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RESEARCH ARTICLE
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Hydroxyapatite Derived from Blood Clam Shells (*Anadara granosa*) as a Promising Biomaterial Bone Graft for Accelerating Osteogenesis Post-Surgery

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ABSTRACT

Fractures occur when bone continuity is disrupted by trauma or excessive pressure, often involving soft tissue injury. Bone graft implantation supports healing and should be bioactive, osteoinductive, biocompatible, and bioresorbable. Hydroxyapatite, the main inorganic component of bone and teeth, is widely used to promote bone regeneration. This study evaluated hydroxyapatite derived from blood clam (*Anadara granosa*) shell waste as a potential xenograft to accelerate bone healing through radiographic and histopathological analysis. Twelve male Wistar rats were divided into three groups, with evaluations at the 2nd and 4th weeks post-implantation. Radiographs assessed callus formation, while histopathology examined osteogenic cell activity. Results showed that the *Anadara granosa* hydroxyapatite (AGHA) group has similar effectiveness to the commercial graft and demonstrated better osteoblast and osteoclast distribution than the control group. In conclusion, AGHA shows potential as a safe and effective xenograft alternative for accelerating bone regeneration.

Keywords: *Anadara granosa* hydroxyapatite, Bone Graft, Fracture, Osteogenesis.

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INTRODUCTION

Bone is a mineralized connective tissue that provides structural support, protects vital organs, stores important minerals such as calcium and phosphorus, and plays a role in the process of hematopoiesis (Su et al., 2019). Bone homeostasis is maintained through coordinated interactions between osteoblasts, osteoclasts, and osteocytes (Silva et al., 2015). Fractures are one of the most common orthopedic disorders, caused by trauma, excessive mechanical stress, or certain pathological conditions, and their incidence continues to increase, potentially causing long-term disability in humans and animals (Alhawas & Alghamdi, 2023). The bone healing process occurs through the inflammatory phase, the reparative phase (callus formation), and the remodeling phase, but this process can be disrupted if the bone fragments are widely displaced or inadequately fixed (Zhang et al., 2025). In such conditions, bone grafting is necessary to provide scaffolding, cellular support, and osteoinductive signals to stimulate tissue repair. An ideal graft should be osteoconductive, osteoinductive, biocompatible, bioactive, and biodegradable (Wang & dan Yeung, 2017). Synthetic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which is similar to natural bone minerals, has been widely used as an alloplastic material; however, various obstacles still exist in autografts, allografts and xenografts, including limited availability, donor site morbidity, and the risk of complications (Munakata et al., 2024).

Recent developments show a shift in attention towards biomaterials derived from natural marine sources, particularly mollusk shells, which contain high amounts of calcium carbonate and have a tiered microstructure that is beneficial for biomedical applications (Muntean et al., 2024). *Anadara granosa* (blood clam), one of the mollusk species widely consumed in Southeast Asia, produces shell waste rich in $CaCO_3$ that can be converted into hydroxyapatite through controlled thermal or

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wet-chemical processes (Khiri et al., 2016). In South Sulawesi alone, blood clam shell waste reaches around 13.5 tons annually (Rozirwan et al., 2023), demonstrating its potential as a sustainable and low-cost source of biomaterial. The high calcium content and natural mineralization characteristics of *Anadara granosa* shells further support their suitability for biomedical applications, as demonstrated by structural analysis comparing hydroxyapatite from blood clam shells with conventional sources (Idulhaq et al., 2024).

Hydroxyapatite can be synthesized through wet-chemistry approaches such as precipitation at controlled pH and sintering temperatures, which determine morphology, crystallite size, and Ca/P ratio (Rodríguez-Lugo et al., 2018). Sintering at controlled temperatures can increase density and reduce porosity (Bohner et al., 2020; Babalola et al., 2023), while hydrothermal processes allow the formation of pure and homogeneous hydroxyapatite crystals with better biocompatibility potential (Rodríguez-Lugo et al., 2018). Recent studies have optimized similar synthesis techniques for marine-based hydroxyapatite, emphasizing the influence of process parameters on crystallinity, porosity, and bioactivity (Etinosa et al., 2024), including hydroxyapatite derived specifically from *Anadara granosa* shells (Khiri et al., 2016). Additionally, global interest in sustainable bioceramics is increasing, with marine-derived HAp recognized for its low toxicity profile, high osteoconductivity, and favorable mechanical properties (Wang et al., 2024; Patil et al., 2025).

Investigations using animal models further reinforce these findings. Hydroxyapatite derived from *Anadara granosa* shells has been shown to increase osteoblast and osteoclast activity without causing toxic or immunological reactions, as demonstrated by histopathological and radiographic evaluations (Cahyaningrum et al., 2020; Hikmah et al., 2023). Additional preclinical studies on other mollusk-based hydroxyapatite, including hydroxyapatite composites in green mussels and blood clams, also reported accelerated callus formation, increased mineralization, and better tissue integration (Hadi et al., 2024; Hermanto et al., 2024; Tjandra et al., 2023). Further physicochemical analysis confirmed that hydroxyapatite from shells can achieve crystallinity and Ca/P ratios comparable to synthetic HAp, reinforcing its potential as a xenograft material (Oladele et al., 2023). Although these findings are promising, comprehensive studies integrating radiographic and histopathological assessments to evaluate the effectiveness of hydroxyapatite from *Anadara granosa* shells in fracture repair are still limited. Therefore, the purpose of this study was to evaluate the effectiveness of hydroxyapatite derived from *Anadara granosa* shells as a xenograft material in promoting bone healing in Wistar rats, through radiographic and histopathological analysis.

MATERIALS & METHODS

This study evaluated the potential of hydroxyapatite synthesized from blood clam shells (*Anadara granosa*) using diammonium hydrogen phosphate ($(NH_4)_2HPO_4$), referred to as *Anadara granosa* hydroxyapatite (AGHA). Its performance was compared with a commercial bovine xenograft-hydroxyapatite (BX-HA) marketed as Batan Graft®. Assessments included Ca/P ratio analysis with SEM-EDX characterization, and post-implantation radiographic and histopathological evaluation of bone defects treated with AGHA.

Preparation of *Anadara granosa* Hydroxyapatite (AGHA)

The AGHA implant was produced from household waste-derived biomaterial through raw material preparation, high-temperature calcination, and mineral phosphate synthesis. Blood clam shells were cleaned, cut into 2–3cm fragments, and immersed in hydrogen peroxide (H_2O_2) for 3–4 days for deproteinization. The samples were then sintered at 1000°C for 2 hours (10°C/min heating rate) and ground into powder, producing calcium carbonate ($CaCO_3$) as the main phase.

Synthesis of Hydroxyapatite with Diammonium Hydrogen Phosphate

A 25mg portion of calcite powder was dissolved in 250mL of deionized water and heated to 70°C with stirring, followed by the dropwise addition of a 0.68M diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) solution. The pH was maintained at 8–9 using ammonium hydroxide (NH_4OH). The resulting product was autoclave-dried, sintered again at 1000°C for 2 hours (10°C/min), and sterilized with gamma irradiation at 1kGy (2.5kGy/sec) before storage (Daneshvar et al., 2019).

SEM-EDX Analysis

This test analyzed the surface morphology, particle and pore size, and mineral composition of AGHA powder, focusing on the Ca/P ratio. Scanning electron microscopy (SEM) was performed at 200kX, 400kX, 600kX, and 800kX magnifications to observe particle morphology and pore structure. Mineral composition and Ca/P ratio were determined using EDX attached to the SEM system (Haris et al., 2025).

FTIR and XRD Analysis

Fourier Transform Infrared Spectroscopy (FTIR) was performed by irradiating the sample with infrared radiation and recording absorption spectra across a specific wavenumber range, which corresponds to vibrational frequencies of chemical bonds in the material. The resulting spectra provide characteristic information about functional groups present, enabling qualitative identification of molecular composition. X-ray diffraction (XRD) analysis was conducted by exposing the sample to monochromatic X-rays to detect the diffraction patterns generated by the crystal lattice. These patterns were analysed using Bragg's law to determine crystallinity, mineral phase, crystallite size, and quantitative material purity.

Animal Model Testing

Twelve male Wistar rats were used as animal models. The animals were acclimatized for 14 days and provided

ad libitum access to standard feed and water. The rats were randomly assigned into three groups. The negative control (NC) group did not receive any graft implantation and served as the untreated control. The bovine xenograft–hydroxyapatite (BX-HA) group received graft implantation using Batan Graft®, a bovine-derived hydroxyapatite material. Meanwhile, the *Anadara granosa* hydroxyapatite (AGHA) group was implanted with hydroxyapatite derived from *Anadara granosa* (blood clam) shells. This grouping design was intended to compare the effects of each graft material on the evaluated experimental parameters.

Surgical implantation was performed by opening the right femur area of the animal model with a 3mm diameter drill. A defect was created in the proximal metaphyseal region of the femur (Fig. 1). This region was chosen due to its wider cross-sectional area and larger diameter, which reduces the risk of post-drilling fracture and supports stability during the postoperative recovery phase.

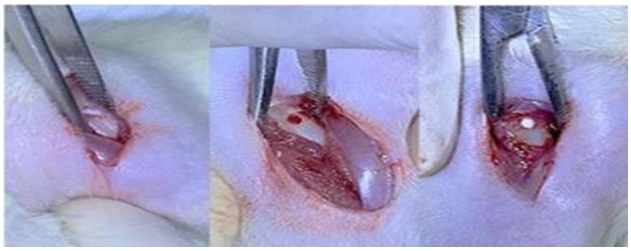

Fig. 1: Identification of the proximal metaphyseal region of the femur, defect creation, and AGHA graft implantation procedure.

Radiographic Examination

Radiographic assessment was performed at two and four weeks post-surgery. The animal models were positioned in a mediolateral view, with imaging focused on the femur bone at the implantation site. Radiographic findings were evaluated both descriptively and using a modified scoring system based on Oryan et al. (2015), assessing bone formation, radiographic union, implant absorption, and remodelling.

Histopathological Examination

Histological evaluation was conducted at two and four weeks post-surgery after euthanasia. Femur segments containing the implantation defect (segment length approximately 2–3 cm) were harvested and fixed in 10% neutral buffered formalin for 48 hours. After fixation, each femur segment was grossed into smaller blocks with a thickness of approximately 2–3 mm to allow adequate penetration of fixative and processing reagents. The tissue blocks were then processed routinely, paraffin-embedded, sectioned (4-5µm), and stained with hematoxylin and eosin (H&E) for microscopic analysis. Osteoblast and osteoclast activity was assessed under a light microscope at 400× across five fields per slide, with results averaged and compared statistically using SPSS. AGHA success was determined by evaluating cellular morphology and bone tissue architecture.

RESULTS

SEM-EDX Analysis

SEM-EDX analysis of AGHA revealed a rough, porous morphology with irregularly distributed granular particles at magnifications of 2.00kX, 4.00kX, 6.00kX, and 8.00kX (Fig. 2). This microstructure indicates particle aggregation with micro-porosity, supporting osteoblast adhesion, proliferation, nutrient diffusion, and osseointegration (Čandrlić et al., 2024). The rough surface and porous architecture facilitate cell penetration, extracellular protein adhesion, and mineralization through calcium and phosphate ion release. The absence of large cracks suggests good micro-scale mechanical stability, consistent with typical biocompatible hydroxyapatite scaffolds that promote osteogenesis and bone regeneration (Amini & Lari, 2021; Niu et al., 2021; Pan et al., 2022; Barba-Rosado et al., 2024; Elahi et al., 2025; Zhang et al., 2025).

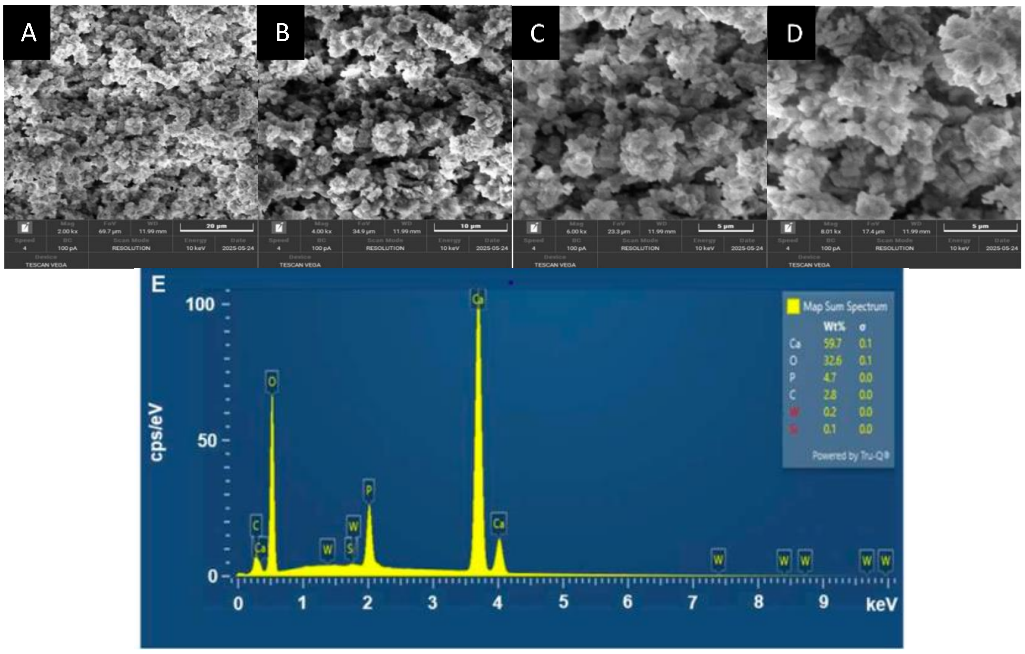

Fig. 2: Scanning electron microscopy (SEM) examination of the AGHA implant material at various magnifications: 2.00kX (A), 4.00kX (B), 6.00kX (C), and 8.00kX (D). Elemental composition analysis using energy-dispersive X-ray spectroscopy (EDX) (E).

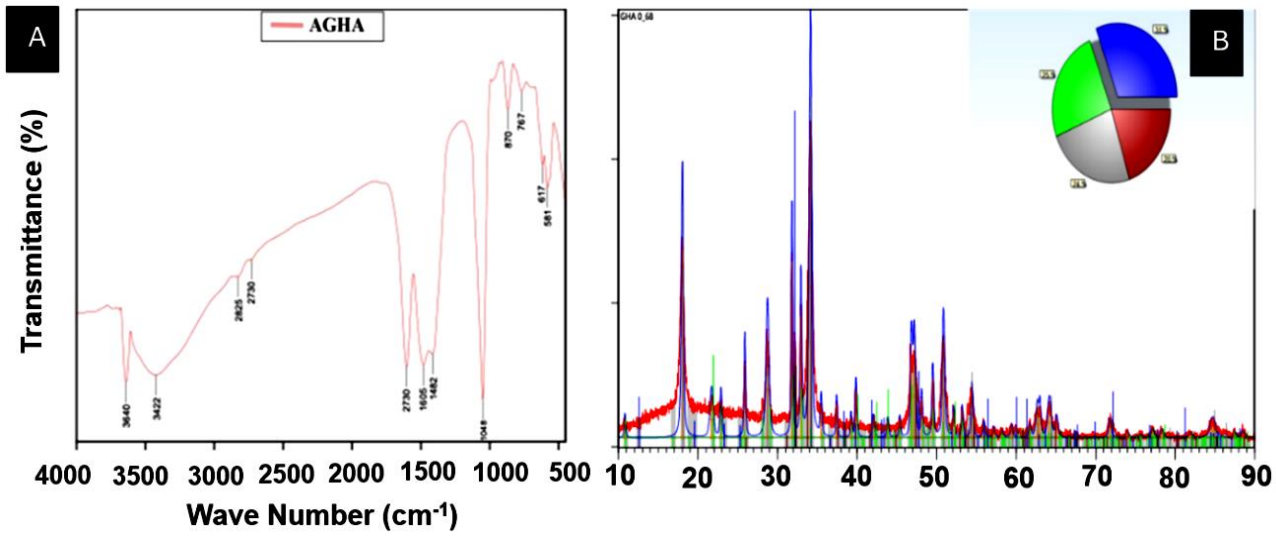

Fig. 3: FTIR plot (A) and XRD analysis (B) of the AGHA bone graft material.

EDX analysis of AGHA showed a dominant composition of calcium (Ca) and phosphorus (P), with a Ca/P molar ratio close to the stoichiometric value of hydroxyapatite (1.67) (Fig. 2E), closely mimicking natural bone and enhancing biocompatibility and bioactivity (Roldan et al., 2023). The presence of oxygen confirmed hydroxyl and phosphate groups within the crystal structure, reinforcing material stability and strength. These results indicate successful synthesis of high-purity hydroxyapatite free from heavy metal contaminants, confirming that AGHA possesses a stable structure resembling natural bone mineral and strong potential as a safe and effective bone graft biomaterial (Jerbić Radetić et al., 2021; Hamada et al., 2022; Demir-Oğuz et al., 2023; Furtado et al., 2025).

FTIR and XRD Analysis

Fourier transform infrared spectroscopy (FTIR) was employed to identify functional groups based on the absorption of infrared radiation. This technique generates a spectrum that represents molecular vibrational frequencies, serving as a distinctive fingerprint of molecular structure. In contrast, X-ray diffraction (XRD) was used to analyse the crystalline structure of
a material by examining the scattering of X-rays by its crystal lattice.

The FTIR spectrum of AGHA hydroxyapatite (Fig. 3A) shows characteristic phosphate (PO_4^{3-}) peaks at 1,040–1,020cm^{-1} and 600–560cm^{-1}, and hydroxyl (OH^-) peaks at 3,400–3,600 cm^{-1}, critical for stability and bioactivity, with a minor carbonate (CO_3^{2-}) peak at 1,600–1,650 cm^{-1} indicating carbonate substitution similar to natural hydroxyapatite. These peaks confirm the successful transformation of clam shell calcium carbonate into pure hydroxyapatite, and the dominant hydroxyl groups enhance biological function and chemical stability, validating AGHA's effectiveness as a biomaterial scaffold for bone regeneration (Amini & Lari, 2021; Feroz & Dias, 2021; Alonso-Fernández et al., 2023; Aminatun et al., 2024).

XRD analysis (Fig. 3B) revealed main diffraction peaks at 31.7°, 32.9°, and 25.9°, matching the standard hydroxyapatite pattern (JCPDS No. 09-0432) and confirming high crystallinity and pure hydroxyapatite phase with nano- to micro-scale crystal sizes suitable for bone applications. Combined SEM, EDX, FTIR, and XRD results show that AGHA closely resembles natural bone in morphology, elemental composition, chemical structure, and crystallinity, supporting its biocompatibility, osteoinductivity, osteoconduction and controlled biodegradation, highlighting its potential as a sustainable and effective scaffold for veterinary and clinical orthopaedic use (Amini & Lari, 2021; Jerbić Radetić et al., 2021; Pan et al., 2022; Roldan et al., 2023; Diansari et al., 2025; Elahi et al., 2025; Furtado et al., 2025).

Radiographic Examination

Radiographic assessment at the 2nd week showed that the negative control (NC) group formed only a thin, low-density callus along the fracture edge, while the BX-HA and GHA groups exhibited uniform, well-defined thin callus across the defect with higher radiopacity (Fig. 4), indicating accelerated early callus formation and osteointegration. Callus formation, a critical stage in fracture healing, begins as fibrocartilaginous tissue that gradually mineralizes into immature bone, providing temporary support before remodelling into mature bone (Jo et al., 2024).

Analysis at the 4th week showed that the negative control group still had incomplete defect closure, though the defect size had decreased. In contrast, the BX-HA and AGHA groups demonstrated complete defect closure with evenly distributed bone density and formation of a well-organized, hard callus, indicating significant mineralization and a clear transition from soft to bony callus. Adequate blood supply and fracture stability are essential for this transition (Einhorn & Gerstenfeld, 2015), and grafts provide a supportive matrix for osteogenic cell proliferation, accelerating bone formation (Markel, 2019). Although the grafts were not fully resorbed, they remained stable scaffolds supporting new bone growth, highlighting their efficacy in bone regeneration (Einhorn & Gerstenfeld, 2015; Sudimartini et al., 2019).

Radiographic scoring (Table 2) showed significant improvements in bone formation, union, and remodelling over time, while implant resorption remained stable, indicating AGHA's structural stability (Chacon et al., 2023). AGHA results were comparable to BX-HA and superior to the negative control, highlighting its osteoconductive potential (Hikmah et al., 2023). This demonstrates that blood clam shell-derived hydroxyapatite effectively supports fracture healing.

Table 1: Radiographic scoring in each treatment group

Scoring Time		Treatment Group		
		NC	BX-HA	AGHA
2nd week	Bone Formation	1.00±0.00^{a}	2.00±0.00^{b}	2.00±0.00^{b}
	Total Radiographic Unification	1.00±0.00^{a}	2.00±0.00^{b}	2.00±0.00^{b}
	Implant Absorption	1.00±0.00^{a}	2.00±0.00^{b}	2.00±0.00^{ab}
	Remodelling	1.00±0.00^{a}	2.00±0.00^{b}	2.00±0.00^{b}
4th week	Bone Formation	2.50±0.71^{a}	3.50±0.71^{b}	4.00±0.00^{b}
	Total Radiographic Unification	2.50±0.71^{a}	2.50±0.71^{b}	3.00±0.00^{b}
	Implant Absorption	1.00±0.00^{a}	2.50±0.71^{b}	2.00±1.41^{ab}
	Remodelling	1.50±0.71^{a}	3.00±0.00^{b}	3.00±0.00^{b}

NC: Negative control; BX-HA: Bovine xenograft–hydroxyapatite; AGHA: *Anadara granosa* hydroxyapatite. Different superscript letters within the same row indicate significant differences between treatment groups at the same time point (P<0.05). Values sharing at least one common letter are not significantly different.

Time treatment interaction was significant only for bone formation, showing AGHA's increasing efficacy over time (Sudimartini et al., 2019). At week 4, AGHA achieved outcomes comparable or slightly superior to the commercial graft in bone formation and remodelling. These results confirm AGHA as a promising xenograft alternative for accelerating fracture healing.

Histopathological Observation and Osteogenic Activity

At the second week post-implantation, the negative control group (NC) showed minimal trabecular formation and a low number of osteoblasts (8.75±2.47) (Fig. 5). In contrast, the BX-HA (23.50±2.12) and AGHA (25.25±2.47) groups had a statistically significant increase in osteoblasts (P<0.05, two-way ANOVA with Duncan's test), enhancing bone matrix deposition and trabecular network development. These results align with previous studies demonstrating the bioactive potential of naturally derived hydroxyapatite to stimulate bone-forming cells (Rizzo et al., 2025), and in the staining HE showed that the presence of residual AGHA graft material indicates seamless integration without persistent foreign material (Fig. 6).

By the fourth week post-implantation, osteoblast numbers declined compared to week 2, yet the AGHA (11.50±0.71) and BX-HA (17.25±1.06) groups remained significantly higher than the control (5.60±0.85) (P<0.05). Increased trabecular thickness and the presence of osteocytes within lacunae in the treatment groups indicate ongoing bone maturation and structural organization, while the control group exhibited suboptimal organization,

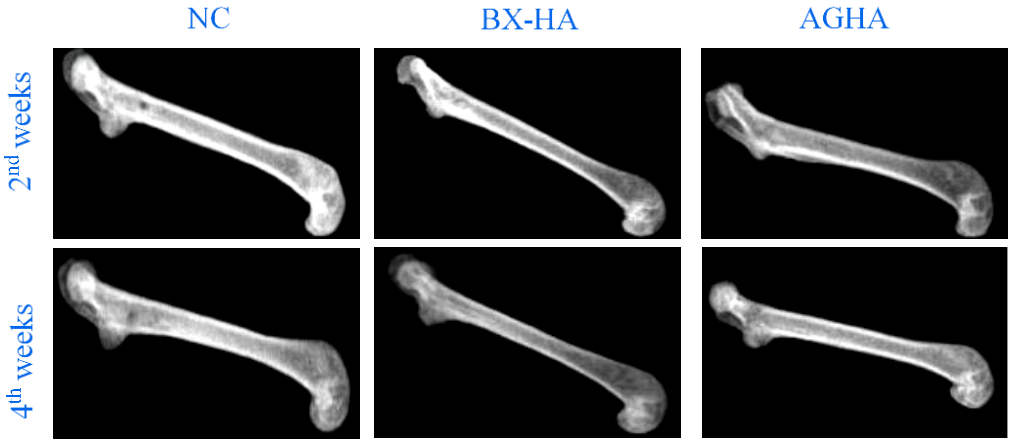

Fig. 4: Postoperative radiographic images of bone implantation at the 2nd and 4th weeks. The radiographs illustrate the progression of osteogenesis in the three groups: Negative Control (CN), bovine xenograft–hydroxyapatite (BX-HA), and *Anadara granosa* hydroxyapatite (AGHA). Improved bone formation is visibly more prominent in the BX-HA and AGHA groups compared to the control.

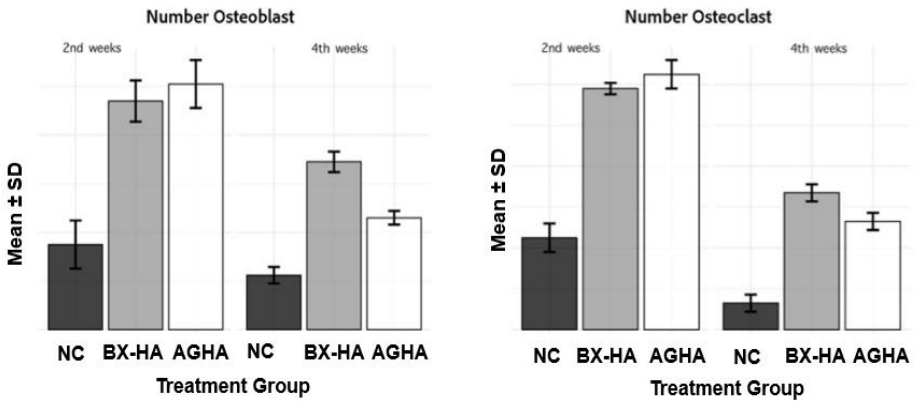

Fig. 5: Comparison the number of osteogenic cells following implantation.

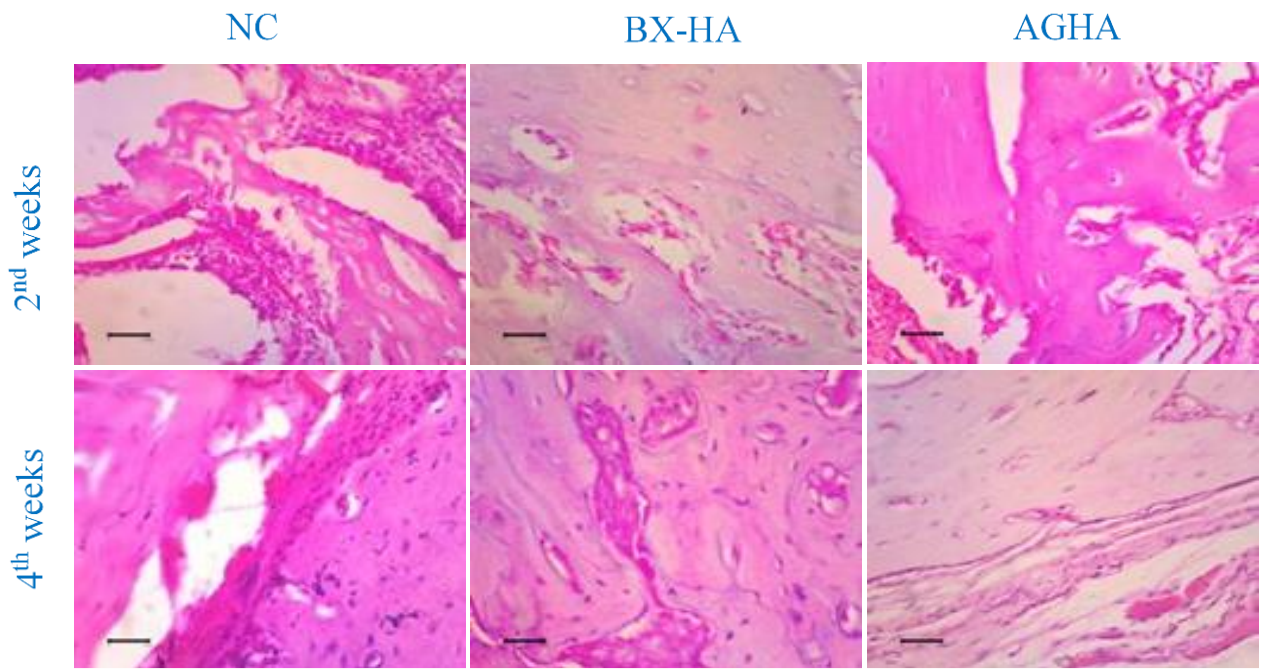

Fig. 6: Histological images stained with hematoxylin and eosin (HE) at 40X magnification on the second and fourth post-implantation weeks across all treatment groups. Enhanced osteogenic activity and trabecular growth were notably observed in the BX-HA and AGHA groups compared to the control. Scale bar: 25µm.

highlighting the need for biomaterial support or mechanical stability (Fig. 6). Osteoblast and osteoclast activities followed a dynamic pattern, peaking at week 2, with osteoclast numbers in BX-HA (5.90±0.14) and AGHA (6.25±0.35) significantly higher than the control (2.25±0.35), and remaining elevated at week 4 (BX-HA 3.35±0.21; AGHA 2.65±0.21; control 0.65±0.21) ($P<0.05$), reflecting balanced bone formation and resorption.

These findings demonstrate that AGHA derived from blood clam shells is a promising biomaterial for bone regeneration, significantly enhancing osteoblastic and osteoclastic activity while being fully resorbed. It provides mechanical stability necessary to prevent micromovement and fibrous tissue formation, supporting endochondral ossification (Einhorn & Gerstenfeld, 2015). Stability also promotes improved blood perfusion and vascularization at the fracture site, facilitating nutrient and growth factor delivery for regeneration (Menger & Laschke, 2022). These results are further supported by Yadav et al. (2024), showing that carbonate apatite from clam shells promotes more organized osteoblastic arrangements and advanced mineralization compared to controls without implants.

These findings are consistent with previous studies using animal models, which show that hydroxyapatite derived from natural materials can accelerate bone healing through increased osteoconduction and mineral deposition. Cahyaningrum et al. (2020) and Hikmah et al. (2023) reported increased osteoblast activity in mouse models after implantation of hydroxyapatite from shellfish shells, and these results are consistent with the increase in osteoblast numbers in week 2 in the AGHA group in this study. Additionally, Hamada et al. (2022) and Elmeshreghi et al. (2025) demonstrated that hydroxyapatite scaffolds implanted in rabbits and mice resulted in strong trabecular formation and early mineralization, which is consistent with our radiographic results showing complete defect closure and callus maturation at week 4.

The increase in mineralization observed in the AGHA-implanted femurs is also consistent with the findings of Chacon et al. (2023), who reported that hydroxyapatite chitosan composites enhance radiographic union and early remodeling in an ovariectomized rat model. Furthermore, the increase in osteoclast numbers in the AGHA group reflects bone turnover balance as described by Jerbić Radetić et al. (2021) in xenograft-treated orthopedic defects, confirming that controlled osteoclast activity is an important part of normal remodeling, rather than indicating pathological resorption.

This study also supports the findings of Yadav et al. (2024), who reported that carbonate apatite derived from shellfish shells enhances long bone healing in goats, resulting in a more regular osteoblast arrangement and faster mineralization. The similarity of results across various animal species, including mice, rabbits, and goats, reinforces the translational potential of AGHA as an effective xenograft biomaterial.

Conclusion

Hydroxyapatite derived from the blood clam shells (*Anadara granosa*) shows potential as a bone graft biomaterial with effectiveness comparable to commercial grafts in radiographic and histopathological evaluations. This material possesses good biomaterial characteristics, displaying a uniform distribution of osteoblasts and osteoclasts as well as significant trabecular-formation activity. Bone remodelling in the AGHA treatment group is more optimal than in the negative-control group, yielding significant results that are on par with Batan graft.

These findings indicate that the blood-clam-shell-based material is safe, biocompatible,

and capable of accelerating bone regeneration without provoking an immune response.

DECLARATIONS

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Conflict of Interest: The authors declare no potential conflict of interest.

Data Availability: Data will be available upon request.

Ethics Statement: This research has received ethical approval from the Animal and Research Ethics Commission, Hasanuddin University on August 28, 2025 with number 017/UN4.1.RSHUH/B/PP36/2025.

Author's Contribution: Muhammad Zulfadillah Sinusi, Dwi Kesumasari Sari, Dian Fatmawati, Rini Amriani, Andi Rifqatul Ummah, Musdalifah designed and conducted the research. Saiful Rahman and Cika Maharani carried the data analysis. Nurul Sulfi Andini and Aniek Setiya Budiatin as a histopathologist and characterization material experts, respectively. All authors contributed to manuscript preparation and final review, approved the final manuscript for submission.

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