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Original Article

Influence of L-arginine on hydroxyapatite-based ovine bone graft - An *in vitro* evaluation of surface characteristics and cell viability

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ABSTRACT

Background: Current challenges in bone grafting revolve around the limited availability of autografts and the complications associated with their use. Promising alternatives include osteoinductive substances stimulating stem cells to mature into bone-forming osteoblasts. However, existing products lack optimal characteristics of a bone graft. The study aimed to evaluate the impact of L-arginine treatment on hydroxyapatite (HA) derived from ovine bone and compare its surface and mechanical properties to that of the commercially available xenograft-Bio-Oss.

Materials and Methods: The research was structured as an *in vitro* investigation, wherein HA was formulated from ovine bone. The sintering process involved heating it to 360°C for 3 h and adding the amino acid L-arginine. Different tests were done which included scanning electron microscopy (SEM), X-ray diffractometry, Fourier-transform infrared spectroscopy (FTIR), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The goal was to compare these results with a commercially available bone graft called BioOss, especially regarding their physical and chemical characteristics. Data were analyzed in SPSS software using one way ANOVA test, significant level at 0.05.

Results: A bone graft made of HA and L-arginine displayed a complex and interconnected pore structure, indicating that the sintering process had a significant impact. SEM confirmed this. FTIR analysis identified peaks at 650–700 cm^{-1} and 1000–1100 cm^{-1}, confirming the presence of HA and L-arginine. X-ray Diffraction assessments also confirmed the existence of both substances in the sintered specimens, supporting their suitability for various biomedical applications.

Conclusion: The study presents a novel approach, deproteinizing a bone graft followed by sintering at 360°C with L-arginine. Physicochemical analyses confirmed desired mechanical attributes and surface characteristics. Further investigations are needed to evaluate cellular adherence, immunological response, and osteogenesis in relevant animal models.

Key Words: Autograft, BioOss, hyalouronic acid, hydroxyapatite, L-arginine, osseondutivity, ovine bone graft

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INTRODUCTION

Bones possess remarkable self-repairing capabilities, efficiently healing fractures or disruptions through natural processes. However, severe bone traumas resulting from trauma, neoplastic growths, any pathological or inherent malformations present significant therapeutic challenges beyond the reach of traditional interventions.[1] As a result, the search for effective bone regeneration techniques remains a top priority in the fields of orthopedics and reconstructive surgery.[2] A bone graft is a material or tissue that helps regenerate bone when placed in a bony defect, either alone or combined with other materials. The use of bone grafts and alternatives has been a common practice in medicine for many centuries.[3] In 1682, historical records show the first use of bone grafts when a defect in the skull was successfully reconstructed using a graft from a deceased dog's skull. According to the US Food and Drug Administration guidelines, bone grafts are classified into Class II devices for replenishing skeletal defects and Class III devices that include grafts with pharmaceutical agents. In dentistry, bone graft and substitute materials are mainly categorized based on either their tissue origin or their composition.[4] Nonetheless, materials for bone grafts can be broadly classified into natural bone graft and substitute materials, synthetic bone alternatives, substitutes enriched with viable osteogenic cells, growth factor-centric bone alternatives, and composite bone replacements.[2] While autografts have long been considered the gold standard in bone grafting, showcasing exceptional effectiveness, they are not without challenges. The primary issue revolves around the limited availability of suitable bone donor sites, which is further compounded by the associated morbidity linked to the donor site.[5] These challenges underscore the pressing need for innovative interventions that can match or even surpass the effectiveness of autografts.

Osteoinductive agents have a unique ability to stimulate the maturation of native stem cells into osteoblasts, the key cells responsible for bone tissue formation.[6] This natural ability makes them top candidates for improving bone revitalization efforts. By guiding stem cells into the osteogenic pathway, these substances effectively start the complex process of bone repair, ensuring faster and superior bone regeneration.[7] Despite the widespread use of bone graft and substitute materials, there are ongoing challenges associated with the current options. The primary constraints revolve around allografts, which involve transferring grafting components between genetically different individuals, and autografts which require moving grafting substances from one anatomical site to another within an individual.[8] These practices inevitably increase the risks of complications at the donor site, greater morbidity, anatomical distortion, and noticeable scarring, often requiring additional surgical interventions.[9] Currently available products in the marketplace do not encompass all the ideal attributes for optimal bone replacement material. These attributes include minimal patient distress, easy manipulation, reduced immunological reactivity, cost-effectiveness, and the ability to stimulate vascular growth.[10] Autografts face challenges due to limited graft material, discomfort, donor site issues, and the need for two surgeries. Allografts have limitations related to possible tissue rejection and concerns about infectious diseases. Despite limited research on indications and safety profiles, there is a noticeable increase in efforts to develop innovative bone grafting materials, especially bone graft substitutes.[11] With an increasing demand for bone grafts, there is a clear need for thorough exploration and innovation in creating new materials with ideal qualities for various bone grafting methods.

This study aimed to assess the impact of L-arginine on hydroxyapatite (HA) from ovine bone and compare its properties with a commercial bone graft material (Bio-Oss). The main objective is to gather insights on the osteoinductive properties of bone graft materials and strive for innovative alternatives that match the effectiveness of autogenous bone grafts, recognized as the gold standard in bone restoration. Null hypothesis is framed as there is no difference between prepared novel bone graft with L-arginine and existing commercially available Bio-Oss in terms of physical and osteoconductive properties.

MATERIALS AND METHODS

Study design

The present *in vitro* study was carried out at Saveetha Dental College and Hospitals, Chennai, India. The study was approved by the Scientific Review Board approval number-(SRB/SDC/PROSTHO-2105/22/011).

Preparation of bone graft and isolating hydroxyapatite

Bone contains both organic and inorganic components. The organic part comprises of lipids and

proteins, while the inorganic part includes compounds such as calcium carbonate, HA, calcium oxides, and phosphates. Hence, the first step in obtaining inorganic HA is to remove the organic components. The preparatory procedure involved the extraction of the organic constituents: segments of the ovine femur's central region were sectioned into substantial slices, with any residual soft-tissue meticulously excised. Subsequently, to remove the intrinsic fluids, including marrow and soft tissues, the bone slices were subjected to a boiling regimen at 100°C for a duration spanning 12 h. Subsequent to the process of cleaning, the specimens were stored at a temperature of 4°C for an interval of 12 h, after which they were subjected to a thermal desiccation phase in a hot air oven set at 100°C, spanning another 12-h duration. Leftover soft tissue and unnecessary material from the samples were carefully removed. Subsequent chemical procedures aimed at the eradication of lipids, adipose content, and proteins through thermal extraction technique. Following a 12-h immersion phase, the specimens underwent a meticulous ethanol rinse to eliminate residual organic solvents. Subsequent to this ethanol cleansing, the samples were subjected to a thermal desiccation process in a hot air oven, maintained at 100°C, for an additional 12-h period. To thoroughly remove organic materials, 150 mL of Ethylene diamine (Cat. No: 70790, Sisco Research Laboratories Pvt. Ltd., Mumbai, India) was used as an extra solvent (HME 250, Labquest, Mumbai, India). This procedure was also conducted over 48-h duration. The specimens underwent dual ethanol washes [Figure 1]. Following the chemical treatment regimen, the samples were subjected to a sintering process, achieving a temperature of 320°C, sustained for a duration of 3 h (Aadarsh Technologies, Mumbai, India).[12] 173 g of L-Arginine was added to 20 g of this prepared HA-based bone graft material. Hyaluronic acid was used as a binder (Himedia, Chennai, India).

Surface characterization

Scanning electron microscopy

Scanning electron microscopy (SEM) serves as the modality for evaluating parameters such as particle dimensions, structural morphology, and surface characteristics. The samples were measured to ensure optimal placement and stability on the specimen stage subsequent to this, the granular entities underwent a sequential purification process involving acetone rinsing, deionization using distilled water, followed by air-drying phase to eliminate any residual moisture content. Utilizing a high-energy electron beam, SEM (Hitachi S-3400N, New Delhi, India) imagery of the specimen was procured. When electrons interacted with the sample, it caused the release of secondary electrons, electron back-scattering, and unique X-ray emissions. These signals were captured by detectors, and subsequently comprehensive visual representations were created. The surface intricacies of the samples were observed at 1 μm and 5 μm. JSM–IT800 NANO SEM apparatus (JEOL Benelux in the Netherlands), was used. Subsequent data analysis was done using the Image J software to ascertain the mean particle dimensions inferred from the SEM images.

X-ray diffractometry

X-ray diffraction (XRD) spectroscopy was used to study the mineralogical phases and the degree of crystalline structure in the samples from both the experimental and control groups. The finely ground samples were fixed onto the analytical holder, and the analysis was done using a D8 Advance Diffractometer (Bruker AXS GmbH, Karlsruhe, Germany), spanning a 2 θ angular range extending from 10° to 80°. X-ray emissions originated predominantly from a cathode ray tube, subsequently undergoing filtration processes to yield monochromatic radiation directed at the specimens. This resulted in generation of diffracted rays, which were captured, analyzed, and quantified. The X-ray wavelength was meticulously calibrated to approximate the bond distances inherent between atoms within the crystalline lattice, thereby producing a distinctive pattern reflective of the atomic configuration within the positioned samples. Subsequent analysis compared these findings with established benchmarks to ascertain the phase consistency and crystalline integrity. The resultant data sets were graphically represented using the origin software platform.

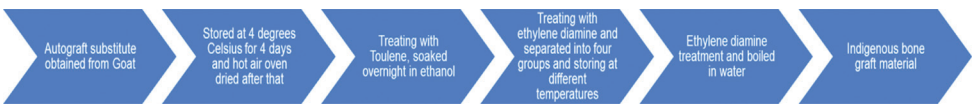

Figure 1: Preparation of hydroxyapatite-based bone graft.

Fourier-transform infrared spectroscopy

The sample was exposed to infrared radiation, and the resulting absorbance spectrum is obtained using a Fourier Transform Infrared Spectroscope (ALPHA II Compact Bruker, Billerica, Massachusetts). Key settings include spectral resolution and scanning range. The mechanism involves measuring the interferogram, from which the spectrum is derived, offering insights into molecular composition.

Cell viability

The samples were crushed and made into fine powder to assess for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay was carried out to assess cell viability and proliferation (MTT cell growth assay kit, Sisco Research Laboratories, Mumbai, India). In the 6-well plate, 1 mL of complete culture medium per well was added. Next, 0.5 mg/mL MTT was added to the bottom well. The plate was then incubated at 37°C for 4 h. After incubation, the culture medium was aspirated from the insert well, and the resulting formazan crystals were eventually solubilized by adding 100 µL of dimethyl sulfoxide (DMSO) solution per well. Finally, 100 µL of the colored DMSO was transferred from each insert and each well to a new 96-well plate to quantify cell viability. Absorbance at 450 nm was measured using a microplate reader.

Percentage cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = (A_{450\ \text{od of sample}}/A_{450\ \text{od of control}}) \times 100$$

Bone formation assay (alizarin red staining)

In a differentiation medium containing DMEMF12, 10 mM B-glycerophosphate, 0.05 mM ascorbic acid, and magnesium nanoparticles, the MG63 cells, osteoclast cells, were grown for 14 days using an osteogenesis kit (catalog N 5533-25G, Sigma-Aldrich, Burlington, United States). Calcium deposition was identified using alizarin red staining. The cells were once more stained with 2% alizarin red solution (catalog N 5533-25G, Sigma-Aldrich, Burlington, United States) for 10 min after 2 weeks. The cells were subsequently given two washes in 1X PBS. Each well received 200 µL of DMSO for quantitative analysis, which was incubated for 1 h. Using a spectrophotometer, the amount of alizarin was determined at 405 nm.

Statistical analysis

All experiments were performed in at least triplicates. The results are presented as mean ± standard deviation (SD) statistical analysis was done using SPSS software (Version 26.0; SPSS, Inc., Chicago, IL, USA). The errors of each data point were obtained from a minimum of three independent replicate tests, from which the mean and SDs were calculated. Comparative measurements between independent data sets were performed using one way ANOVA, significant level at 0.05.

RESULTS

HA-based bone graft samples, enhanced with L-Arginine, were examined alongside the commercial bone graft product BioOss. The analysis was done using SEM, XRD, Fourier-transform infrared spectroscopy (FTIR), and MTT assay.

Micromorphological analysis

The SEM examination of BioOss revealed a cohesive matrix characterized by closely interwoven particles with bioactive surface coating. The synthesized specimens exhibited a distinct pore arrangement coupled with a compact structure, showcasing a meticulously delineated surface topography when compared with BioOss. SEM assessments revealed a meticulously organized porosity, underscoring the synthesis of HA originating from ovine osseous material. In addition, the SEM images showcased a notably sleek surface texture accentuated by strong interparticle cohesion, further emphasizing the compact L-Arginine and HA matrix [Figure 2].

X-ray diffractometry

Upon analysis of the XRD data, distinct crystalline phases of HA with the composition Ca4.854 (H0.126(PO4) 3) (OH) 0.834) and calcium oxide phosphate with the formula CA5(PO4) 3O0.5) were detected, accompanied by a presence of silica, L-arginine. Graphically, pronounced peaks of elevated intensity were seen at an approximate angle of 42.5o, 43.7o and 49.4o [Figure 3]. The graphical representation of BioOss similarly exhibited three prominent peaks, indicative of the crystalline manifestation of HA. These peaks spanned an angular range from 34o to 40o, mirroring the patterns observed in HA-based L-arginine-modified bone graft specimens. Based on the XRD findings, the crystalline characteristics of HA-based L-arginine-modified bone graft samples closely aligned with those of the commercially accessible xenograft, BioOss.

Fourier-transform infrared spectroscopy

HA is identified by the presence of phosphate and carbonate groups. In FTIR analysis, peaks at

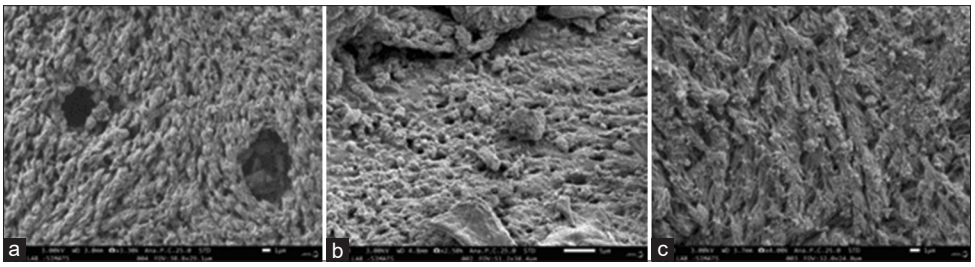

Figure 2: Scanning electron microscopy analysis. (a) Hydroxyapatite-based bone graft modified with L-arginine (Scale at 1 μm), (b) Hydroxyapatite-based bone graft (Scale at 5 μm), (c) BioOss (Scale at 1 μm).

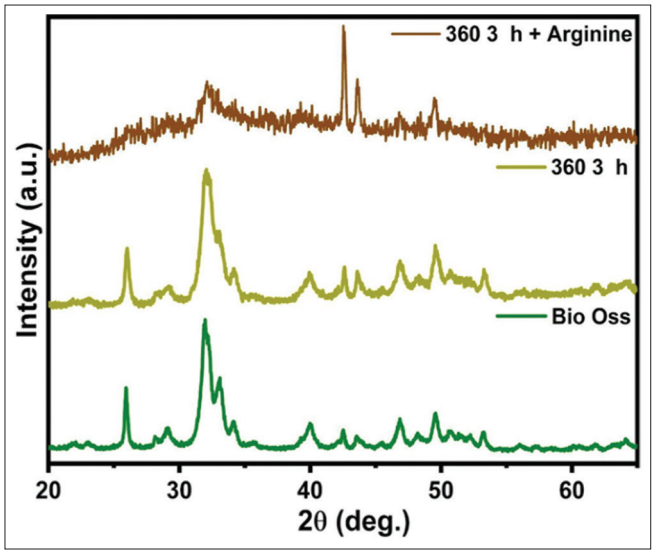

Figure 3: X-ray diffractometry analysis showing peaks of elevated intensity and confirming the presence of hydroxyapatite in commercially available bone graft-BioOss, ovine bone graft processed at 3600 C and Ovine bone graft + Arginine.

900–1200 cm^{-1} and 1400–1500 cm^{-1} confirm these groups, affirming the presence of HA. Samples showed bands in this range, confirming HA presence. An additional peak at 3200 cm^{-1} indicated L-arginine presence, suggesting its binding and dispersion in the HA matrix [Figure 4].

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

When considering the results of the MTT assay, all the samples showed biocompatibility and growth of MG-63 cells at 24 h [Figures 5 and 6].

Bone formation assay (alizarin red staining)

HA-based L-arginine-modified bone graft showed an increased amount of aggregation of the MG-63 cells after a period of 24 h at 25 and 50 μg/mL concentration of bone graft. This can greatly affect and also enhance the osteogenic differentiation potential [Figure 7].

DISCUSSION

Within the confines of this research, the influence of L-arginine on HA sourced from ovine osseous material was meticulously explored. The synthesized HA specimens were compared with commercially available BioOss using advanced analytical modalities including SEM, XRD assessments, FTIR and MTT assay. The SEM examination of BioOss revealed a cohesive matrix with closely interwoven particles and bioactive surface coating. SEM assessments showed distinct pore arrangement and compact structure, with meticulously delineated surface topography. XRD analysis detected crystalline phases of HA and calcium oxide phosphate, aligning with BioOss peaks. FTIR confirmed HA presence with characteristic bands, and L-arginine binding indicated by a peak at 3200 cm^{-1}. MTT assay indicated biocompatibility and MG-63 cell growth, with enhanced cell aggregation at 25 and 50 μg/mL concentrations of HA-based L-arginine-modified bone graft, potentially enhancing osteogenic differentiation potential. The results indicated similar characteristics as that of BioOss, supporting the potential use of L-arginine-modified HA bone graft in clinical scenarios after thorough and meticulous *in vivo* trials.

Diverse studies have been carried out to establish various methodologies associated with the fabrication of HA derived from bovine bone. Notably, HA was synthesized from bovine bone utilizing a triphasic hydrothermal treatment approach.[13-15] For instance, Parisi *et al.* employed an alkaline hydrothermal hydrolysis approach to isolate Hydroxyapatite (HAp) from bovine bone. Subsequent thermal treatment at 250°C for 5 h yielded nanoflake-shaped HAp with a calcium-to-phosphorus (Ca/P) ratio measuring 1.86.[16] Ruksudjarit and colleagues utilized a hybrid approach, integrating calcination with the vibro-milling method to derive HAp from bovine bone. Their findings

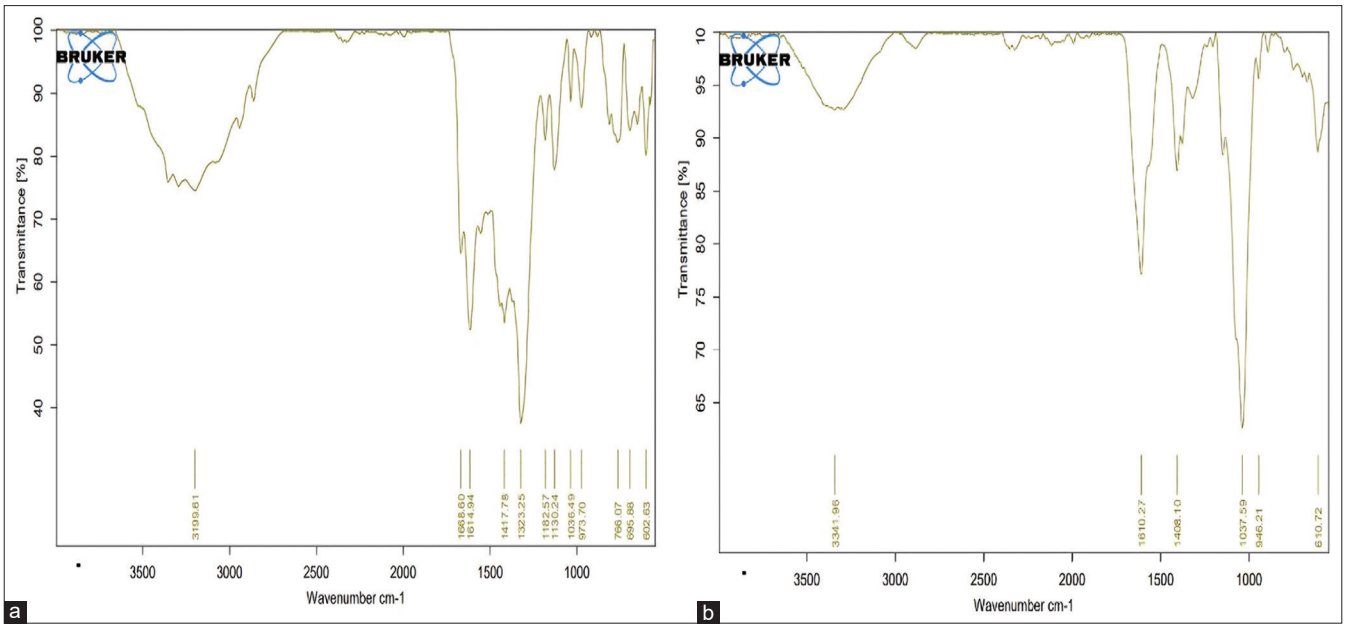

Figure 4: Fourier-transform infrared spectroscopy analysis (a) Hydroxyapatite-based bone graft modified with L-Arginine (b) BioOss showing peaks at 900–1200 cm^{-1}, 1400–1500 cm^{-1} and 3200 cm^{-1}.

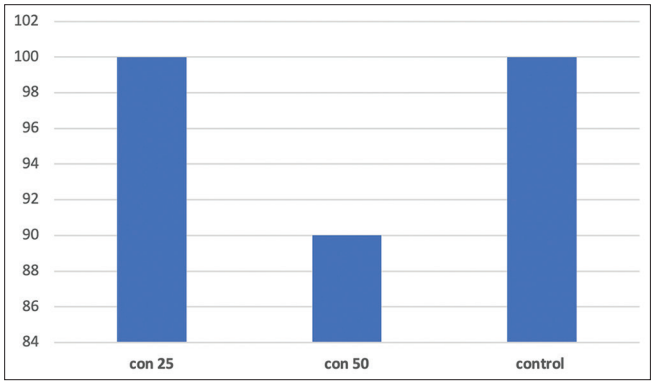

Figure 5: Mean cell aggregation at 25 and 50 µg concentration of bone graft at 24 h.

highlighted the emergence of HAp in a needle-shaped morphology, characterized by a diameter < 100 nm and a Ca/P ratio recorded at 1.66.[17] There is a noticeable scarcity of research on acquisition of HA specifically from ovine bone. While there exists some literature on HA sourced from fish bone, which underwent sintering at various temperatures ranging from 800°C to 1200°C. These studies mainly highlight concerns about reduced mechanical strength, increased brittleness, and overall decreased resilience.[18] Effective application of graft in implant dentistry can be a great choice mentioned by previous literatures.[19-21]

The influence of amino acids on bone grafts is a complex aspect studied in the field of biomaterials and tissue engineering. Incorporating L-arginine into bone graft material holds promising implications for cellular responses, tissue regeneration, and clinical success. Studies have indicated that L-arginine, as an essential amino acid, can positively influence osteoblast activity, enhance collagen synthesis, and promote angiogenesis.[22] These biochemical effects are crucial for fostering a favorable microenvironment for bone formation and regeneration. Furthermore, the presence of L-arginine has demonstrated potential anti-inflammatory properties, suggesting its role in mitigating inflammation during the healing process.[23,24]

While our investigation provided valuable understanding regarding the influence of L-arginine on HA sourced from ovine bone, it is crucial to recognize a few inherent constraints. Primarily, our emphasis is directed toward the presence of potential variables such as the intrinsic chemicals and contaminants present in the initial stage of bone substrate processing. In addition, while our chosen analytical methods (including SEM, XRD, and FTIR assessments) offered insights into the microstructure, crystalline properties, and biological relevance of the sintered HA, there exists a scope for the exploration of even more sophisticated characterization methodologies. Emphasis on *in vitro* cell study at different concentrations, time duration and effect of bone graft on various cell lines could also be carried out to further advance and bring to light the various osteoinductive and/or

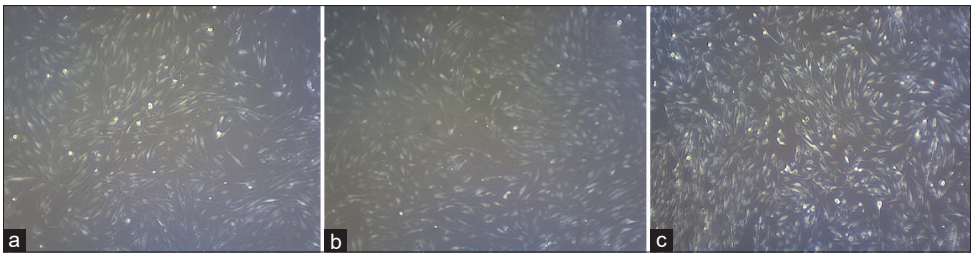

Figure 6: Cell viability at 24 h showing cell aggregation (a) at 25 µg/mL concentration, (b) at 50 µg concentration, (c) control.

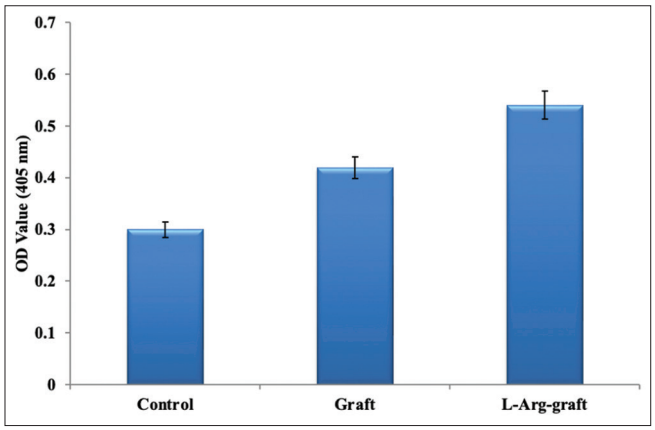

Figure 7: Alizarin red staining (405 nm) for bone formation in dental pulp stem cells (25 µg/mL).

osteoconductive properties inherent in the bone graft. Sophisticated fabrication methods, such as additive manufacturing or three-dimensional printing, to craft detailed architectures utilizing sintered HA could be explored in future. Exploring the addition of bioactive substances, growth factors, or specific drug release methods could enhance the regenerative capabilities of the material. Moreover, thorough examinations on how the material breaks down and assessments of its strength in natural conditions would provide essential information about its long-term stability and structural strength.

CONCLUSION

Bovine bone xenografts available in the market undergo processing temperatures that span from 300°C to 1200°C. Elevated processing temperatures often result in enhanced crystallinity and a decelerated rate of bone resorption during the healing process. Subsequently, the bone graft with amino acid was assessed based on its physicochemical and biocompatible properties. From our observations, we postulate that bone grafts modified with L-arginine yielded a material suitable for grafting by demonstrating satisfactory mechanical attributes and surface features. However, future laboratory tests are essential to evaluate the attachment and growth of various osteogenic cell lines. This should be followed by detailed examinations to measure various additional factors such as immune response, graft breakdown, and the subsequent complications and their alternatives.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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