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RADIOLOGICAL AND CLINICAL EVALUATION OF INFLUENCE OF HYDROXYAPATITE NANOPARTICLES AND DEMINERALIZED AND DECELLULARIZED BONE MATRIX ON DEFECT FRACTURE HEALING OF TIBIA BONE IN GOATS

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ABSTRACT

Implants made of biomaterial and biological scaffolds are the primary means of treating bone defects and encouraging their repair. Researchers are looking into hydroxyapatite (HA) and decellularized bone matrix (DBM) as possible osteogenic agents in the aim of using these materials to stimulate bone growth in various defects in the bone. This study was done to investigate the effect of hydroxyapatite and Demineralized and decellularized Bone Matrix (dDBM) xenograft on fracture healing in goat. Fifteen young goats were used in this study. The animals were divided into three groups (n=5) as the following, control group, Nano-group, and Xeno-group. In all animals, 2 mm removed from the tibia of right hind limp by using bone drill. The bone defect in the control group remained without treatment, while the defect was filled with hydroxyapatite nanoparticles in Nano-group, and xenograft Demineralized and decellularized Bone Matrix (collected from sheep tibia) in Xeno-group. The operation site was closed routinely and the animals were monitored clinically and radiologically along study period. Clinical examination of lameness in all animal groups showed evident lameness right after surgery. Lameness score improved significantly 11 days after surgery in treated groups (Nano and Xeno groups) compared with control group. Comparing the radiological results of the Nano and Xeno groups to the control group at 9 weeks after surgery, the treated groups displayed complete callus development, which was characterized by profuse periosteal response and complete closure of the generated bone gap. The tibial radiographic union scale (RUST) for the treated groups (Nano and Xeno groups) score significant statistical difference ($P<0.05$) compared to the results of RUST in the control group at 6 and 9 weeks after procedure. In conclusions, using of hydroxyapatite nanoparticles and xenograft demineralized and decellularized bone matrix in bone defect fracture has a positive effect on fracture healing through their ability as osteoconductive and osteoinductive biomaterials.

1. Introduction

Tibia, is the weight-bearing bone in the leg and is crucial for the stability, weight carrying, and mobility of the hind limbs [1]. Tibia is at risk for several kinds of fractures. A high energy trauma, diseases, developmental abnormalities, revision surgery, tumor removal, or osteomyelitis can all contribute to big segmental bone defects, also known as critical sized defects, which are extreme conditions in bone healing [2]. Broken or damaged bones recover themselves through a process called bone healing. To reinstate the bone's structural integrity and functionality, a complex biological process involving multiple stages and cellular activity is involved [3]. One of the surgical techniques most frequently employed in orthopedic procedures to promote bone regeneration is bone grafting. The primary combined purposes of bone substitutes are to provide osteo-regeneration and mechanical support. which include osteoconduction, osteoinduction, and osteogenesis—three crucial biological characteristics [4]. Autogenous, allogeneic, xenogenous, and alloplastic bone grafts, such as synthetic hydroxyapatite (HA) and ß-tricalcium phosphate (ß-TCP), are commonly used in bone repair techniques. Any tissue or organ created from a species other than the recipient of the specimen is referred to as a xenograft. Xenografts do have a disadvantage of potentially inducing unfavorable host immunological and inflammatory responses [5].

Generally, xenograft is created through the decellularization of naturally derived tissues, which aims to remove all cellular components of the native tissue while preserving as much of the original extracellular matrix (ECM) structure and composition as feasible [6]. The low immunogenicity scaffold material achieved by physical or chemical removal of cells from the tissue matrix is called decellularized matrix (DECM). DECM is a kind of complex network of macromolecules and a complex component of the material [7]. One of the most significant technological developments of the twenty-first century is nanotechnology, which is producing new materials with a variety of uses in human and veterinary health [8]. Hydroxyapatite (HAp) is calcium mineral occurs naturally and accounts for around half of the mass of bone. There is no denying its superior osteoconduction and osteointegration qualities. When implanted in vivo, they encourage the adhesion, growth, and differentiation of bone cells. They also aid in revascularization, which triggers the production of new bone. In addition to having good cell conductivity and enabling a good fibrin network structure, synthetic HAp is typically employed in bone transplant procedures and exhibits an outstanding biological response [9]. The current study aims to assess the effectiveness of two distinct graft biomaterials (HAp and DBMX) in tibia bone defects regeneration utilizing clinical indicators (lameness score), and the radiographic union scale in tibial fracture (RUST).

2. MATERIALS AND METHODS

Animals: Fifteen young female goats (local breed) weighing (20-25) kg and aged (6-7) months were used in this study. The animals were housed in the College of Veterinary Medicine's animal farm house in individual cages. The University of Basra's College of Veterinary Medicine's Ethics Committee for the Use of Experimental Animals gave its approval to this work.

Preparation of xenograft: The tibia was obtained from 6–8-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% volume [10]. 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 [11]. 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 [11].

Sterilization of xenograft (DBM): The DBM was sterile in an autoclave. Sterilization cycle at 134°C at 2,16 bars for 3.30 min. of sterilization and 5 min. of drying [12]; prior to being implanted in the bone defect.

Surgical preparation: Before the procedure, all goats fasted for 8 hours. The right lower leg was clipped and shaved from the knee to the ankle. The area was then thoroughly cleaned with distal water, and the surgical site was scrubbed for two to three minutes using diluted liquid soap. Next, an antisepsis (ethanol alcohol 70%) was applied to the entire clipped area, and lastly 2.5% tincture iodine was applied to the incision site.

Surgical procedure: The animals were anesthetized using anesthetic protocol utilizing a combination of xylazine (0.05–0.1 mg/kg) and ketamine (5–10 mg/kg) IM. A subcutaneous injection of 1 mL of 2% lidocaine was also administered around the operating site [13]. The surgical site in each goat was prepared using aseptic techniques. The tibia bone was exposed by a 3–5 cm long incision made cranio-laterally through the skin and soft tissue. In all experimental animals, a 2mm core lesion was extracted using a bone drill from the tibia's mid-shaft. sterile warm isotonic saline was dropped during the drilling process [14]. 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. In Xeno-group, the bony defect was filled with xenograft demineralized and decellularized bone matrix taken from sheep tibia (Figure1). The surgical site was closed routinely in all experimental goats.

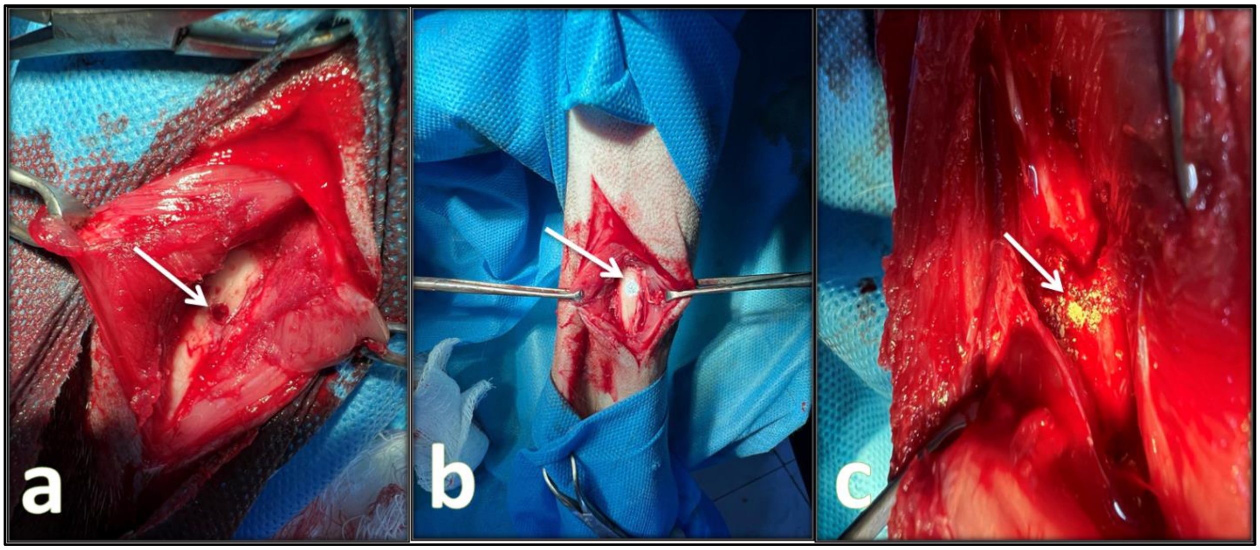

Figure 1: a: control group, b: Nano-group c: Xeno-group

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/ was administered intramuscularly for7 days after the surgery.

Clinical evaluation: The animals were monitored daily for three weeks post-surgery to evaluate lameness status. The lameness score was assessed according to gait scoring description by [15].

Table 1: Description of the 5-point gait scoring system previously developed for use in dairy goats [15].

Gait scoring system		Assessment criteria					
Category	5-point	Limp	Moving forward	Weight bearing	Head nod	Identify affected leg(s)	Other descriptors
Normal gait	1	no	Yes	Yes	No		Even stride on all four legs, tracking up, walks with a fluid motion.
Uneven gait	2	no	Yes	Yes	No	No	Shorter stride, not tracking up, joints slightly stiff, inward or outward swinging of a hoof at each stride
Mildly lame	3	yes	Yes	Yes	No	Possibly	One or more legs may be affected. Observer may not be able to determine affected leg(s). Mild limp
Moderately lame	4	yes	Reluctant	Reluctant	Possibly	Yes	One or more legs may be affected. Moderate limp or slight goose stepping
Severely lame	5	yes	Unwilling/ unable	Unable	Yes	Yes	One or more legs may be affected. Severe limp or walking on knees, or pronounced high goose stepping

Radiological evaluation: The bone healing process of the induced defect was assessed radiologically in the following intervals: post-surgery,14,42, and 64 days. Healing at the lateral cortice was evaluated using the Radiographic Union Scale for Tibial fractures (RUST), modified by [16].

Table 2: Description of the 3-point of radiographic union scale in tibial fracture (RUST) [16].

Criteria for application of Radiological Union Score for Tibia to a radiograph.		
Score	fracture line	callus
Score = 1	Visible fracture line,	without callus
Score = 2	Visible fracture line,	with callus
Score = 3	No fracture line,	with visible callus

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

Clinical observation: There were no signs of local infection and no rejection of biomaterials. However, all tibial defects were healed without signs of complications in all goats in the experimental study and all external wounds healed normally within 10-12 days' post-surgery.

Lameness: Lameness scoring was recorded according to [15]. On day zero (pre- operation), all animals showed normal walking. The pathognomonic signs (lameness) on the affected limb were noticed in all goats immediately postsurgical operation, then increased during 24-48 hrs., while at days 3, 7, 11 and 14, There was a gradual improvement in the treated groups and there was a statistically significant difference ($P<0.05$) in treated groups comparing with control group (Figure 2).

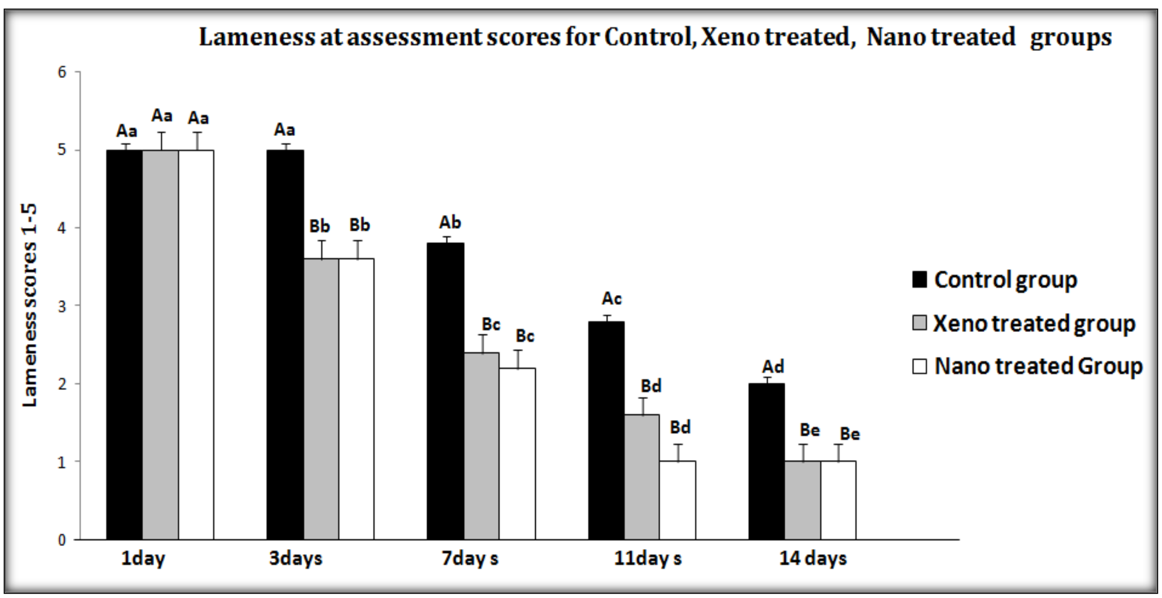

Figure 2: Clinical study showed lameness assessment scores for Control, Xeno treated, Nano treated groups during different periods (1, 3, 7, 11 and 14 days post-surgery). AB Different letters among groups indicates significant differences ($P<0.05$). abcde Different letters within group in different times indicates significant differences ($P<0.05$).

Radiological evaluation: Immediately post-operation, the radiographic findings in all groups, revealed well clear of the radiolucent of tibia defect, and no signs of periosteal reaction around the fracture gap (Figure 3)

Figure 3: All groups immediately post-operation a: control group b: Nono-group c: Xeno-group

Two Week Post-operation: The radiographic findings of both treatment groups after Nano and Xeno therapy revealed high soft callus formation and bony mineralization at bone gab, and increased the periosteal reaction compared with the control group which showed clear radiolucent in the gap of the tibia bone and slight periosteal reaction (Figure 4). RUST findings reveal no statistically significant variation between the groups (Figure 7).

Figure 4: All groups two weeks post-surgery a: control group b: Nono-group c: Xeno-group

Six Week Post-operation: The radiographic findings at 6th week's post-operative in treatment groups revealed hard callus formation and radiopaque of bone defect with good signs of healing process in Nano and Xeno groups. Compared to the control group, which showed little amount of the new callus formation (Figure 5). For the RUST test, there was a statistically significant difference ($P<0.05$) between the treated groups and the control group (Figure7).

Figure 5: All groups 6 weeks post-surgery a: control group b: Nono-group c: Xeno-group

Nine Week Post-operation: The radiographic findings at 9th week's postoperative in treatment groups revealed complete hard callus formation and disappeared of bone defect (radiopaque) with good signs of healing process in Nano and Xeno groups. Compared to the control group, which showed little amount of the new callus formation (Figure 6). When comparing the treated groups to the control group, there was statistically significant differences for the RUST test (Figure 7).

Figure 6: All groups 9 weeks post-surgery a: control group b: Nono-group c: Xeno-group

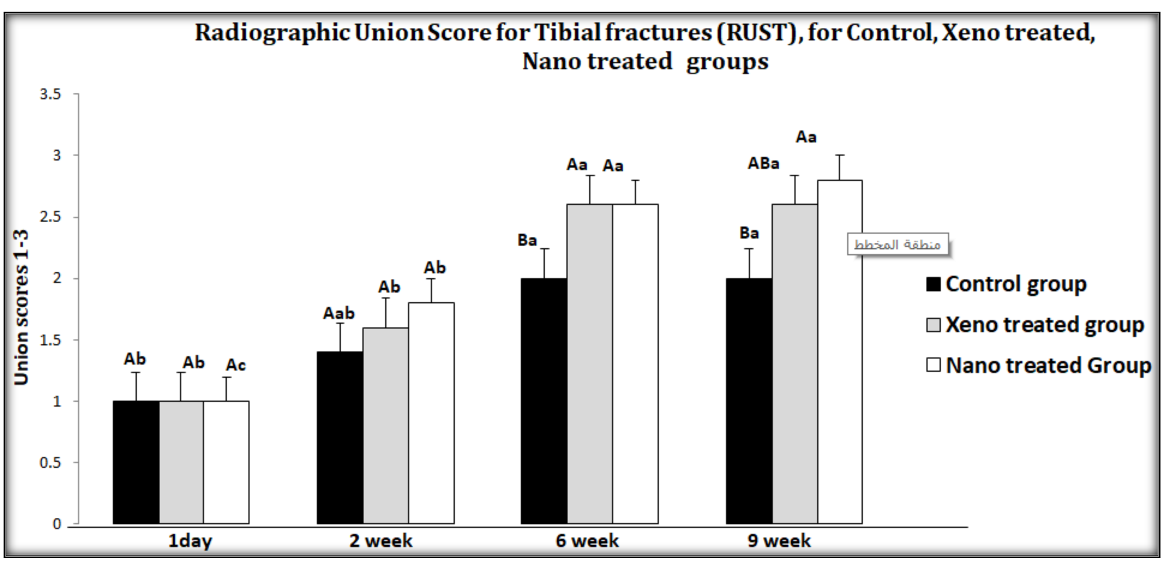

Figure 7: Radiological study showed radiographic union score for tibial fractures (RUST) for Control,

Xeno treated, Nano treated groups during different periods (1 day , 2, 6 and 9 weeks post-surgery). AB Different letters among groups indicates significant differences ($P<0.05$). abc Different letters within group in different times indicates significant differences ($P<0.05$).

4. DISCUSSION

Bone grafting has evolved to meet the needs of bone reconstruction. Hydroxyapatite and xenograft decellularized bone matrix (XDBM) are both materials that have shown promise in promoting bone healing, each with its unique properties and applications. The performance of XDBM and hydroxybitite as bone substitutes was investigated in vivo in the current study. At 1, 3, 5, 9, and 11 days following surgery, the rate of lameness was evaluated using the gait score description provided by [15]. At 2, 6, and 9 weeks following surgery, the rate of bone formation was assessed radiographically using the Radiographic Union Scale for Tibial Fractures (RUST), which was modified by [16]. The use of hydroxyapatite nanoparticles and Xenograft decellularized bone matrix could be one possible strategy providing all the necessary characteristics for bone repair and regeneration. They had an effective role and showed a significant effect of the treatment compared to the control group, and this was evident through radiological evaluation and examination of lameness.

Clinically, when bacterial and viral infections occur, that lead to uncontrolled immune damage and lead to implants failure [17]. During the study there were no signs of local infection. All external wounds healed normally within 10-12 days' post-surgery. However, [18] reported that, It is possible to create a highly immunocompatible decellularized scaffold by employing the efficient decellularization method.. There were no xenograft rejections in the xeno group during the research, which is evidence of the efficacy of the decellularization technique that used. No indications of hydroxyapatite rejection were observed during this investigation. Nevertheless, [19] revealed that, since hydroxyapatite (HAp) shares chemical similarities with the mineral that forms up bones, it is a bioceramic that has been extensively explored. Additionally, it is thermodynamically stable, biocompatible, and bioactive in bodily fluids.

Pathognomonic signs (lameness) on the affected limb were noticed in all goats' immediately postsurgical operation then increased during 24-48hrs. Pain during the postoperative phase may cause by tissue congestion. It is thought that greater capillary permeability raises intracellular pressures, irritating nearby nociceptors in the process [20]. At days 3, 7, 11 and 14; there was a significant improvement in the treated groups. This might be because the inflammatory phase of the fractures healed more quickly in the treated groups, even though the implantation of hydroxyapatite or xenografts is thought to speed up the osteoinduction process. Thus, [18] reported that, decellularized extracellular matrix, facilitates particular biomechanical conditions and biochemical signals that impact adhesion, proliferation, and the determination of cell fate. Interestingly reported by [21], the Bovine hydroxyapatite-based scaffold and synthetic hydroxyapatite accelerated the inflammatory phase by a high number of M2 (a protein is expressed on the membrane of macrophages and monocytes, particularly M2c macrophages), which possess anti-inflammatory activity [22] at the early phase of implantation. These results support our findings by showing that hydroxyapetite may enhances weight bearing by accelerating up the inflammatory process. The findings of this investigation corresponded with those of [23] who used huacaya alpaca, wherein a debrided fracture site was treated with a bovine xenograft in order to encourage and utilize osteogenesis in situ. which stated that 10 days after operation, the alpaca progressively improved its weight bearing once the bandages were removed. In the same way after surgery, all rabbits had lameness and decreased appetite; however, following a week of implantation of Bone-like hydroxyapatite/poly amino acid, the rabbits gradually recovered [24]. Another study conducted by [25] investigated the use of xeno-bovine bone implantation in rabbits with femoral defects and shown that, at the end of the fourth week following surgery, the animals were able to bear weight, walk, and run properly with no complications or bodily rejection.

Radiography has historically been the most widely used method for assessing fracture healing because of its inexpensive cost, widespread availability, and relatively low radiation profile. The two processes that are easiest to spot on radiographs when assessing fracture healing are the development and growth of an exterior callus and the bridging of the fracture line by callus [26]. Following the procedure, all groups' radiographic results demonstrated a radiolucent image of the tibial defect and no evidence of periosteal response surrounding the fracture gap. This results from the defective area's lack of X-ray absorption. However, the hydroxyapatite implant is visible as a radiopaque region in the Nano-group on x-rays. This is probably because hydroxyapatite nanoparticles contain calcium, phosphorus, oxygen, and hydrogen atoms. Calcium, which is a major component, has a relatively high atomic number (20). In X-ray imaging, materials with higher atomic numbers tend to absorb more X-rays, leading to reduced transmission of X-rays through the material [27]. This result has a lighter appearance on the X-ray image, making it radiopaque. The same finding showed a much higher absorption of X-rays for Fluorapatite and Hydroxyapatite bone substitutes immediately post-operation [28]. Additionally, [29] also found that the hydroxyapatite's radiodensity was comparable to that of the host bone. Demineralized bone may lose structural rigidity (due to processing) and the inability to visualize the material radiographically (due to its inherent radiolucency) [30]. These observations are consistent with the radiological images immediately after operation for the Xeno-group in this study, as the images showed radiolucent area of the defect implanted with demineralized and decellularized bone matrix. At two Weeks after Surgery: the radiographic results for both treated groups showed increased periosteal reaction, high soft callus formation, and bony mineralization at bone gab. This can be as a result of the several ways that hydroxyapatite quickens the production of new bone. Bioactively binding to bone tissue, hydroxyapatite can also stimulate bone cells during the process of bone formation (osteoconductive). As a biomaterial that is conductive to host cells and has the ability to promote cell growth, differentiation, and migration, hydroxyapatite serves as a scaffold [31]. Because decellularized extracellular matrix has unique osteoinductive and ostioconductive qualities, it may also hasten the process of bone formation. It has been documented that localized bone differentiation results by implanting coarse demineralized extracellular matrix powders (74-420 μm) under the skin [32]. Following the osseograft's insertion into the osseous defect, mesenchymal-type cells sequentially differentiate to produce bone and cartilage [33].

Additionally, it's possible to observe that hydroxyapetite was not fully absorbed. The current study's findings agree with those of [34], in which Dense hydroxylapatite appears to be attacked by macrophages and TRAP-positive multinu- cleated cells but the resorbing rate is so low that no decrease in the biomaterial volume could be found after six months. The composition and crystallinity of the HA determine how quickly it dissolves. Biodegradation is significantly impacted by a number of factors, including the Ca/P ratio, impurities like F- or Mg2+, the degree of micro- and macro-porosities, and defect structure. The type and concentration of the surrounding solution, the solution's pH, its saturation level, the ratio of solid to solution, and the duration of the suspension in the solution are all factors that affect the rate of dissolution [29], [35]. After six weeks of surgery, the radiography results showed that the Nano and Xeno groups had good signs of healing. By the ninth week, the bone defect had completely vanished, and a complete, hard callus had formed. this could be because the decellularized bone matrix at Xeno-group preserves the microstructural features like micropores and surface roughness as well as the macrostructural topographies like highly porous structure and geometry [18], which significantly improve the osteoinductivity. This study's findings concurred with those of [36] which mentioned that HA composites are thought to be bioactive and have shown promise in treating bone defects in both human and animal patients, while [29] used Bengal goats. Radiographs taken on day 90 showed that the cortical defect that filled with a HAp strut was completely bridged throughout the axis of the radius. Also [37] reported a zone of callus formation surrounding the bone graft and complete healing with increased radiopacity were observed in the treatment group receiving hydroxyapatite nano gel. Hydroxyapatite has been shown in studies on dogs [38] and cats [39] to be able to stimulate bone development, making it appropriate for use in small animal surgery. The current study's findings corroborated those of [34], who found that xenogeneic bone T 650 was a good osteoconducting and resorbable material. It seems to repair architecture and bone mass similarly to an autograft.

According to [40], Xeno bone implantation can support body weight, fill in gaps, and encourage healing with minimal immunogenicity. Bovine-xenograft used to treat bone defects in rabbits [41] and Xeno-Sheep implants in rabbits [25] who showed interested results. Another study [25] demonstrates that the xeno-bovine bony implantation (XBBI) exhibits abundant external callus production along with a notable increase in the density of the sclerotic area. Additionally, New Zealand white rabbits were used in the investigation by [42]. demonstrate how xenografts like InterOss® and Bone + B® can both enhance bone growth.

5. CONCLUSIONS

Using of hydroxyapatite nanoparticles and xenograft demineralized and decellularized bone matrix in tibia bone defect fracture has a positive effect on fracture healing through their ability as osteoconductive and osteoinductive biomaterials. Demineralized and decellularized bone matrix and hydroxyapatite nanoparticles improve the regeneration of damaged bone. The use of hydroxyapatite nanoparticles and demineralized and decellularized bone matrix from xenografts can accelerate the healing process of tibia bone fractures and facilitate the restoration of tissue structure and function. Two different treatment approaches were employed in this study: xenograft demineralized and decellularized bone matrix and hydroxyapetite. As far as clinical treatment of tibial bone defects is concerned, hydroxyapitite appears to be a simple, easy, low-cost option compared to the more complex and costly preparation of demineralized and decellularized bone matrix.

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CONFLICT OF INTEREST

There are no conflicts of interest, according to the authors.

AUTHORS CONTRIBUTIONS

AHA and AAA –Development of the Methodology, preparing and writing the initial draft, review and editing the manuscript and analyze the data. AHA and AAA –Preparing animals, surgical operations, laboratory analysis, and analyze the data. AAA and WMMS –Looked over the document, review and editing the manuscript, provided feedback, and approved the final version.

ETHICAL APPROVAL

This research was designed and done in accordance with all applicable national and international regulations as well as ethical principles followed by the Animal Care and Use Committee/College of

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