essential for host cell migration. Scaffolds must provide a microenvironment surrounding the cells and support cell adhesion, colonization, proliferation, and migration (12). Tissue engineering consists of a scaffold or biodegradable carrier, cells, and signals for cellular recruitment (13). A suitable porous architecture, sufficient porosity, and interconnections between the pores of a scaffold provide an environment to promote cell infiltration, migration, vascularization, nutrient and oxygen flow, and removal of waste materials, while being able to withstand external loading stresses (14). The porous scaffold begins to degrade within the body, while the migrated host cells within the structure continue to grow and produce new matrix until eventually, new bone tissue is formed.

Starch is one of the most common natural polymers used for constructing scaffolds in tissue engineering (15). Starch is composed of carbohydrates and has unique properties, including a hydrophilic structure, biodegradability, low cost, and non-toxicity (16). Although starch-based composites in bone tissue engineering have some limitations, such as low mechanical strength, difficulty in scaffold fabrication, and long-term stability issues, they increase the likelihood of success due to their ability to be chemically modified with cross-linking agents, which enhances their mechanical strength (15). Starch-based composites have been suggested as an appropriate ECM to promote bone regeneration (17, 18) that improves osteogenic differentiation of the loaded mesenchymal stem cells (19). Autologous bone grafts can damage the donor site, making xenogenic bone tissue a preferable alternative. Decellularized tissues retain essential ECM components necessary for functional tissue regeneration (20). Studies have shown that decellularized bone can accelerate repair (20). Both decellularized scaffolds and hydroxyapatite are inorganic ECM components exhibiting osteogenic properties (21, 22). Although obtaining human

decellularized ECM and hydroxyapatite is challenging, xenogenic sources are increasingly used. The FDA has approved the clinical application of decellularized scaffolds, with bovine bone commonly employed in orthopedics (23). Bovine bone is currently used in orthopedic treatment. Decellularization effectively removes xenogenic antigens, reducing the risk of immunological rejection (23). In the current study, we used a starch-based paste with decellularized bone powder and hydroxyapatite extracted from bovine bone to fabricate an easy-to-use bone substitute for dental application.

2. Material and method

2.1 Bone preparation

The pelvic bone from a young bovine was used to prepare the decellularized powder and extract HA. Bovine pelvic bone was obtained from Fars Livestock Slaughterhouse, a licensed slaughterhouse, as a by-product of routine meat production. The animal was slaughtered for human consumption under halal standards and was not sacrificed for research purposes. No anaesthesia or euthanasia was performed for research purposes.

The steps included crushing, fat removal, demineralization, decellularization, and the removal of residual detergents.

To remove fat, the crushed bones were placed in 5% hydrogen peroxide for about 60 to 90 minutes with regular shaking, and then they were incubated in diethyl ether for 48 hours. To demineralize the bone, all samples were placed in 10% nitric acid solution for about 8 days. To decellularize the bone, they were crushed and treated with different percentages of SDS and Triton X-100 as depicted in Table 1. Finally, all samples were washed with PBS. In the next step, it was crucial to remove residual solvents and dry the samples. Therefore, the powder was added at a ratio of 1:40 to double-distilled water and kept for 1 hour at 25°C to 30°C. Then, it was washed 5 times and kept overnight in double-distilled water. Finally, all samples were dried overnight at 40°C in an oven. They were then sterilized for 15 minutes with UV light.

Table 1: Different decellularization procedures

Procedures :	Step 1	Step 2	Step 3
Procedure1 :	SDS 1% - 24 h	Triton 3%- 4h	Wash with PBS
Procedure2 :	SDS 1% - 48 h	Triton 3%- 4h	Wash with PBS
Procedure3 :	SDS 0.5% - 48 h	Triton 3%- 4h	Wash with PBS
Procedure4 :	SDS 1.5% - 48 h	Triton 3%- 8h	Wash with PBS

Procedure5 :	SDS 1% - 72 h	Triton 3%- 24h	Wash with PBS
Procedure 6: intact	No treating	No treating	No treating

2.2 Hydroxyapatite (HA) extraction

First, the pelvic bone was broken down into smaller pieces. To remove excess tissues, they were washed multiple times and finally boiled in water for 2 to 3 hours. Then, the bone fragments were defatted by incubating them in 5% hydrogen peroxide (H_2O_2). To ensure H_2O_2 penetrated all crevices and spaces within the tissue, the samples were immersed in ethanol for 24 hours. Afterward, the samples were heated at 100°C overnight. Finally, the bone tissue was calcined in a furnace at 450°C for 6 hours. The powder was passed through a 500 µm sieve.

2.2.1 Scanning electron microscopy (SEM)

To measure the particle size of hydroxyapatite, 1 g of hydroxyapatite was dissolved in 1 mL of PBS 1X and dispersed by ultrasonic bath (SnorexDigitec) at 60°C for 20 minutes. Then, a small drop of the dispersed hydroxyapatite solution was placed on a 1 cm × 1 cm slide and left to dry at room temperature for 3 to 4 days. Then, gold coating was performed using a Q150R-ES sputter coater (Quorum Technologies, UK). Electron micrographs were taken using a VEGA electron microscope (TESCAN, Czech Republic) at 10 kV.

2.2.2 X-ray Diffraction (XRD)

To verify the purity of the extracted hydroxyapatite, a small amount of the sample was carefully mounted onto the sample holder of the XRD instrument. The diffraction pattern was obtained using a Bruker D8 Advance operated at an accelerating voltage of 40 kV and a current of 40 mA. Data were collected over a 2θ angular range of 5° to 75°, with a scanning speed of 0.07°/min. The diffraction patterns were then analyzed to identify characteristic peaks and confirm the phase composition of the material. The data were compared with XRD data from commercial HA. The size of the HA crystal was evaluated by ImageJ software (http://mac.softpedia.com/get/Graphics/ImageJ.shtml).

2.3 Decellularized bone characteristics

To characterize the decellularized tissues, they were first fixed in 10% formalin. The samples were dehydrated, cleared, and embedded in paraffin using a tissue processor (Didsabz DS200). The slides were stained with H&E, Masson's trichrome, colloidal iron, and periodic acid-Schiff (PAS) staining.

2.3.1 DNA Quantification

46 µg of each sample was placed in 2 mL microtubes and immersed in 400 µL alkaline lysis solution containing 0.5 M NaOH. The tubes were vigorously agitated by a vortex mixer for about 1 minute. Then, 20 µL proteinase K (10 mg/mL) was added, and the tubes were incubated at 70°C. Samples were vortexed gently after 5 minutes and incubated for about 160 minutes to complete the lysis process. The pH was adjusted using 100 µL of Tris-HCl solution (pH = 8.3) at a final concentration of 100 mM. The mixtures were centrifuged at 14,000 rpm for 2 minutes. The supernatants were transferred to a new tube and diluted with 250 µL absolute cold ethanol for 2 minutes. The obtained suspension was completely transferred to a spin filter column and centrifuged at 12,000 rpm for 1 minute. The columns were washed with 80% ethanol and centrifuged at 12,000 rpm for 1 minute. The washing step was repeated twice with absolute ethanol. Finally, the DNA was eluted in 100 µL elution buffer. The DNA quantity was evaluated by a microvolume spectrophotometer (Thermo Scientific, NanoDrop 1C, USA) and stored at −20°C.

2.4 Preparation of a bone-like composite (paste)

To prepare a bone-like composite, a starch-based scaffold was first fabricated as follows. Unmodified granular edible corn starch, consisting of 30% w/w amylose and 70% w/w amylopectin, was prepared by Glucozan Company (Iran) (24). 3% starch was prepared in distilled water by heating in a water bath at 90°C for 20 to 30 minutes. During this time, the tube was repeatedly shaken to prevent starch sedimentation.

Next, the solution was cooled to 65°C, mixed with 15% glycerol, and shaken thoroughly. Subsequently, the temperature was raised again to 90°C, and 30% citric acid was added to the mixture, which was kept in the water bath for 20 minutes (25, 26).

Then, the pH was adjusted to 7 by adding 10x NaOH. Finally, 20% decellularized bone powder and 20% HA were added to the mixture, and the mixture was shaken thoroughly. Before use, the paste-like mixture was exposed to air for 48 hours. The procedure is summarized in Figure 1.

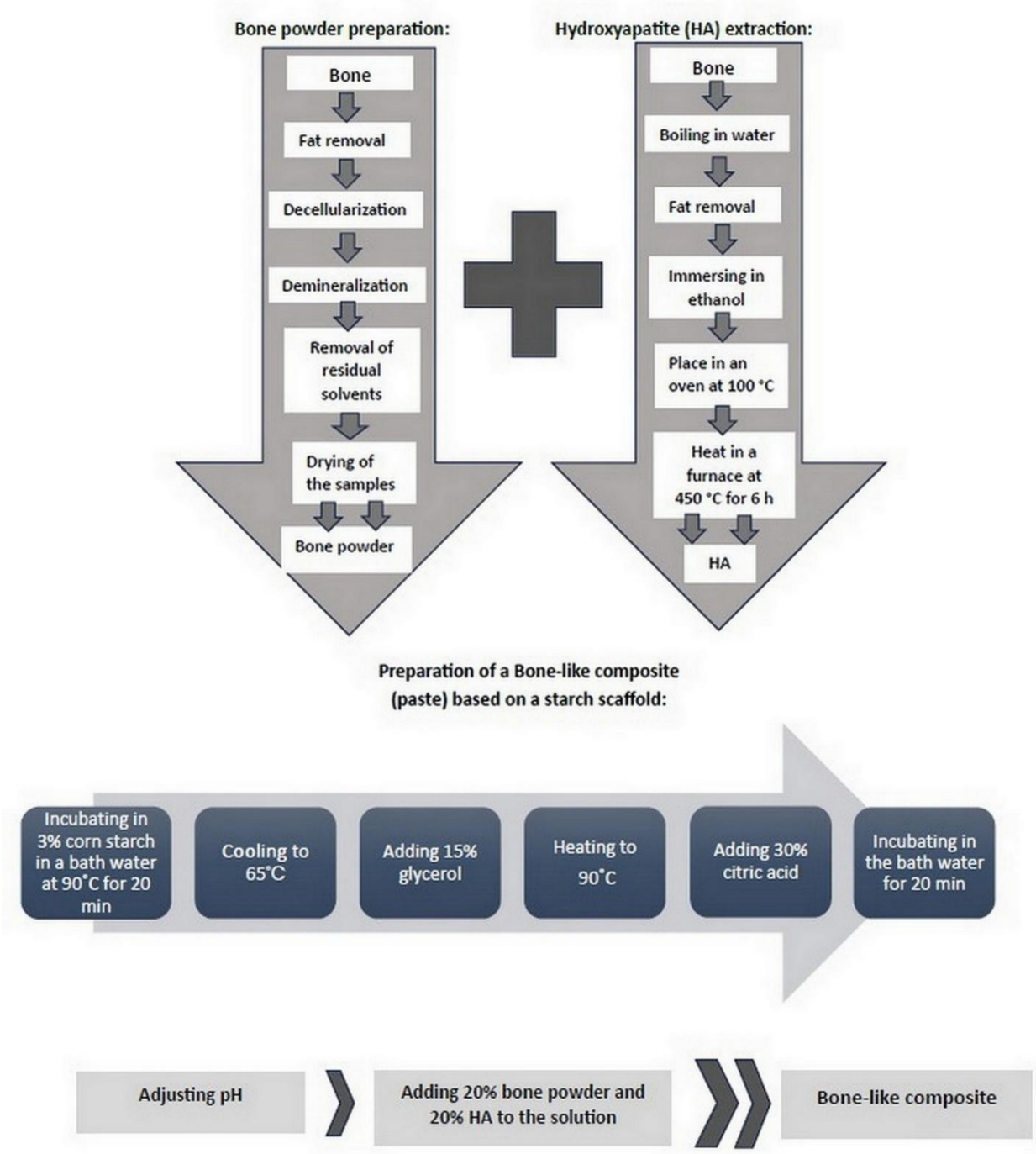


Figure 1

Schematic depiction of bone-like composite preparation

2.5 Evaluation of the Properties of Bone-Like Composite

Pore size and porosity percentage were evaluated by analyzing the SEM micrographs based on the aforementioned procedure.The images were analyzed using ImageJ software.

2.6 Degradation test

To determine the biodegradability, the bone-like composite, along with the same amount of appropriate controls (Table 2), was placed in a 4 cm diameter plate, and 4 mL of 1X PBS was added. The test was performed under humidified conditions at 37°C. Every day, the scaffolds were weighed.

Table 2 : Different groups of Degradation Test and their ingredients

Groups	Ingredients
Group 1	Starch 3%, Glycerol 15%, Citric acid 30%
Group 2	Starch 3%, Glycerol 15%, Citric acid 30 %, Bone powder
Group 3	Starch 3%, Glycerol 15%, Citric acid 30%, HA
Group 4	Starch 3%, Glycerol 15%, Citric acid 30%, Bone powder, HA

Invitro tests

2.7 Cell adhesion test

The MG63 osteoblast cell line was initially obtained from the Pasteur Institute, Iran. They were cultured in DMEM/F12 (Shellmax) containing 10% fetal bovine serum (FBS, Biosera) and 1% penicillin/streptomycin (Biosera).

Cell seeding at a density of 1×10^4 was done for 1 hour. Then, the culture media was removed, and the number of unattached cells was counted. The number of unattached cells was subtracted from the initial number of the seeded cells.

2.8 Indirect MTT assay

To evaluate the toxicity of the scaffold's ingredients, an indirect MTT assay was performed. Initially, an equal amount of scaffold ingredients, including HA, bone powder, starch, or citric acid and NaOH, was added to DMEM/F12 containing 1% penicillin/streptomycin and 20% FBS. First, four groups—bone powder, hydroxyapatite, starch, and a combination of citric acid and NaOH—were used in equal amounts to coat the bottom of a well. They were then incubated for 24 hours in DMEM/F12 medium. Then, the supernatant was removed and diluted with fresh culture medium at 11%, 22.5%, and 45%. The mixture was added to osteoblast cultures at a density of 1×10^4 cells for 24 hours. After that, the MTT assay was performed by adding MTT for 4 hours. The MTT was replaced with DMSO, and the amount of eluted formazan was evaluated by measuring optical density at 570 nm wavelength.

2.9 Direct MTT assay

The cells were seeded at a density of 1×10^4 cells/well of a pre-coated 24-well culture dish with the same concentrations of the aforementioned ingredients for 24, 48, and 72 hours. Each experiment was performed in triplicate. The osmolarity was checked and optimized (315 mOsm, (27)) after adding the ingredients. The optimal osmolarity supports normal cellular functions, metabolic activity, and overall viability. Cells were cultured on the culture dish without any pre-coating, which was considered a control. To enable comparison of results, the same number of cells were also cultured on a piece of untreated bone, and an MTT test was performed.

Due to the high water solubility of starch and controlled osmolarity, the pre-coated plates were incubated in an oven at 37°C for 24 hours.

3.Results

3.1 Gross and histological analysis

Gross examination of the decellularized bone showed a white, soft powder. Histological analysis of the samples decellularized with various methods revealed that nuclei were still clearly present in samples treated with 1% SDS for 24 hours and 0.5% SDS followed by incubation in Triton for 4 hours. Increasing the SDS concentration to 1.5% and

extending the exposure time, along with immersion in Triton for up to 8 hours, led to appropriate cell removal. However, since lower detergent concentration and shorter exposure time minimize the washing out of ECM components, the optimal condition was achieved using 1% SDS for 48 hours and Triton for 4 hours.

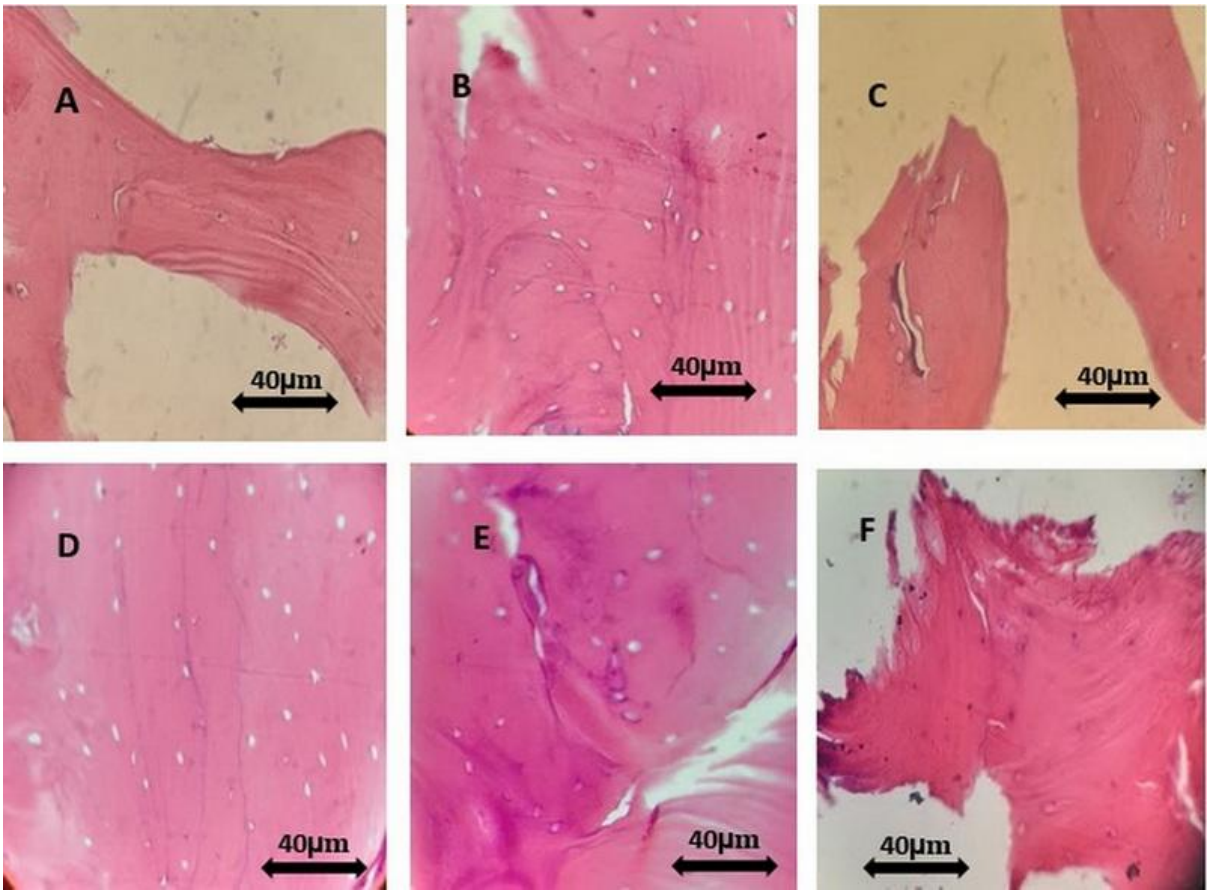


Figure 2

Histological sections of the bones decellularized with various methods and stained with H&E. The representative samples were treated with SDS 1% for 24 hours and 3% Triton for 4 hours (A), 1% SDS for 48 hours and 3% Triton for 4 hours (B), 0.5% SDS for 48 hours and 3% Triton for 4 hours (C), 1.5% SDS for 48 hours and 3% Triton for 8 hours (D), SDS 1% for 72 hours and 3% Triton for 24 hours (E), and the intact untreated bone section (F). The presence of nuclei in images A and C is clearly visible, which does not confirm effective decellularization. In the other groups, the empty lacunae indicate appropriate cell removal.

Histological examination indicates that the effective methods of bone decellularization are procedures 2, 4, and 5 (Fig 2). While procedure 2 involves a shorter processing time and lower detergent consumption, we used this method to obtain decellularized bone powder for bone-like paste fabrication. Consequently, ECM retention of the decellularized tissues

was evaluated in the samples prepared with procedures 2 and 4 by different histochemical staining and compared with the intact tissue, as shown in Fig 3.

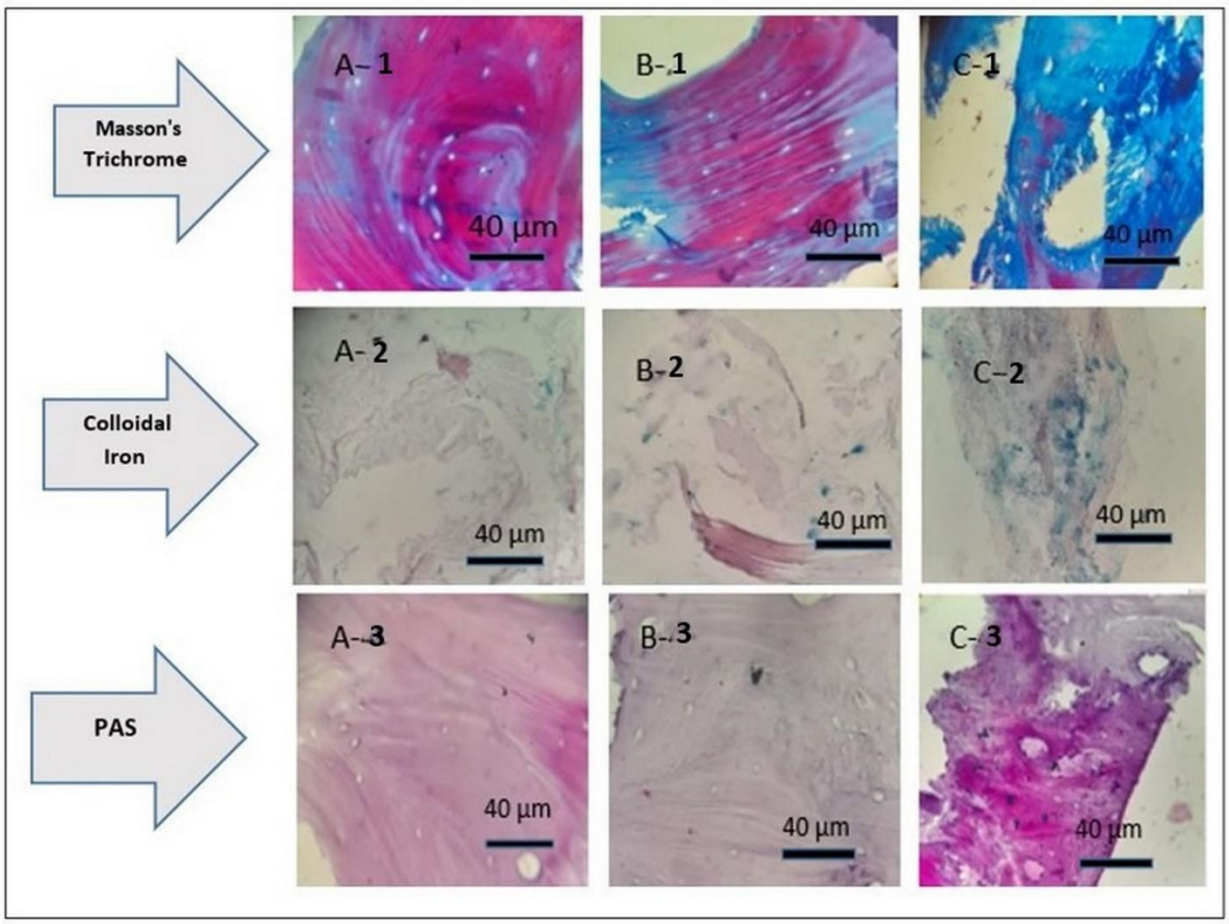


Figure 3

The histochemical staining of the decellularized bone with 1% SDS for 48 hours and 3% Triton for 4 hours (A1–3) and 1.5% SDS for 48 hours and 3% Triton for 8 hours (B1–3) showed partial washing of ECM in both samples when they were compared with matched staining of intact bone (C1–3). However, superior ECM preservation was observed in the samples treated with 1% SDS for 48 hours and 3% Triton for 4 hours. Masson's trichrome, colloidal iron, and PAS staining detect collagen, glycosaminoglycans, and neutral carbohydrate content of ECM, respectively.

Masson's trichrome, colloidal iron, and PAS staining demonstrated that collagen, glycosaminoglycans, and neutral carbohydrates were partially preserved in the decellularized scaffold; however, some degree of unavoidable ECM washing occurred due to the detergent and washing procedures.

3.2 DNA Quantification

DNA quantification serves as an indicator of proper decellularization. Fig. 4 compares DNA levels in samples 2 and 4 with intact bone. The permissible limit for DNA content in decellularized tissue should be less than 50 ng per milligram (28).

The DNA content of both decellularization procedures was lower than the allowable amount, and both procedures were statistically similar in terms of DNA residue removal.

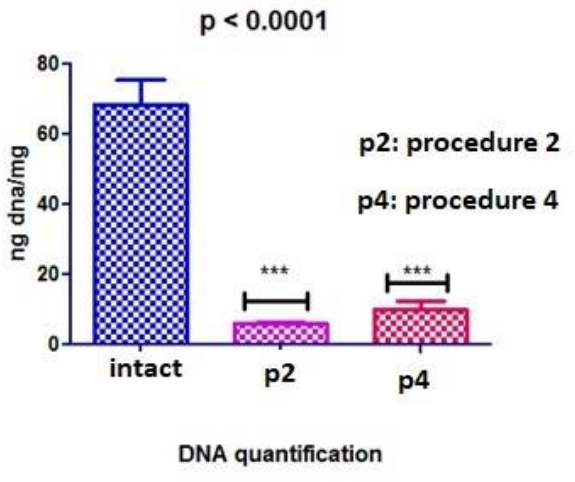


Figure 4

Comparing DNA quantification of the decellularized bones with intact tissue revealed that both procedures were effective in DNA removal. This graph compares the DNA levels in the intact bone and the two effective decellularized samples (2 and 4). ***Significant difference compared with intact bone ($P < 0.001$).

3.3 XRD (X-ray diffraction)

The XRD spectrum of purified HA from bovine pelvic bone was compared with that of

commercial HA (Sigma). The diagrams indicate that the purification method of HA was accurate, and the resulting spectrum matches the commercial sample's pattern (Fig 5).

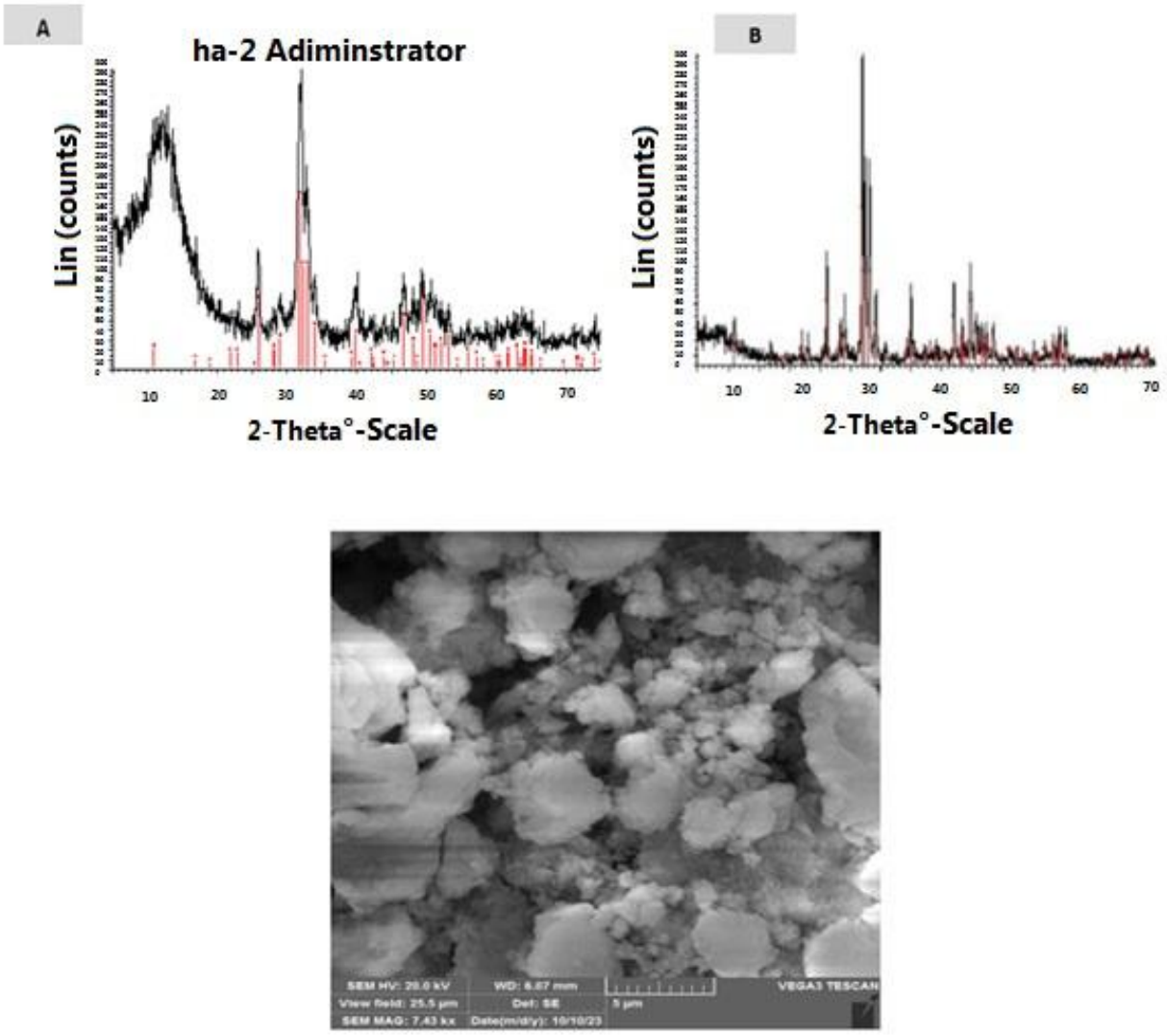


Figure 5

XRD spectrum of HA extracted from bovine pelvic bone (A) and commercial source (B). Comparing these two diagrams confirms the validity of the HA purification.

The electron micrograph shows the structure of HA extracted from bovine pelvic bone. Over 50 granules were measured to determine their size, providing a detailed view of the particle morphology and distribution.

3.4 Scanning electron microscopy

The size of the extracted HA particles was analyzed using electron microscopy images with ImageJ software. After calibrating the measurements and analyzing over 50 different granules, the average particle size was found to be 3.28 ± 2.56 μm (with three diameter measurements taken for each granule).

SEM micrographs from the fabricated paste showed the surface morphology, particle distribution, and microstructure. The images likely reveal interconnected porosity, mineral deposits, and fibrous and biomimetic features that mimic the architecture of real bone, indicating potentially good integration of the composite.

The average porosity was estimated as 76.8% ± 2.99. Observations indicated that the pore sizes were not uniform. While the average pore size has been reported as 3.25 ± 20.15 μm, some pores were found to exceed 100 μm in diameter; at the same time, micropores were abundantly observed. The median pore size was 75.5 nm ± 6.24, which indicated that at least half of the pores have a pore size of more than 75.5 nm (Fig. 6).

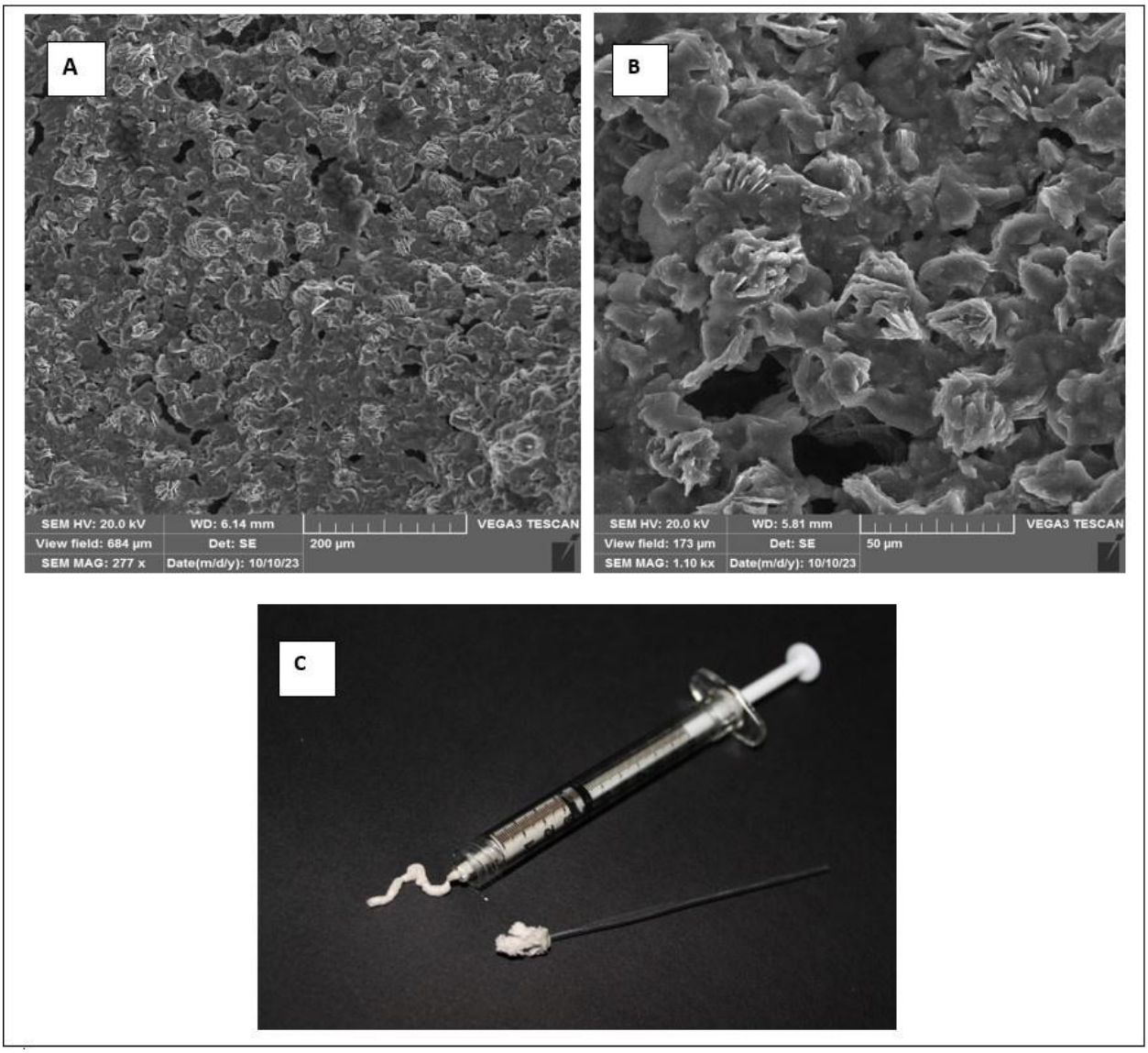


Figure 6

The ultrastructure and porosity of a bone-like composite. A: SEM image (bone-like composite) at 200 µm magnification. B: SEM image at 50 µm magnification. C: bone-like composite. It can be shaped rather than forming a rigid structure. Pore sizes were examined, and the average was 3.25 ± 20.15 µm. The average porosity was 76.8% ± 2.99.

3.5. Cell adhesion test

The ability of cell adhesion to the bone-like composite was evaluated and compared with 2D conventional culture conditions in polyester culture dishes.

The data showed that cell adhesion to these 3D scaffolds was statistically similar to that in 2D conventional conditions on the polyester discs (Fig 7).

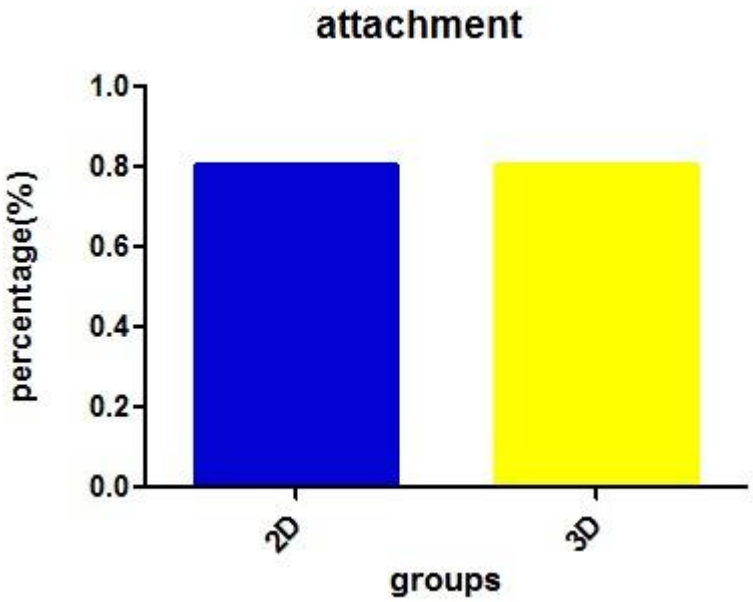


Figure 7

Cell adhesion in two-dimensional culture (polyester dishes) and bone-like composite wassimilar.

3.6 Cytotoxicity assessments

The indirect MTT test showed a significant difference between the groups ($P < 0.001$). Regardless of the concentrations, adding bone powder, HA, and starch significantly increased cell viability (for all, $P < 0.001$); in contrast, citric acid and NaOH, which were added as crosslinkers, had no significant impact on cell viability. The most effective supplemented ingredient was starch, since adding it to the culture media led to a significant increase in cell viability compared to those exposed to the same concentration of bone powder ($P < 0.001$) and HA ($P = 0.022$ for 11% and $P < 0.001$ for the other concentrations). Bone powder and HA showed statistically the same impact on cell viability. The data indicated that cell viability was not dose-dependent (Fig. 8).

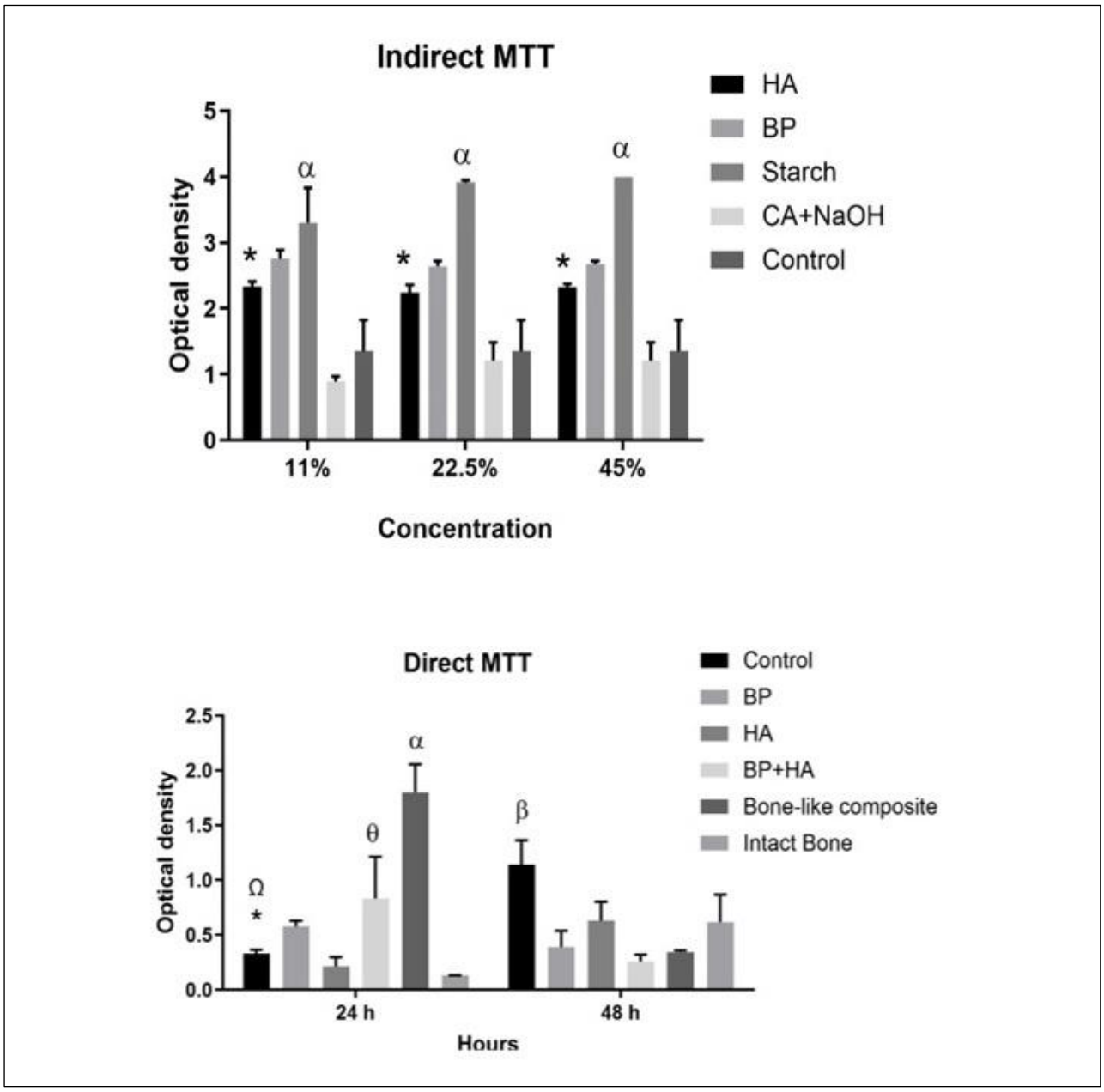


Figure 8

In the indirect MTT assay, cells were incubated for 24 hours. Then, the supernatant was removed and diluted with fresh culture medium at 11%, 22.5%, and 45%. Results showed that cell viability was higher in the group exposed to starch compared to all the other groups ($P < 0.00001$). The presence of hydroxyapatite and bone powder also elevated cell viability, while the crosslinker had

no statistically significant impact on cell viability. *Significant difference compared to the HA, BP, and starch groups ($P < 0.001$). Significant difference with BP and HA groups ($P < 0.001$).

Direct cytotoxicity assessment showed that the scaffolds were not toxic to the cells and supported cellular proliferation in a short time. *Significant difference with BP+HA and bone-like composite at 24 hours ($P < 0.001$). αSignificant difference with all other groups at 24 hours ($P < 0.001$). βSignificant difference with all other groups at 48 hours ($P < 0.05$). θ Significant difference with bone-like composite and intact groups ($P < 0.001$). Significant difference with the matched group at 48 hours ($P < 0.001$).

Direct MTT

At 24 hours of culturing, the cell viability of the control cultures was statistically similar to the cultures on the scaffolds fabricated from HA ($P = 0.430$) or bone powder ($P = 0.107$). The highest cell viability was detected for the cells cultured on the bone-like composite (for all, $P < 0.001$). The cells cultured on the scaffolds fabricated from the mixture of bone powder and HA ($P = 0.002$), as well as the bone-like composite ($P < 0.001$), showed significantly higher cell viability compared to the control culture. The cells cultured on the bone-like composite also showed significantly higher cell viability compared to those exposed to either bone powder or HA (for both, $P < 0.001$). After 48 hours, all culture conditions significantly decreased cell viability compared to the control condition ($P < 0.001$ for all except bone powder and HA, $P = 0.048$). The cells cultured on the bone-like composite showed a significant reduction in cell viability compared to the corresponding group at 48 hours ($P < 0.001$), which indicated stopping of cell growth under this condition. The number of cells cultured on the HA and intact bone increased significantly compared to the matched conditions at 48 hours ($P = 0.009$ and $P = 0.003$, respectively; Fig 8).

3.7. Degradation assay

The degradation assay indicated that the bone-like composite exhibits sufficient integrity in the initial days, supporting its potential use as an implant material. The increase in scaffold weight on the first day can be attributed to water absorption due to its porosity. Degradation remained statistically constant during the first 10 days, after which biodegradation progressed, such that by day 20 the scaffold had degraded completely (Fig 9).

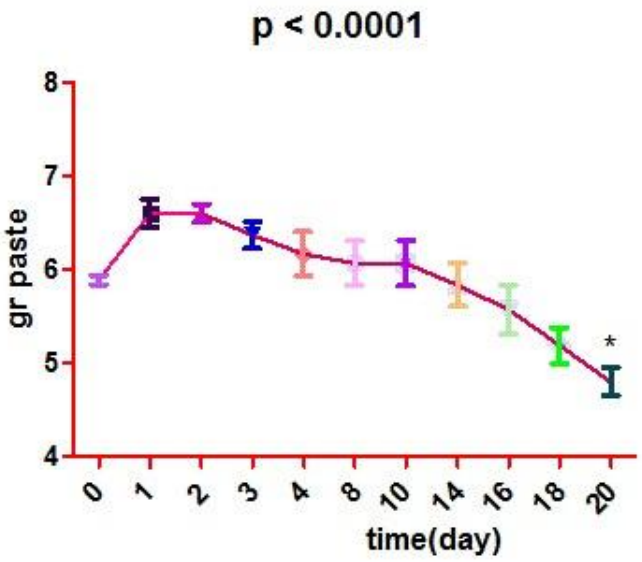


Figure 9

The degradation assay indicated that the scaffolds remained stable for 10 days, and after that, they started degrading until the 20th day, when they were completely destroyed.

Discussion:

The present study focuses on developing a substitute material for accelerating alveolar repair to prepare the dental socket for implantation. Bone grafts made of natural or synthetic materials implanted at the site of a bone defect not only replace lost bone but also support bone regeneration (28, 29). Bovine bone xenografts (BBX) are widely used for promoting bone regeneration in dental applications due to their unique characteristics, such as wide availability from donors and low production costs compared to autografts and allografts (30, 31). BBX has been thoroughly tested in both preclinical and clinical studies, with strong evidence supporting its effectiveness in maxillary sinus floor augmentation, guided bone regeneration, and alveolar ridge preservation (32). Since tissue-specific extracellular matrix enhances the functional performance of tissue-specific cells in vitro (33), in this study, bovine bone was used to harvest the ECM. Decellularized bone ECM, which contains bone-specific ingredients such as osteoinductive and osteoconductive factors and cytokines, is effective in osteogenesis and can be used as a main component for bone regeneration (34, 35). The removal of cells and the washing out of residual cellular debris are among the most important steps in obtaining a

decellularized matrix. SDS and Triton X-100 were used to find the most effective and cost-efficient method for complete cell removal. Studies have shown that proper removal of cellular antigens is carried out by SDS and Triton X-100 detergents (36-38). The current study demonstrated effective decellularization using 1% SDS for 48 hours and 3% Triton for 4 hours. This procedure offers the shortest processing time and minimal detergent use, making it suitable for generating bone-derived powder for paste fabrication.

Histochemistry revealed that key ECM components—such as collagen fibers, neutral carbohydrates, and acidic glycosaminoglycans—were well preserved, indicating the quality of the decellularized matrix. These preserved components are crucial for bone tissue regeneration (39). Both collagen and glycosaminoglycans increase osteogenesis capacity in mesenchymal stem cells (40). Collagen also increases cell adhesion (41), and our data also show that cells can attach to the scaffold properly. The findings suggest that the resulting composite has the potential to reduce preparation time for bone socket procedures, facilitating quicker patient readiness for dental implants.

Hydroxyapatite is the principal mineral in bone, playing a vital role in bone regeneration and biological functions (42). Calcium phosphate (CaP) materials are especially important for hard tissue reconstruction, thanks to their excellent biocompatibility, bioactivity, non-immunogenicity, non-toxicity, and non-inflammatory properties (43, 44). As a key component of natural bone, it elevates local Ca^{2+} levels, which can stimulate osteoblast proliferation and enhance the growth and differentiation of mesenchymal stem cell (45-47). The MTT results obtained from this study showed that the presence of hydroxyapatite particles in the fabricated scaffold was not only non-toxic but also significantly enhanced osteoblast proliferation. This finding indicates that osteoblasts exhibit a particular affinity for growing in proximity to HA particles.

Starch, as a vehicle, is a promising option due to its safety and compatibility with the body (48). In line with our data, studies show that starch/HA scaffolds are non-toxic to mammalian cells. Adding starch improves scaffold flexibility and moldability by forming a network structure that interacts with HA nanoparticles, enhancing the scaffold's mechanical strength through adhesive forces (25, 49, 50). Research has shown that three-dimensional porous structures with appropriate porosity facilitate the three-dimensional growth of cells (51). This study also confirms that starch-based composites provide good integrity and a platform for cell attachment. Crosslinking is a commonly used method to modify the biodegradability of starch polymers. Adding citric acid enhances thermal stability, water resistance, and fluidity by forming strong hydrogen bonds with starch. Initially, it depolymerizes starch granules in the presence of water and glycerol, which increases its flexibility (24). This study used a starch-based scaffold crosslinked with citric acid and glycerol to enhance strength, flexibility, and stability. Results showed that starch significantly protects osteoblast growth, especially in the first 24 hours. It may indicate

that osteoblasts can use starch as an energy source to support their proliferation (52). Besides, the incorporation of starch not only provided the necessary plasticity for clinical handling but also enhanced the scaffold's hydrophilicity, which is crucial for protein adsorption and early-stage cell attachment in the alveolar socket.

Starch-based biomaterials have also been used for bone healing. The composite of starch and polycaprolactone showed osteoconductive properties in an animal model (17, 53). A recent study demonstrated that the starch-HA composite enhanced osteoblast proliferation and bone marker expression in the tooth socket of alveolar bone in a rat model (54). Starch was also used for fabricating an injectable composite for bone healing (55). A combination of starch and chitosan is printable, which is suitable for drug delivery and enhancement of osteogenesis (56). All these studies have shown that starch can be a proper base for fabricating different forms of bone-inducing scaffolds. In the current study, we also showed that starch is appropriate for fabricating a moldable composite in the form of a paste that is more user-friendly than previous products.

The scaffold designed in this study has adequate porosity to support nutrient flow, waste removal, and cell growth. Its three-dimensional, porous structure may potentially promote cell migration and attachment, as confirmed by SEM images. Pore size can be divided into two different groups: microporous (<5 μm pores) and macroporous (>100 μm pores) (57, 58). The median pore size of the fabricated moldable bone-like composite was 75.5 nm ± 6.24, which indicated that it has both macro- and micropores. In vivo bone regeneration involves recruitment and penetration of cells from the surrounding bone tissue, as well as vascularization into the transplanted bone substitutes. Higher porosity is expected to enhance osteogenesis, and numerous studies have verified this hypothesis. These results were likely due to the larger surface area, which resulted in higher ion exchange and bone-inducing factor adsorption (59-61).

The average pore size that supports osteoblast proliferation ranges between 20 and 300 μm (62)(62). Although the bone-like composite designed in this study does not possess the ideal mean value of pore size, its paste-like and non-fixed structure allows for variable porosity. Besides, the median pore size showed that it has a large enough pore size to be used for vasculogenesis and osteoblast migration. While the micropores can be used for nutrient diffusion, they decelerate the fast degradation of the bone-like composite. Since larger pore size reduces the strength of bone substitutes as well, there should be a balance between enhancing osteogenesis and mechanical strength for each artificial bone substitute.

Conclusion

The findings of this study demonstrate that a decellularized bone matrix combined with HA integrated into crosslinked starch was non-toxic and provided a platform for osteoblast attachment. Furthermore, the development of a moldable bone-like composite using a starch-based formulation enhances the ease of application and improves user-friendliness. This moldable paste can conform to the shape of the tooth socket. Since this type of product is available in various forms within the dental market, we recommend producing and offering this essential item as a paste.

Authors declarations

Human Ethics : Not applicable

This study did not involve live animal experimentation. The bovine bone used was obtained as a by-product from a licensed slaughterhouse where animals were slaughtered for human consumption. As no animals were sacrificed for the purpose of this study and only discarded animal by-products were utilized, formal ethics approval was not required. All procedures complied with institutional, national, and international guidelines for the use of animal by-products in research.

Consent to participate : We, as all contributing authors, provide our informed consent to take part in this study.

Consent for publication: Not Applicable

All authors hereby provide their informed and voluntary cosent for the publication of the manuscript.

Data Availability : The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors contributions

M.S. was involved in conceptualization, data collection, and original draft preparation; T.T.K. was involved in experimental design, data interpretation, and the main revision of the manuscript; F.B.G.H. was involved in data interpretation; S.A. was involved in supervision of the project; and M.A. was involved in conceptualization, in experimental design, funding acquisition, data interpretation, and revising the manuscript.

Declaration

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Since this project conducted using a cell line, obtaining an ethics code was not required.

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