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Impact of Hydroxyapatite Nanoparticles, Demineralized and Decellularized Bone Matrix on Tibia Fracture Healing: A Biochemical and Histological Study in Goats

Abdulsamad H. AlBattat [a*] and Abdulbari A. Alfaris [a]

[a] *Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Basrah, Iraq.*

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

At present, using bone grafts is the most successful surgical approach for repairing deficits in the bone structure. This study investigated into how goat tibial defect fracture healing was impacted by using hydroxyapatite (HA) or demineralized and decellularized bone matrix (dDBM) xenograft.
Methodology: In this study, fifteen young goats were employed. Three groups (n=5) consisting of the control group, nano-group, and xeno-group were created from the animals. Bone drill was used to remove 2 mm from the tibia of the right hind limp in all animals. The hydroxyapatite nanoparticles (HA) in the Nano-group and the xenograft demineralized and decellularized bone matrix (dDBM)

**Corresponding author: Email: samadalbattat@gmail.com, abdalbari.sahi@uobasrah.edu.iq;*

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(obtained from sheep tibia) in the Xeno-group were used to fill the bone defect respectively, while the bone defect in the control group remained without treatment, and the surgical site was regularly closed. Blood serum was collected at the following intervals: postoperative, 14, 42, and 64 days for detection of alkaline phosphatase (ALP) and osteocalcin (OC) levels. At 64 days after surgery, all goats were euthanized and the healing was evaluated by specimen histological observation.
Results: Biochemical examination in treated groups (Nano and Xeno groups) showed a statistically significant ($P < 0.05$) increase in the level of ALP and OC. histopathological examination at 64 day post-operation, treated groups shows Completely grown woven bone and the fracture callus composed of mature fibro-osteoid mixture. In conclusion, We demonstrate that the healing process accelerated and the bone defect can be filled by using hydroxyapatite (HA), or dDBM, as a scaffold for the damaged tibia bone.

Keywords: Bone gap; hydroxyapatite nanoparticles; demineralized and decellularized bone matrix; bone graft; goat.

1. INTRODUCTION

bone is an essential organ for support and movement. Bone defects can be caused by degenerative diseases, infections, trauma, and malignancies. Larger defects beyond a critical size require bone substitutes to allow effective healing, but small defects like fractures can heal on by themselves [1].

The goal of bone tissue engineering, a modern and difficult scientific field, is to improve bone regeneration and repair after bone is lost by a disease or injury, eventually restoring the bone's natural shape [2].

Currently, a variety of bone transplant materials are used, such as autografts, allografts, xenografts, ceramic- and polymer-based alternatives to bone grafts. Every variety has advantages and disadvantages of its own [3].

Because autologous bone transplants are limited, more bone matrix materials must be created to heal bone abnormalities. It is expected that xenograft bone will be used in clinical treatment due to its ideal structural resemblance to native bone and high degree of biocompatibility [4].

Scaffolds based on decellularized extracellular matrix (dECM) are becoming more prevalent in the field of regenerative medicine. Tissue homeostasis and regeneration are facilitated by the extracellular matrix (ECM), a complex environment with superior biochemical, biophysical, and biomechanical qualities that can control cell adhesion, proliferation, migration, and differentiation [5].

Because bone tissue consists of both organic and inorganic phases, both mineral and organic biomaterials are typically used to create the scaffolds used in bone tissue engineering applications. Hydroxyapatite (HA) is one of the most often employed minerals in bone scaffolds. HA is a key component of bone regeneration and makes up around 60% of the inorganic material in dry bone. In order to aid in bone healing, HA is a resorbable bioactive mineral having osteoconductive and osteointegrative qualities [6].

The current study aims to assess the effectiveness of two distinct graft biomaterials hydroxyapatite (HA) and demineralized and decellularized bone matrix (dDBM) in tibia bone defects regeneration. utilizing Biochemical indicators alkaline phosphatase ALP and osteocalcin OC, and the histological Evaluation.

2. MATERIALS AND METHODS

2.1 Animals

In this investigation, fifteen young female goats of the local breed, mean age 7±0.57 months and mean weights 22±0.49 kg, were utilized. The animals were kept in separate cages in the animal farm house of the College of Veterinary Medicine.

2.2 Preparation of Xenograft (dDBM)

The tibia was obtained from 7±0.34 month old sheep from the Basra slaughterhouse. Before demineralization, Using a diamond blade cutting saw, the cortical bone was cut to dimensions of 4 mm x 4 mm from the mid-diaphyseal portion along the longitudinal direction. The samples were then immersed in 20 mL of 0.5 M HCl for 7 hours at room temperature. The samples were placed on a rocking chair to ensure agitation.

After being rinsed with deionized water, the samples underwent five minutes of dehydration in water/ethanol solutions with ethanol concentrations progressively increased 25%, 50%, 75%, to 100% vol. [7].

Bone blocks decellularization were started with using a PBS plus wash. Subsequently, four thermal shock cycles were executed, wherein a 20-minute step at 121°C was followed by a 16-hour freezing duration in liquid nitrogen (-196°C). Bidistilled water (ddH2O) was used to submerge bone blocks throughout these passages; after every cycle, the solution was changed. After that, bone blocks were cleaned for eight hours in 1% TritonX-100 and then for sixteen hours in 0.1% TritonX-100 to eliminate any remaining cellular debris. TritonX-100 was dissolved in ddH2O. To remove any last traces of detergent, bone samples were twice washed in ddH2O for a duration of 24 hours. These steps were all carried out using a rotatory shaker in continuous shaking mode at room temperature (RT). At this point, the Semi-Automated Rotary Microtome M-240/Myr- Spain was used to reduce the decellularized bone blocks into granules with diameters of 420–840 mm. Granules of bone were dehydrated for two hours at room temperature using a series of graded ethanol concentrations (50, 70, 96, and 100%). After their transfer to cell culture dishes, bone granules were allowed to dry at room temperature under a sterile laminar flow hood [8].

2.3 Sterilization of Xenograft (dDBM)

The DBM was steriled in an autoclave. Sterilizing cycle for 3.30 minutes of sterilization and 5 minutes of drying at 134°C and 2,16 bar prior to being implanted in the bone defect [9].

2.4 Surgical Preparation

Prior to the procedure, every goat was fasting for eight hours. From the knee to the ankle, the right lower leg was shaved and clipped. After using distal water to thoroughly clean the area, the surgical site was scrubbed for two to three minutes with diluted liquid soap. After that, a 70% ethanol alcohol antisepsis was administered to the entire clipped area, and the incision site was treated with 2.5% tincture iodine.

2.5 Surgical Procedure

The animals were anesthetized using an anesthetic method that included intramuscular ketamine (5–10 mg/kg) and xylazine (0.05–0.1 mg/kg). Around the operation site, a 1 mL subcutaneous injection of 2% lidocaine was also given [10]. Each goat's surgery site was prepped with aseptic methods. A 3–5 cm long cranio-lateral incision into the skin and soft tissue revealed the tibia bone. A bone drill was used to remove a 2 mm core lesion from the mid-shaft of the tibia in each experimental animal. During the drilling procedure, sterile warm isotonic saline was dropped [11].

The goats split up into three groups(each five goats): control group, Nano-group, and Xeno-group. In control group the bony defect was left without any treatment, while in Nano-group the bony defect filled with hydroxyapatite nanoparticles powder as filling material were purchased from (US Research Nanomaterials, Inc.3302 Twig Leaf Lane /Houston, TX 77084, USA). In Xeno-group, as filling material of powdered xenograft demineralized and decellularized bone matrix from sheep tibia was used to fill the bony defect (Fig. 1). The surgical site was closed routinely in all experimental goats.

Following the procedure, the goats were put back in their cages and given free rein to move around without any external or internal fixation. Every 12 h, ceftriaxone 20 mg/kg body weight was administered intramuscularly for7 days after the surgery .

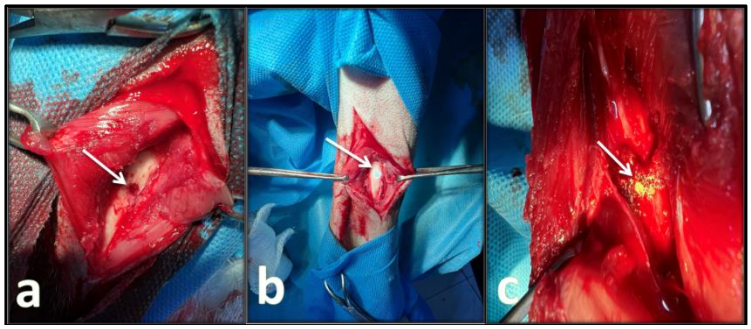

Fig. 1. a: control group, b: Nano-group c: Xeno-group

2.6 Blood Serum Biochemical Parameters

2.6.1 Blood serum collection method

Blood serum collection was gathered according to Nagyová et al. 2016 [12] in the following intervals :post-surgery,14,42, and 64 days. Step 1: A 5 ml blood volume was obtained using a vacutainer^{tm} serum separator tube (SST), aseptically from peripheral vein. The vacutainer^{tm} blood tubes were left at room temperature for 30 – 60 minute before centrifuging the tube to separate the serum. Step 2: After centrifuging and separating the serum samples, they were put into plastic freezer vials with leak-proof screw covers. The serum samples were labeled and keept at -20^{o}C till use.

2.7 ELISA Kit for Osteocalcin

the detection of osteocalcin in serum samples was performed using a Bioassay Technology Laboratory (TB LAB) Kit (BT, China) following the manufacturer's instructions. In summary, 120ul of stock concentration (128ng/mL) was reconstitution with 120ul of a standard diluent to provide a stock solution starting with 64ng/mL. The stock solution was farther serially diluting a ratio of 1:2 to create duplicate standard curve point at 32ng/ml, 16ng/ml, 8ng/ml and 4ng/ml. the wash buffer was diluted to 1X with deionized water and kept in a clean glass bottle till use. The samples and the standards were transferred to the ELISA plate using a multichannel pipette. After the initial incubation , series of washing and incubation were conducted following the manufacturers procedure. Finally, the stop solution was added, and the plate was measured at 450nm wavelength using an 800 TS absorbance ELISA plate Reader (Biotek, USA). The data analysis was conducted using Gen5 software (Biotek, USA).

2.8 Measurement of Alkaline Phosphatase (ALP) Enzyme Activity

The assay kit, which was already made and produced by Biolab in France, was utilized with a spectrophotometer (Aple, Japan) set to 505 nm. The standard curve that was made specifically for this purpose was employed to extract the ALP enzyme activity (U/L).

2.9 Histopathological (Microscopic) Evaluation

All goats were euthanized by being exsanguinated by neck cutting at 64 days after surgery, and specimens were taken around the defect site from every animal in the study . The bone samples have been fixed in a 10% buffered formalin for 48 hours (2 days) and remove the calcium by a decalcification solution (formic acid 10%). Then samples irrigate by distill water, then dehydrated with ethyl alcohol, washed with xylene first, later, embeded in paraffin wax. 4–5 µm thickness of transverse sections was harvested from the site of tibial defect then stained by using H&E stain [13].

2.10 Statistical Analysis

The statistical test was done by SPSS (2022) protocol for statistical comparison of different events in the research examination. The chi-square analysis for comparison of the percentage with the least significant difference; the LSD test (ANOVA) to study the significant comparison between means level of the present project, this level ($P \leq 0.05$) was considered significant.

3. RESULTS

3.1 Biochemical Evaluation

3.1.1 Alkaline phosphatase kit

ALP examination conducted immediately following the procedure revealed a distinct similarity in the ALP levels across the groups. While on days 14, 42 and 64, there was a statistically significant ($P < 0.05$) increase in the level of ALP in the treated groups compared with the control group (Fig. 2).

3.1.2 Osteocalcin test

Serum OC concentrations for each of the control, Nano, and Xeno groups are shown in (Fig. 3). We note that across periods, there were no significant differences between groups. However, there was a significant difference ($P<0.05$) between the values of the two treatment groups (Nano and Xeno groups) on the first day after the operation compared to those during the 14 days after the operation. While there was no significant difference in the control group during the same period.

3.2 Histological Evaluation

After 64 days of induction of fracture, the changes observed in the control group included the osteocytic dilation of the bone, the fracture callus composed of immature hyaline cartilage and fibro-osteoid mixture. Woven bone with osteoblastic rimming in a reactive fibrovascular stroma (Fig. 4).

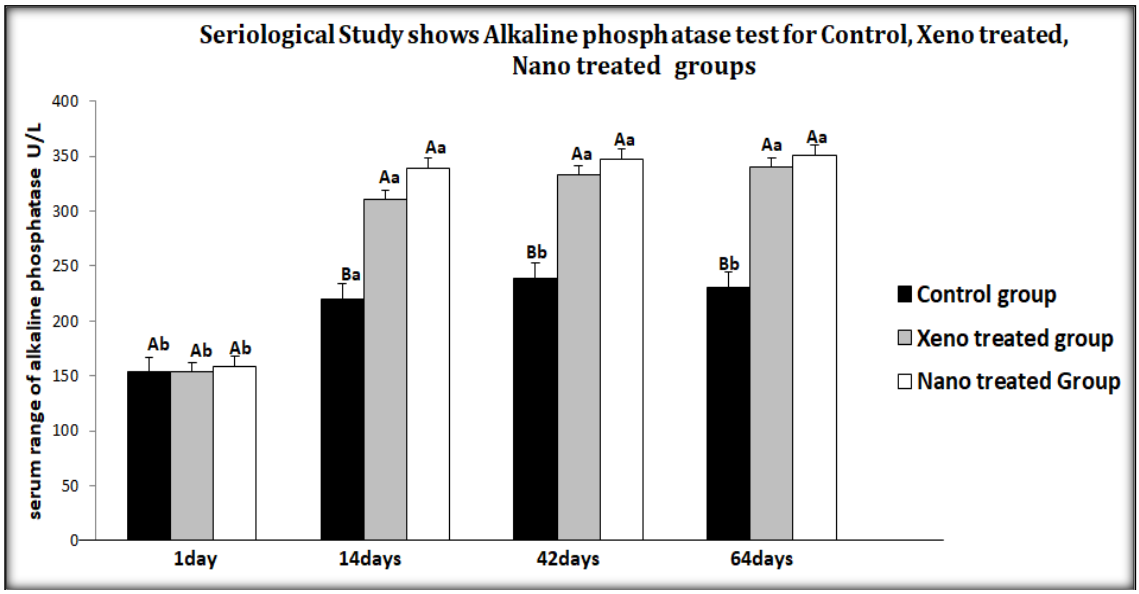

Fig. 2. Alkaline phosphates test. AB Different letters among groups indicates significant differences (P<0.05). ab Different letters within group in different times indicates significant differences (P<0.05)

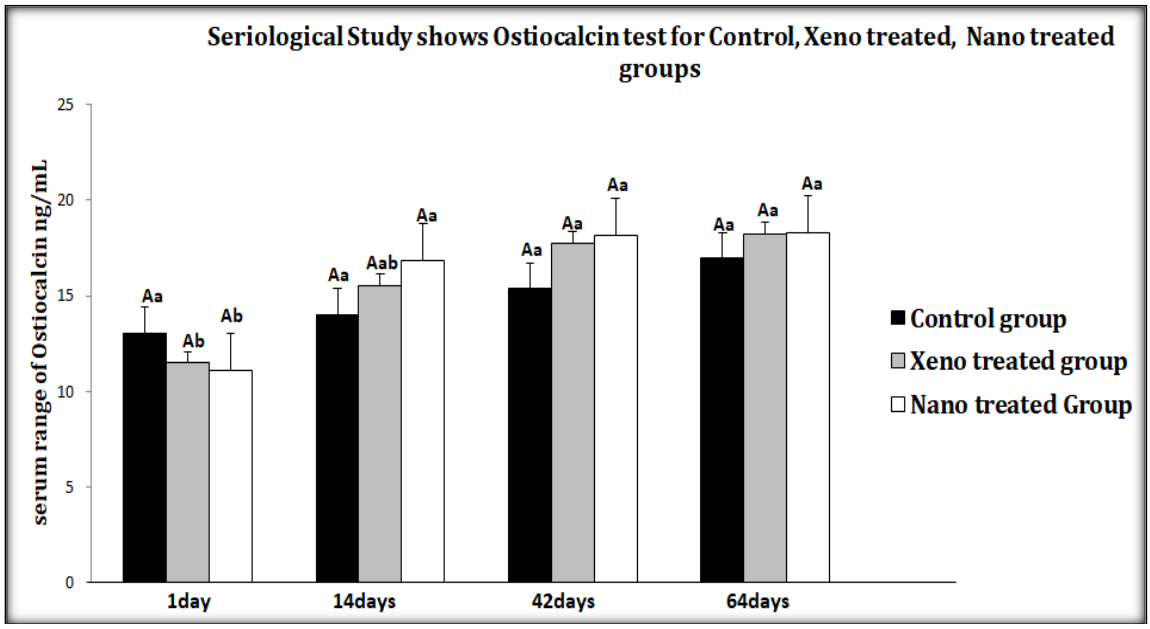

Fig. 3. Octiocalcin test. AB Different letters among groups indicates significant differences (P<0.05). ab Different letters within group in different times indicates significant differences (P<0.05)

Nano- group after 64 days of induction of fracture showed presence of the obvious junction between newly formed bone and old bone. Nearby the original "osteotomy cut lines", remodeling lamellar bone (well-formed) might be located, evenly distributed trabecular bone. The nano-group showed formation of well distributed woven bone, bone remodeling was recognized by presence well-recognized woven bone, Haversian canal, osteoblast, osteocytes (Fig. 5).

Xeno-group shows Completely grown woven bone with regular collagen fibers deposition in the fibrovascular stroma, and the fracture callus composed of mature fibro-osteoid mixture (Fig. 6).

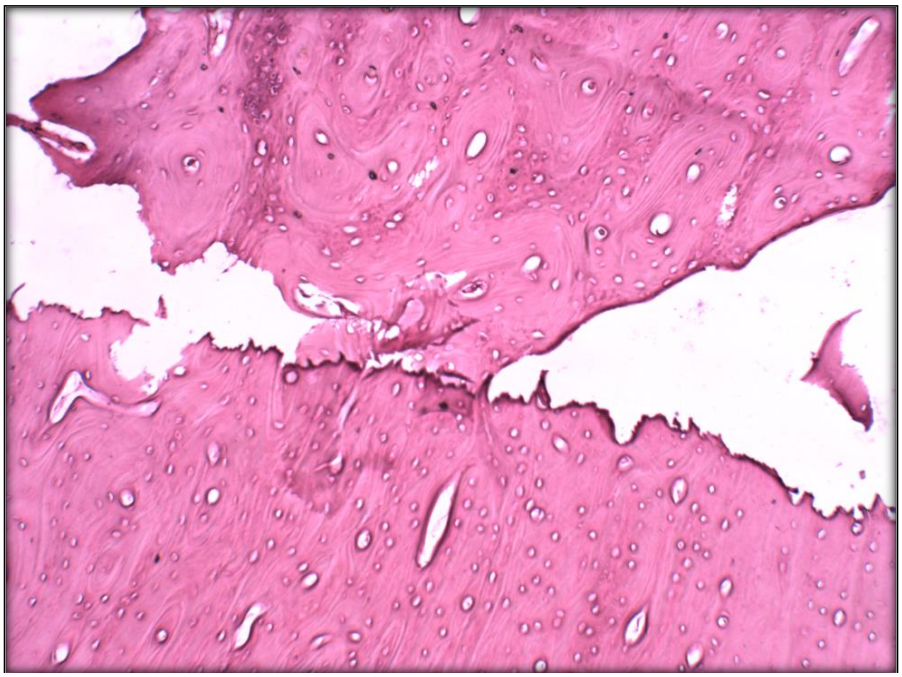

Fig. 4. The histopathological section of bone of the control group shows osteocytic dilation of the bone, woven bone with osteoblastic rimming in a reactive fibrovascular stroma, the fracture callus composed of immature hyaline cartilage and fibro-osteoid mixture,formation of newly havresian systems , between them some areas have noncompete form. H&E stain. 10X

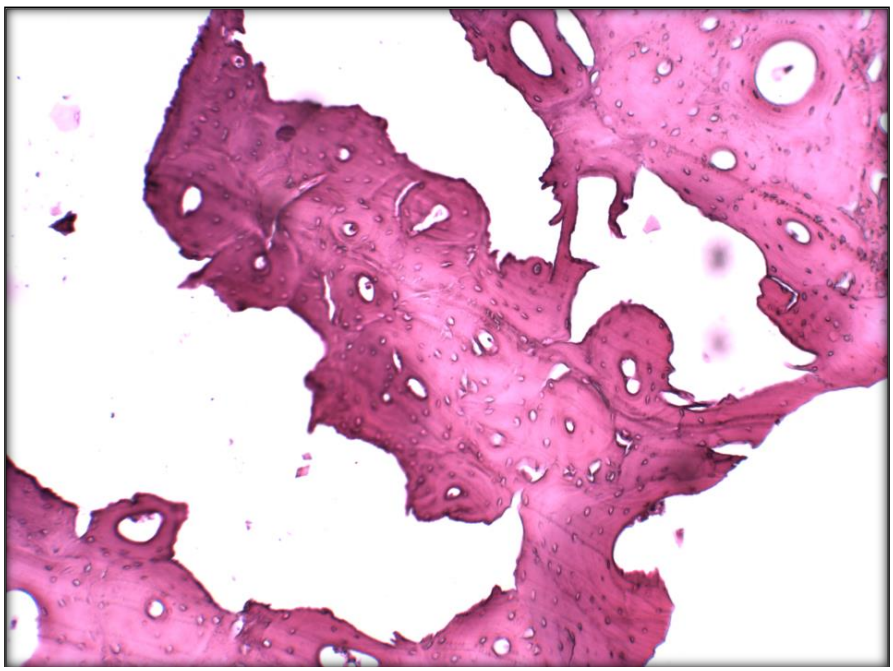

Fig. 5. The histopathological section of bone of the Nano-group shows completely grown woven bone with regular collagen fibers deposition in the fibrovascular stroma, and the fracture callus composed of mature fibro-osteoid mixture ,the artifact detachment is around the repair formation. H&E stain. 10X

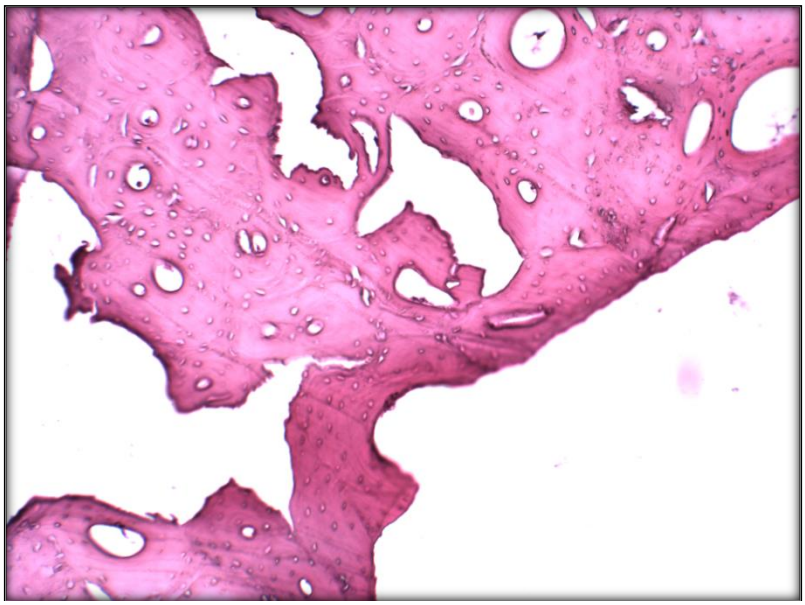

Fig. 6. The histopathological section of bone of the Xeno-group shows woven bone with regular collagen fibers deposition in the fibrovascular stroma, and the fracture callus composed of mature fibro-osteoid mixture, the artifact detachment is around the repair formation. H&E stain. 10X

4. DISCUSSION

The present study keeps pace with developments in regenerative medicine and tissue engineering, offers novel and innovative strategies to managing goats with tibial bone defects. biochemical and histological analysis results demonstrated that the use of HA or DECM as implantation improved bone repair. The acquired results showed that in the injured long bone area, HA and DECM induce a positive reaction.

4.1 Discussion of Histological Evaluation

Histological evaluation did not show any fibrous encapsulation or inflammatory processes at the bone–scaffold interface in both treated groups.

Based on the results of the histopathological examination, the animals treated with HA or dDBM had better healing than the animals in the control group. This is because the animals in the treatment groups for HA and DECM demonstrated more advanced healing criteria. Natural bone minerals contain hydroxyapatite, a crystalline form of calcium phosphate that has showed significant promise as an implant material due to its initial mechanical stiffness and structure, as well as its osteo-conductive and angiogenic qualities in vivo [14,15,16].

In the treated groups, newly generated osteoid tissue with fully developed Haversian canals nearly healed the bone deficit. It supported the results of prior investigations in which HA was implanted in rabbit ulnae and rat skulls [17,18]. According to Simmons [19] and Alexander [20], the porous HA in these cases served as a scaffold for the ingrowth of vasculature and subsequent deposition of new bone.

The Xeno-group bone histological section reveals woven bone with regular collagen fiber deposition in the fibrovascular stroma and a fracture callus made of a mature fibro-osteoid combination. decellularized extracellular matrix as scaffold encourages capillary ingrowth, osteoblast migration and proliferation, and osteogenesis [21].

According to Bigham [22] research, a group of low-molecular-weight glycoproteins, including bone morphogenetic proteins (BMPs), are the main osteoinductive element of DBM. These osteoinductive growth factors are hidden inside the mineralized matrix and become visible when cortical bone decalcifies, which accelerates the process of bone production. These proteins encourage mesenchymal cells to differentiate into chondroblasts, which is followed by endochondral osteogenesis, which produces new bone.

When dDBM and synthetic HA were used to compare the regeneration of bone defects in goat tibia bone, it was shown that there was no difference in bone neoformation between the two. The same thing was seen in Pires [23], where there was no statistically significant difference in the histomorphometric evaluation between group 2 (regenerated with a bovine xenograft) and group 1 (regenerated with synthetic hydroxyapatite).

4.2 Discussion of Bone Markers

There are various ways to use certain bone markers to analyze newly produced bone. Alkaline phosphatase (ALP) and osteocalcin (OC) are markers of bone production; ALP is selective for bone formation, but only in the absence of liver or bile duct disease in the patient. OC is another particular osteoblast product , which comes in a variety of active forms [24].

4.3 Alkaline Phosphatase (ALP)

The enzyme which synthesized by osteoblast, play crucial role in processes of bone development and mineralization. The measurement of alkaline phosphatase activity in blood stream can serve as an indicator of the rate at which bone is being formed. This is because the quantity of alkaline phosphatase produced by osteoblast may be reflected in the blood level of this enzyme [25].

The treated groups had significantly higher serum alkaline phosphatase (ALP) concentrations than the control group, which may suggest that using dDBM or HA can raise ALP levels. Increased osteoblast activity could be the cause of the raised serum ALP level.

When comparing the Xeno-group to the control, there was a higher rise in ALP. A possible reason for this could be the placement of the osteoinductive biomaterial dDBM at the fracture site, which stimulates osteoblastic activity even more. According to Umashankar and Ranganath [26], An substantial increase in serum ALP levels indicates elevated osteoblastic activity. Large amounts of ALP are secreted by osteoblasts, and this enzyme is necessary for the creation and mineralization of bone matrix.

Osteoblasts can differentiate on almost all of HA surfaces, according to (Shu et al., [27]. This is consistent with HA capacity to facilitate osteoblast adhesion and encourage bone growth inside implants. Moreover, alkaline phosphatase activity showed that HA promotes osteoblasts' early development.

In a study of femur fracture repair in dogs, Singh et al., [28] also observed a significant increase in mean serum alkaline phosphatase values in the demineralized bone matrix xenograft group. This study strongly supports our findings.

4.4 Osteocalcin (OC)

When there is a rapid turnover of bone, osteoblasts make more osteocalcin. Osteocalcin is thought to be a marker of bone turnover rather than a particular indication of bone formation because it is released into the bloodstream during bone resorption from the matrix. and considered specific marker of osteoblast activity [29].

In this investigation, 14 days after the surgery, osteocalcin levels in the treated groups increased significantly ($P<0.05$). This could suggest that implantation of dDBM or HA into the tibial bone defect can raise blood osteocalcin levels considerably. elevated osteoblast activity and callus formation at the fracture site could be the cause of this.

Osteocalcin increased gradually during the healing of femur neck fractures, starting in the first week following surgery and increasing significantly by the eighth week, according to Ohishi et al., [30]. Osteoblast cell activity and callus formation at the fracture site are the reason of this little increase in OC [31].

Wang et al., [32] discovered that HA particularly the HA nanospheres, may significantly improve the osteoblastic development of rat mesenchymal stem cells.

According to Zhou et al., [33], HA size has a significant impact on how bone-associated cells behave biologically. According to research by Yang et al., [34] and Huang et al., [35], suggesting that smaller HA may be more effective than bigger HA in promoting osteogenic differentiation of MSCs. These outcomes agree with the research we conducted, which showed that 50 μm nanoparticle size boosted more osteoblast activity and, consequently, elevated levels of ALP and osteocalcin.

This study agrees with Yang et al., [34] findings, which show that the high concentration of $Ca2+$

released by HA may have an impact on the osteogenic differentiation of stem cells. It has been demonstrated that calcium ions (Ca2+) influence stem cell development and osteogenic differentiation [36]. The degree of cell mineralization was considerably enhanced by higher Ca2+ concentrations [37]. According to Zayzafoon [38], Ca2+ is crucial for preserving cell development and functionality. Additionally, the MAPK signaling pathway, which is crucial for promoting cell differentiation, can be activated by Ca2+ [39].

dDBM may raise OC levels by promoting osteogenesis. Because non-collagenous matrix proteins and osteogenic factors (such as BMPs) are preserved in the DBM and become visible after partial demineralization,. Moreover, osteoinduction can be a reaction to collagen type I directing MSC differentiation along the proper lineage. Through α2β1 integrin binding, collagen type I is known to trigger osteoblastic differentiation of bone marrow cells [40].

Additionally, Sawkins et al., [41] found that throughout the in vitro scaffold breakdown process, component chemicals released from the bECM material promote cell proliferation.

Similar findings to Liu et al., [42], who demonstrated that Dioxide Decellularized Bone Matrix (scDBM) by itself significantly ($p < 0.05$) raised osteocalcin expression in comparison to control groups, suggesting that scDBM stimulated osteocalcin expression in bone regeneration and new bone production.

5. CONCLUSION

We may conclude that our study's use of HA and dDBM had a favorable effect on the development of new bone. Both biomaterials had the potential to be osteoinductive and were very biocompatible, osteoconductive, and well-integrated.

After 64 days following surgery, histological results showed that the HA and DECM material had better and faster bone growth.

To sum up, HA powders are an incredibly easy way to adjust for the best osteoinductive and conductive responses in vivo. In line with its physiologic osteoconductive characteristic, HA material encouraged the growth of bone over the defect. The implanted substance did not cause any inflammation to be noticed.

Notwithstanding the limitations of this investigation, it was shown that both synthetic HA and DECM enhanced bone defect repair. Nonetheless, additional research needs to be done to validate these findings.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ETHICAL APPROVAL

This work was approved by the Ethics Committee for the Use of Experimental Animals (17/37/2024), College of Veterinary Medicine, University of Basra.

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The authors are grateful to Bio-vet Veterinary Laboratories, Basrah, Iraq for granting them permission to use their facilities for this experiment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Walsh WR, Oliver RA, Christou C, Lovric V, Walsh ER, Prado GR, Haider T. Critical size bone defect healing using collagen–calcium phosphate bone graft materials. PloS one. 2017;12(1):e0168883.
2. Nair MB, Varma HK, Menon KV, Shenoy SJ, John A. Tissue regeneration and repair of goat segmental femur defect with bioactive triphasic ceramic-coated hydroxyapatite scaffold. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2009;91(3):855-865.

3. Kumar P, Vinitha B, Fathima G. Bone grafts in dentistry. Journal of Pharmacy and Bioallied Sciences. 2013;5(Suppl1) :S125-S127.
4. Wang Y, Zong Y, Chen W, Diao N, Zhao Q, Li C, He Z. Decellularized antler cancellous bone matrix material can serve as potential bone tissue scaffold. Biomolecules. 2024;14(8):907.
5. Zhou S, Zhang S, Jiang Q. Extracellular matrix-based materials for bone regeneration. racing for the surface: Antimicrobial and Interface Tissue Engineering. 2020;489-533.
6. Emami A, Talaei-Khozani T, Tavanafar S, Zareifard N, Azarpira N, Vojdani Z. Synergic effects of decellularized bone matrix, hydroxyapatite, and extracellular vesicles on repairing of the rabbit mandibular bone defect model. Journal of translational medicine. 2020;18:1-18.
7. Pang S, Su FY, Green A, Salim J, McKittrick J, Jasiuk I. Comparison of different protocols for demineralization of cortical bone. Scientific Reports. 2021;11 (1):7012.
8. Gardin C, Ricci S, Ferroni L, Guazzo R, Sbricoli L. Decellularization and delipidation protocols of bovine bone and pericardium for bone grafting and guided bone regeneration procedures. 20151–26. Available:https://Doi.Org/10.1371/Journal. Pone.0132344
9. Giudice RLo, Rizzo G, Centofanti A, Favaloro A, Rizzo D, Cervino G, Squeri R, Costa BG, Fauci VLa, Giudice G Lo. Steam sterilization of equine bone block : Morphological and Collagen Analysis; 2018.
10. Smith JS, Schleining J, Plummer P. Pain management in small ruminants and camelids: Analgesic Agents. Veterinary Clinics of North America - Food Animal Practice. 2021;37(1):1–16. Available:https://doi.org/10.1016/j.cvfa.2020.12.001
11. Azam MQ, Sadat-Ali M. Quality of life in sickle cell patients after cementless total hip arthroplasty. Journal of Arthroplasty. 2016;31(11):2536–2541. Available:https://doi.org/10.1016/j.arth.2016.04.025
12. Nagyová V, Arfuso F, Rizzo M, Zanghì E, Nagy O, Piccione G. Stability of total proteins and their electrophoretic fractions in goat serum (Capra hircus), maintained under different condition. Small ruminant research. 2016;144:145-148.
13. Wick MR. The hematoxylin and eosin stain in anatomic pathology—An often-neglected focus of quality assurance in the laboratory. In Seminars in diagnostic pathology. WB Saunders. 2019;36(5):303-311.
14. Kilian O, Wenisch S, Karnati S, Baumgart-Vogt E, Hild A, Fuhrmann R, Franke RP. Observations on the microvasculature of bone defects filled with biodegradable nanoparticulate hydroxyapatite. Biomaterials. 2008;29(24-25):3429-3437.
15. Appleford MR, Oh S, Oh N, Ong JL. In vivo study on hydroxyapatite scaffolds with trabecular architecture for bone repair. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2009;89(4):1019-1027.
16. Yoshikawa T, Ohgushi H, Nakajima H, Yamada E, Ichijima K, Tamai S, Ohta T. *In vivo* osteogenic durability of cultured bone in porous ceramics: A novel method for autogenous bone graft substitution. Transplantation. 2000;69(1):128.
17. Singh S. Reconstruction of segmental ulnar defect with tricalcium phosphate calcium hydroxyapatite and calcium hydroxyapatite bone matrix combination in rabbit (Doctoral dissertation, IVRI); 1998.
18. Uchida A, Nade SM, McCARTNEY ER, Ching WILLIAM. The use of ceramics for bone replacement. A comparative study of three different porous ceramics. The Journal of Bone & Joint Surgery British Volume. 1984;66(2):269-275.
19. Simmons DJ. Fracture healing. Fundamental and clinical bone physiology. Philadelphia, PA: JB Lippincott. 1980;283-330.
20. Alexander H, Parsons JR, Ricci JL, Bajpai PK, Weiss AB. Calcium-based ceramics and composites in bone reconstruction. CRC Critical Reviews in Biocompatibility. 1987;4(1):43-77.
21. Amini Z, Lari R. A systematic review of decellularized allograft and xenograft–derived scaffolds in bone tissue regeneration. Tissue and Cell. 2021; 69:101494.
22. Bigham AS, Dehghani SN, Shafiei Z, Torabi Nezhad S. Experimental bone defect healing with xenogenic

demineralized bone matrix and bovine fetal growth plate as a new xenograft: radiological, histopathological and biomechanical evaluation. Cell and tissue banking. 2009;10:33-41.
23. Pires JLDS, de Carvalho JJ, Pereira MJDS, Brum IDS, Nascimento ALR, Dos Santos PGP, Fischer RG. Repair of critical size bone defects using synthetic hydroxyapatite or xenograft with or without the bone marrow mononuclear fraction: A histomorphometric and immunohistochemical study in rat calvaria. Materials. 2021;14(11):2854.
24. Rowe P, Koller A, Sharma S. Physiology, bone remodeling; 2018.
25. Ansari S, Ito K, Hofmann S. Alkaline phosphatase activity of serum affects osteogenic differentiation cultures. ACS omega. 2022;7(15):12724-12733.
26. Umashankar S, Ranganath L. Osteomedullographic studies during fracture healing in dogs; 2008.
27. Shu R, McMullen R, Baumann MJ, McCabe LR. Hydroxyapatite accelerates differentiation and suppresses growth of MC3T3-E1 osteoblasts. Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials. 2003;67(4):1196-1204.
28. Singh P, Dwivedi DK, Kushwaha RB, Kumar S, Sharma A, Gupta P, Bhardawaj HR. Clinical evaluation osteogenic properties of demineralised bone matrix (DBM) xenograft in femur fracture in dogs. Indian Journal of Animal Research. 1, 6.
29. Delmas PD, Eastell R, Garnero P, Seibel MJ, Stepan J, Committee of scientific advisors of the international osteoporosis foundation. The use of biochemical markers of bone turnover in osteoporosis. Osteoporosis International. 2000;11(6):S2.
30. Ohishi T, Takahashi M, Kushida K, Hoshino H, Tsuchikawa T, Naitoh K, Inoue T. Changes of biochemical markers during fracture healing. Archives of Orthopaedic and Trauma Surgery. 1998; 118:126-130.
31. Pedersen BJ, Schlemmer A, Hassager C, Christiansen C. Changes in the carboxyl-terminal propeptide of type I procollagen and other markers of bone formation upon five days of bed rest. Bone. 1995;17(1):91-95.
32. Wang J, Yang G, Wang Y, Du Y, Liu H, Zhu Y, Zhang S. Chimeric protein template-induced shape control of bone mineral nanoparticles and its impact on mesenchymal stem cell fate. Biomacromolecules. 2015;16(7):1987-1996.
33. Zhou C, Hong Y, Zhang X. Applications of nanostructured calcium phosphate in tissue engineering. Biomaterials Science. 2013;1(10):1012-1028.
34. Yang X, Li Y, Liu X, Zhang R, Feng Q. *In vitro* uptake of hydroxyapatite nanoparticles and their effect on osteogenic differentiation of human mesenchymal stem cells. Stem Cells International. 2018;1:2036176.
35. Huang Y, Zhou G, Zheng L, Liu H, Niu X, Fan Y. Micro-/nano-sized hydroxyapatite directs differentiation of rat bone marrow derived mesenchymal stem cells towards an osteoblast lineage. Nanoscale. 2012; 4(7):2484-2490.
36. Lei Q, Chen J, Huang W, Wu D, Lin H, Lai Y. Proteomic analysis of the effect of extracellular calcium ions on human mesenchymal stem cells: Implications for bone tissue engineering. Chemico-Biological Interactions. 2015;233:139-146.
37. Liu YK, Lu QZ, Pei R, Ji HJ, Zhou GS, Zhao XL, Zhang M. The effect of extracellular calcium and inorganic phosphate on the growth and osteogenic differentiation of mesenchymal stem cells in vitro: implication for bone tissue engineering. Biomedical Materials. 2009;4(2):025004.
38. Zayzafoon M. Calcium/calmodulin signaling controls osteoblast growth and differentiation. Journal of Cellular Biochemistry. 2006;97(1):56-70.
39. Agell N, Bachs O, Rocamora N, Villalonga P. Modulation of the Ras/Raf/MEK/ERK pathway by Ca2+, and calmodulin. Cellular Signalling. 2002;14(8):649-654.
40. Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-α2β1 integrin interaction. Journal of cellular physiology. 2000;184(2):207-213.
41. Sawkins MJ, Bowen W, Dhadda P, Markides H, Sidney LE, Taylor AJ, White LJ. Hydrogels derived from demineralized and decellularized bone extracellular matrix. Acta Biomaterialia. 2013;9(8):7865-7873.

42. Liu KF, Chen RF, Li YT, Lin YN, Hsieh DJ, Periasamy S, Kuo YR. Supercritical carbon dioxide decellularized bone matrix seeded with adipose-derived mesenchymal stem cells accelerated bone regeneration. Biomedicines. 2021;9(12):1825.

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