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Histopathological examination of the effectiveness of nonvascularized tibia and femur bone allografts on bone healing in rat tibia fractures

Examen histopatológico de la efectividad de los aloinjertos óseos no vascularizados de tibia y fémur en la curación ósea en fracturas de tibia de rata

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ABSTRACT

The aim of this study was to histologically evaluate the healing processes of tibia fractures reconstructed with allogeneic bone grafts taken from different anatomical regions (tibia and femur). Twenty-five Sprague-Dawley rats were used in the study. The rats were divided into four groups: fracture control (n = 7), tibia allogeneic bone transplant (n = 7), femur allogeneic bone transplant (n = 7), and donor group (n = 4). Corticocancellous bone blocks (5×5 mm) taken from the donor animals were fixed to the fracture sites created in the tibias of recipient rats with Kirschner wires. At the end of the 4-week healing period, the rats were sacrificed, and the tibias were examined histologically. Tissue samples were fixed in 10 % neutral formalin and decalcified, then embedded in paraffin and stained with hematoxylin-eosin. The new bone formation rate was assessed histomorphometrically and analyzed using the Kruskal–Wallis test. Callus formation was observed at the fracture line in all groups. Callus tissue was more uniform and new bone formation was more dense in the tibia and femur allogeneic transplant groups. The highest new bone formation was observed in the tibia and femur allogeneic bone transplant group when compared with controls ($P < 0,05$ $P = 0.002$). Non vascular allogeneic bone transplantation; tibia and femur derived grafts, was found to significantly increase fracture healing compared to the control group. It was concluded that the use of allogeneic bone obtained from different anatomic sites may be an effective and biocompatible option for bone regeneration.

Key words: Tibia fracture; allogeneic bone graft; femur; tibia; bone healing; rat.

RESUMEN

El objetivo de este estudio fue evaluar histológicamente el proceso de curación de fracturas de tibia reconstruidas con injertos óseos alogénicos procedentes de diferentes regiones anatómicas (tibia y fémur). Se utilizaron veinticinco ratas Sprague-Dawley, divididas en cuatro grupos: control de fractura (n = 7), trasplante óseo alogénico de tibia (n = 7), trasplante óseo alogénico de fémur (n = 7) y grupo donante (n = 4). Bloques de hueso corticocanceloso (5×5 mm) obtenidos de los animales donantes se fijaron a los focos de fractura creados en las tibias de las ratas receptoras mediante agujas de Kirschner. Tras un periodo de curación de cuatro semanas, se sacrificaron las ratas y se examinaron histológicamente las tibias. Las muestras de tejido se fijaron en formalina neutra al 10 %, se descalcificaron, se incluyeron en parafina y se tiñeron con hematoxilina-eosina. La tasa de formación de hueso nuevo se evaluó histomorfométricamente y se analizó mediante la prueba de Kruskal-Wallis. Se observó formación de callo óseo en la línea de fractura en todos los grupos. El tejido calloso fue más uniforme y la formación de hueso nuevo más densa en los grupos de trasplante alogénico de tibia y fémur. La mayor formación de hueso nuevo se observó en el grupo de trasplante de hueso alogénico de tibia y fémur en comparación con los controles ($P < 0,05$; $P = 0,002$). El trasplante de hueso alogénico no vascularizado, mediante injertos derivados de tibia y fémur, incrementó significativamente la consolidación de la fractura en comparación con el grupo control. Se concluyó que el uso de hueso alogénico obtenido de diferentes sitios anatómicos puede ser una opción eficaz y biocompatible para la regeneración ósea.

Palabras clave: Fractura de tibia; injerto óseo alogénico; fémur; tibia; consolidación ósea; rata.

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INTRODUCTION

Bone tissue is a highly organized, multilayered biocomposite structure formed by the integration of an organic matrix and an inorganic mineral phase [1]. This complex structure is continuously renewed by a cellular network composed of osteoblasts, osteoclasts, and osteocytes. In this dynamic process that continues throughout life, a physiological balance is maintained between bone resorption by osteoclasts and new bone formation by osteoblasts. This balance represents the key mechanism responsible for preserving the mechanical strength and metabolic homeostasis of the skeletal system. Bone tissue remodeling occurs as a continuous and adaptive process, allowing the preservation of microstructural integrity while enabling the repair of accumulated microdamage [2, 3].

Bone healing is a dynamic and multistep biological process that depends on the coordinated activity of multiple cell types and signaling pathways. This process progresses through overlapping phases, classically described as inflammation, repair, and remodeling [4, 5]. The inflammatory phase begins immediately after trauma and represents the biological trigger for subsequent healing events [6].

During this stage, the hematoma formed at the fracture site functions not only as a blood clot but also as a transient microenvironment that supports early regenerative signaling. Activated platelets and immune cells within the hematoma release growth factors and cytokines that initiate the inflammatory cascade. These signals promote the recruitment of mesenchymal stem cells (MSCs) to the fracture site and create the cellular framework required for osteogenic differentiation. . As a result, the biological conditions necessary for callus formation and subsequent remodeling are established in the early stages of bone healing [7].

Following the inflammatory phase, bone healing continues with the formation of a soft callus, also referred to as a fibrocartilaginous callus. This phase typically begins within a few days after trauma and is characterized by the migration of mesenchymal stem cells to the fracture. These cells subsequently differentiate into chondrocytes and osteoblasts, leading to the formation of a temporary connective tissue matrix. This matrix constitutes the soft callus and displays a predominantly fibrocartilaginous structure. The developing callus provides early mechanical stability by temporarily maintaining alignment of the fracture ends. In addition, it serves as a biological scaffold that supports subsequent new bone formation [8].

As healing progresses, the initially formed soft callus gradually transitions into a hard, mineralized callus. This stage typically extends over several weeks and reflects the early phase of structural bone remodeling. During this period, osteoblasts produce an extracellular matrix that undergoes progressive mineralization. This process results in the formation of calcified tissue with increasing mechanical strength at the fracture site. The development of a hard callus enhances fracture stability and establishes the biological framework necessary for subsequent remodeling [9].

The final stage of bone healing is the remodeling phase, which extends beyond the early weeks and may continue for several months or even years. During this phase, the hard callus gradually matures into organized lamellar bone, allowing the tissue to recover its original morphology and mechanical properties. This transformation depends on the coordinated interaction of osteoblast-mediated bone formation, osteoclastic resorption, and the supportive contribution of mesenchymal stem cells. Through this balanced cellular activity, tissue homeostasis is progressively re-established, and both microstructural integrity and load-bearing capacity are restored [10].

Recent evidence indicates that bone healing is not driven solely by osteogenic cells, but also involves a significant contribution from the immune system. Among immune-related components, macrophages and bone marrow–derived mesenchymal stem cells (BMSCs) have emerged as key regulators of the early healing environment. These cells contribute to the regulation of inflammation and play an essential role in initiating angiogenesis and tissue regeneration at the fracture site. Importantly, the polarization state of macrophages influences the direction of the regenerative response by modulating osteoblast activity and the rate of new bone formation [11, 12].

A deeper understanding of the molecular and cellular mechanisms involved in bone regeneration is essential for the development of more effective therapeutic strategies. A deeper understanding of the molecular and cellular mechanisms involved in bone regeneration is essential for the development of more effective therapeutic strategies. In addition to these biological factors, an appropriate mechanical environment, sufficient vascular supply, and the regulation of systemic host conditions are critical for the completion of the regenerative process [13].

Both biological and synthetic materials from various sources have been extensively investigated and applied as graft options for the replacement of bone tissue. Autogenous bone grafts are derived from the patient's own bone and remain the reference standard in bone regeneration procedures [14].

In contrast, allogeneic grafts consist of bone tissue harvested from donors and subsequently processed through sterilization and decontamination protocols. These processing steps aim to preserve osteoconductive properties while minimizing the risk of immune-related reactions. Xenografts are produced from animal-derived bone tissues and are generally non-resorbable materials that tend to maintain their volume over time. Alloplastic grafts, on the other hand, are obtained entirely synthetically and offer an alternative option for bone regeneration thanks to their biocompatible structure [15].

Osteoinductivity is defined as the ability of a graft to actively promote new bone formation and facilitate the migration and differentiation of osteoblasts to the site. Osteoconductivity is a skeletal property that, depending on the physical and chemical structure of the graft, allows new bone cells and capillaries to migrate throughout the three-dimensional structure [16].

Autologous bone is still considered the gold standard for bone grafts because of its combination of osteogenic, osteoinductive, and osteoconductive properties. Furthermore, autologous grafts

contain different cell lines capable of supporting new bone formation and remodeling processes [17].

Although autogenous grafts are the most biologically effective graft type, they have disadvantages such as pain at the donor site, morbidity, limited graft volume, and the need for additional surgery. These limitations have increased the demand for alternative graft materials and stimulated the exploration of different biological sources capable of supporting bone regeneration [18].

Allogeneic grafts are among the most commonly used alternatives and may be obtained from either living donors or cadaveric bone sources. Prior to clinical application, these grafts undergo specialized processing to reduce immunogenicity and minimize the risk of disease transmission. Depending on clinical requirements, allogeneic grafts are available in various forms, including cortical, cancellous, and corticocancellous configurations [19].

Allogeneic bone grafts may exhibit osteoinductive potential, largely attributed to their content of type I collagen and bone morphogenetic proteins (BMPs) [20]. Nevertheless, despite originating from the same species, residual genetic differences raise concerns regarding immunogenic reactions, blood compatibility, and the potential transmission of disease or tumor cells. Although allogeneic grafts provide osteoconductive support and limited osteoinductive activity, they lack intrinsic osteogenic capacity. Moreover, processing procedures required for safety and storage may partially compromise both the biological performance and mechanical strength of these grafts [21].

Similar to autologous bone, allogeneic grafts can support vascular ingrowth and new bone formation after implantation, largely due to their porous and reticular architecture. Several studies have reported that fracture healing times associated with allogeneic bone grafting are comparable to those observed with autografts [22]. However, factors such as their tendency to resorption and the risk of immunogenicity can lead to undesirable outcomes in bone healing and limit the long-term stability of the graft [23, 24].

The most basic form of allogeneic bone is a block of unprocessed fresh bone. This bone can then be converted to frozen, lyophilized forms, or used directly as a graft [25]. Because the processing of allografts reduces their mechanical strength, fresh, unprocessed grafts exhibit biomechanically superior properties to engineered grafts. However, the use of fresh allografts may be limited due to concerns about the risk of disease transmission and host immune response [18, 26].

In recent years, numerous biological and material-based methods have been developed to support bone regeneration. However, experimental data comparing the effects of allogeneic bone grafts obtained from different anatomical sites on fracture healing are quite limited. Therefore, the aim of this study was to histologically evaluate the effects of allogeneic bone grafts derived from the tibia and femur on fracture healing in rat tibias. The anticipated findings are expected to address the lack of knowledge regarding the regenerative potential of allogeneic bone from different sources and to provide a scientific basis for future experimental and clinical research.

MATERIALS AND METHODS

This study was approved by the Fırat University Animal Experiments Local Ethics Committee (FÜHADYEK) (Protocol No: 2024/01-10, Date: 09.01.2024). All procedures were carried out in accordance with the ethical principles of the Declaration of Helsinki and the “Guide for the Use of Experimental Animals,” and experimental applications were carried out at the Fırat University Experimental Research Center.

Study design and experimental groups

A total of 25 female Sprague-Dawley rats (Rattus norvegicus), aged 3.5–4 months and weighing 250–300 grams (WL, Shimadzu, Japan), were used. A minimum of seven animals per group was determined to be sufficient based on previous similar studies. The rats were obtained from the Fırat University Experimental Research Center (FUDAM). All animals were housed in specially ventilated rooms throughout the experiment, under a 12-hour (h) light/12-h dark cycle, at a temperature of 25 ± 2 °C, and with free access to food and water.

The study was designed to evaluate the effects of allogeneic bone grafts obtained from different anatomical regions (tibia and femur) on fracture healing in rat tibias. The rats were randomly assigned to four groups: fracture control group (n = 7), tibia allogeneic graft group (n = 7), femur allogeneic graft group (n = 7), and donor group (n = 4).

Surgical procedures

All surgical procedures were performed under sterile conditions. Donor rats were euthanized under deep anesthesia. Bone block grafts, approximately 5 mm × 5 mm in size, were prepared from the corticocancellous portions of the right and left tibia and femur bones from each donor (FIGS. 1 and 2). In the recipient groups, the surgical field was prepared under sterile conditions on the right tibia, a 15-mm incision was made, and the periosteum was dissected. A bone fracture was created in the tibial diaphysis using a rotary instrument cooled with sterile saline. The resulting allogeneic grafts were placed between the two bone fragments at the fracture line and secured with Kirschner wires (FIG. 3). In the control group, only a fracture was created and no graft was applied.

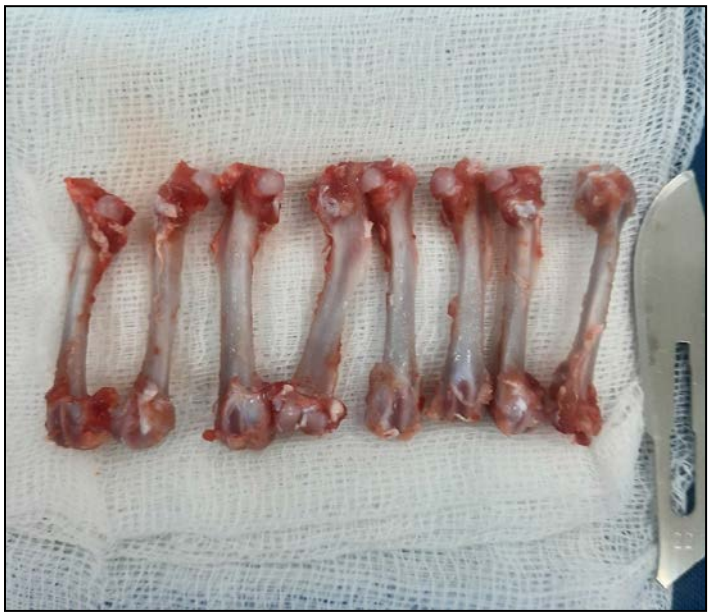

FIGURE 1. Femur bones os rats obtained from donor rats for transplantation.

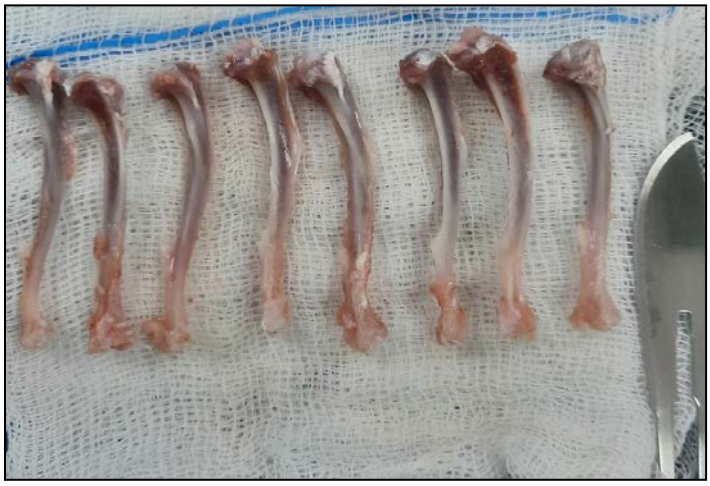

FIGURE 2. Tibia bones of rats obtained from donor rats for transplantation.

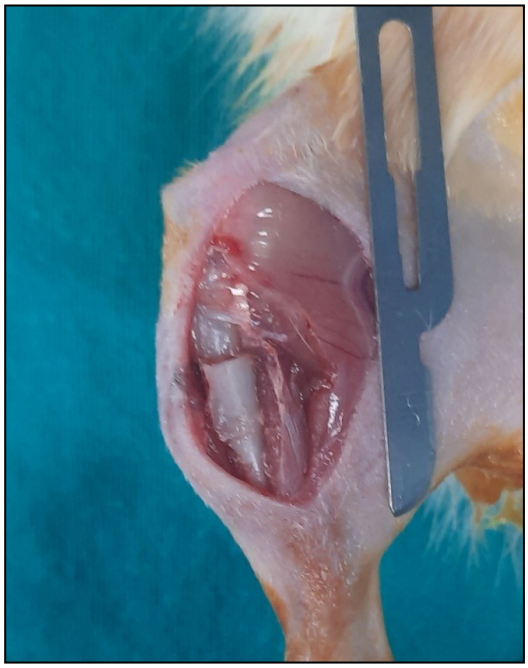

FIGURE 3. Image of rats after the tibia bone transplant.

Following all surgical procedures, the incised soft tissues were closed primarily with 4/0 nonresorbable propilene suture material. Postoperatively, all subjects received antibiotics (Penicillin, 50 mg/kg) and analgesics (Tramadol hydrochloride, 1 mg/kg) intramuscularly for three d to control infection and pain.

Histopathological analysis

At the end of the four-week healing period, all subjects were euthanazied. The right tibia of each subject was carefully excised, including the fracture line and the graft site. The bone samples were fixed in a 10 % neutral formalin solution for at least 72 h. Following fixation, the samples were freed from surrounding soft tissue and decalcified in a 10 % formic acid solution for approximately one week. After decalcification, the samples were processed routinely histologically using an automatic tissue processing device (Leica TP1020, Germany) through ascending series of alcohol, xylene, and paraffin wax. They were embedded in paraffin blocks. Longitudinal sections, 3 micrometers thick, were cut from the prepared blocks using a rotary microtome (Leica RM2125 RTS, Germany) and stained with hematoxylin-eosin (H&E).

Histological examinations were performed using a light microscope (Olympus BX42, Japan). New bone formation (NBF %) areas were assessed in each healing tissue. The percentage of NBF was calculated for each sample separately. Assessments were made by a blinded observer, and comparisons between all groups were analyzed using statistical methods.

Statistical analysis

Statistical analyses of the data obtained from histological evaluations were performed using IBM SPSS Statistics program (Version 22.0, SPSS Inc., Chicago, IL, USA). Shapiro-Wilks, Kolmogorov-Smirnov tests were used to determine whether the data were normally distributed. Data found to be non-normally distributed were analyzed for statistical differences between groups using the Kruskal–Wallis test. When differences between groups were detected, the Mann–Whitney U test was applied. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

This study was conducted using 25 Sprague-Dawley rats: three experimental groups of seven rats each and one donor group. The subjects were divided into three main experimental groups: a fracture control group, a tibia allogeneic graft group, and a femur allogeneic graft group. All surgical procedures were completed without complications, and all specimens obtained from each group were included in the histopathological evaluation.

In the control and experimental groups, varying degrees and types of callus tissue were observed to form and cover the fracture site. New bone formation was observed in all three groups at the fracture healing site. In addition to these, non-resorbed bone fragments were also observed in the control group. In the tibia and femur transplant groups, the callus tissue was more uniform, and NBF tissue was more frequently encountered within the healing callus (FIGS. 4 and 5) .

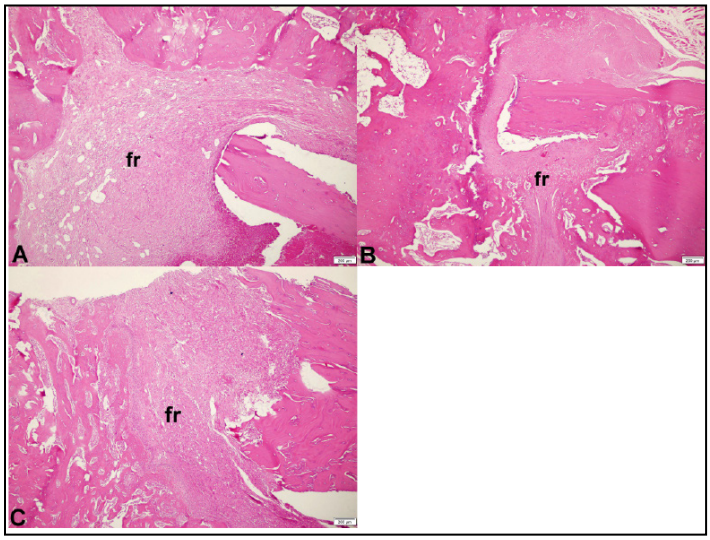

FIGURE 4. General view of the healing area at the fracture site (fr) in the Fracture Control (A) and treatment groups (B: Tibia Transplant and C: Femur Transplant). 4X, HxE.

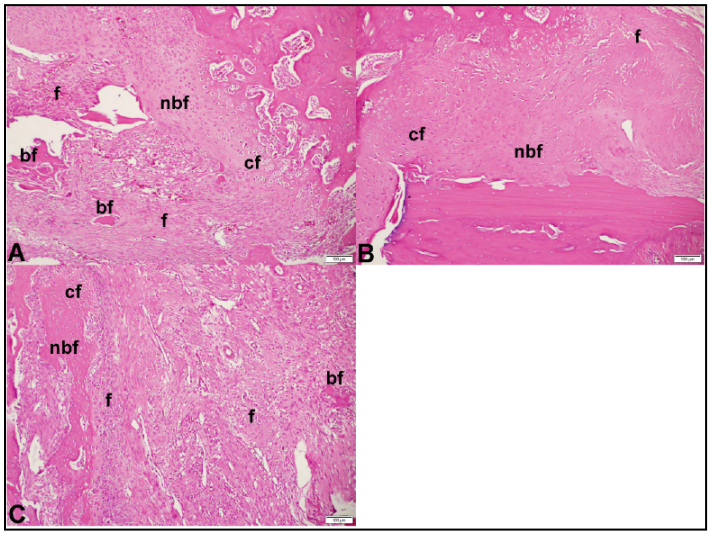

FIGURE 5. New bone formation (nbf), fibrosis or fibrous callus (f), cartilage formation (cf) and bone particles (bf) areas in the fracture control (A) and experimental groups (B: Tibia transplantation, C: Femur transplantation). 10X, HxE

Histological analyses revealed significantly higher NBF% in the tibia and femur allogeneic graft groups compared to the control group. Osteoblastic activity was more intense and the callus tissue exhibited a more regular morphology in the tibia graft group. Similarly, a distinct bone matrix and organized trabecular structure were observed in the femur graft group. Histomorphometric evaluation revealed an average NBF rate of 54.43 % in the tibia allogeneic graft group and 52 % in the femur graft group, compared to 42.57 % in the control group. The difference between the experimental and control groups was statistically significant (P = 0.002) (TABLE I and FIG. 6). Inflammatory responses were similar in all groups, and mature bone tissue was more organized and vascularized in the grafted groups.

TABLE I

New bone formation rates of the groups after the experimental setup. *Kruskal–Wallis Test (P = 0.002). ^{a_1}, ^{a_2}: Mann–Whitney U Test. ^{a_1}: 0.001; ^{a_2}: 0.002 (P < 0.05).

Groups	NBF (%) Mean/Median	Min.	Max	P*
Control (N=7)	42,57 / 41	39	49	0,002
Tibia Allogenic Transplant (N=7)^{a1}	54,43 / 55	49	60	
Femur Allogenic Transplant (N=7)^{a2}	52 / 53	45	57	

New bone formation rates(NBF%).

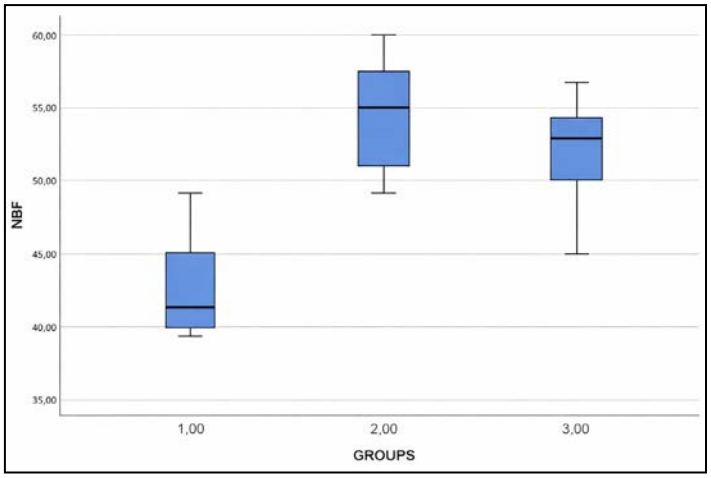

FIGURE 6. Kruskal–Wallis test results after the experimental period. 1: Control group, 2: Tibia allogeneic bone transplantation group, 3: Femur allogeneic bone transplantation group. Kruskal–Wallis Test (P = 0.002).

Bone fracture healing represents a highly regulated and dynamic biological process that progresses through sequential but overlapping stages, including hematoma formation, granulation tissue development, callus formation, and long-term remodeling . In the early phase, the initiation of an inflammatory response and the recruitment ofMSCs to the fracture site establish the biological framework required for subsequent osteogenic activity. This is followed by granulation tissue maturation, osteogenesis, and progressive new bone formation, ultimately restoring tissue continuity. In the later stages, mechanical loading plays a critical role in guiding bone remodeling, allowing the tissue to gradually regain its original morphology and functional integrity [27].

In addition to the sequential stages of fracture repair, the differentiation of MSCs and subsequent bone formation at the fracture site are influenced by a range of local microenvironmental factors [28]. Within this microenvironment, osteoblasts, osteocytes, osteoclasts, and osteogenic precursor cells interact with the extracellular matrix, including type I collagen fibers and non-collagenous components, to regulate bone formation and maintain structural integrity [29].

Sheen *et al*. [27] describe bone fracture healing as a dynamic biological process characterized by hematoma formation, granulation tissue development, callus formation,and long-term remodeling.

Based on this biological sequence, the present study focused on histologically assessing how allogeneic bone grafts harvested

from different anatomical sites contribute to and support the fracture healing process.

Ciobanu *et al*. [30] compared autologous bone grafts with pure bone substitutes and platelet-rich fibrin (PRF)–supported substitutes in a critical-sized rat bone defect model. Their histomorphometric and microimaging analyses demonstrated superior healing outcomes with autologous grafts, while PRF-supported constructs performed significantly better than pure bone substitutes. better results in the PRF-supported group than in the pure graft group. Although autogenous grafts remain biologically advantageous, these findings provide important context for interpreting alternative graft strategies, including the use of allogeneic bone evaluated in the present study. This finding is significant in our study, as we evaluated the effects of allogeneic grafts of different origins (tibia vs. femur) on healing. Although the literature demonstrates superiority of autologous grafts, our study also observed positive effects with allogeneic grafts.

In the review study by Vorontsov and Maltseva [31], the use of animal models in non-union models of long bone fractures emphasized the critical role of modeling in evaluating the effectiveness of biological treatments. This review provides a methodologically strong basis for our study in terms of the choice of experimental model (rat tibia fracture and different graft sources) and reveals how modeling parameters affect research findings in such studies.

In this study, allogeneic bone grafts derived from the tibia and femur were observed to promote bone healing at the fracture site created in the rat tibia. Histological evaluations revealed that callus tissue had a regular morphology in both graft groups, osteoblast activity increased, and new bone trabeculae formed extensively. These findings demonstrate that allogeneic bone tissue, when properly processed, provides an osteoconductive structure and effectively supports the regenerative process at the fracture site.

Similarly, Marongiu *et al*. [32] reported that graft- and cell-based therapeutic approaches were associated with enhanced bone regeneration in the treatment of long bone fractures. In a clinical context , Meiser *et al*. [33] observed that the use of allografts in tibial plateau fractures contributed to improved radiographic and clinical healing outcomes. Together, these observations are in line with the histological findings of the present study and support the concept that allogeneic bone grafts may represent a viable biological alternative for promoting fracture healing.

Furthermore, the findings of the present study suggest that tibia-derived allogeneic bone grafts may support fracture healing more effectively than femur-derived grafts, at least at a numerical level. Histological evaluation demonstrated more mature and organized callus formation in specimens treated with tibia-derived grafts, accompanied by increased osteoblastic activity. This observed difference may be associated with variations in morphological features, cortical density, and mineralization characteristics of the donor bone, which could influence the biological behavior of the graft material.

Zou *et al*. [24] compared autografts, allografts, and artificial graft materials in rat femur defect models and reported significant differences in new bone formation and immunotoxic responses among these graft types. These observations provide supportive context for the findings of the present study, in which differences were noted between allogeneic grafts derived from the tibia and femur. Collectively, such results point toward an emerging perspective in the literature suggesting that the anatomical origin of a graft may influence microstructural organization and cellular events during bone healing.

Animal models are indispensable research tools for understanding the physiological and pathological processes inherent in human biology. Rodent models are frequently preferred because they provide a controlled and reproducible experimental environment, particularly for studying bone healing, regenerative mechanisms, and the effects of systemic factors. Rats, due to their small body size, short reproductive cycle, economical maintenance, and genetic standardization, are widely used in preclinical bone regeneration studies [34]. In this study, the rat model was chosen to evaluate the effects of allogeneic bone grafts obtained from different anatomical sites on the healing process in a tibia fracture model. The high controllability offered by this model allows for reliable comparisons of biological differences between graft sources.

For experimental fracture research, it is essential that animal models be reproducible, well standardized, and capable of demonstrating predictable healing within a defined time frame. Corticocancellous fracture and defect models established in the tibia are widely regarded as reliable experimental systems that satisfy these requirements [35]. Because tibial bone healing predominantly occurs through endochondral ossification, this anatomical site provides a suitable and representative environment for evaluating regenerative responses. Within this framework, the present study offers a comparative experimental approach by assessing the effects of allogeneic bone grafts derived from different anatomical regions using a standardized tibia fracture model.

This study has several limitations that should be considered when interpreting the findings . First, the relatively small number of animals in each group may have reduced the statistical power, limiting the ability to detect biologically relevant differences with statistical significance. Second, the evaluation was restricted to histological analysis . Although histological assessment provides valuable information regarding new bone formation, callus organization, and osteoblastic activity, it does not fully capture parameters such as bone mineral density, microstructural quality, or mechanical strength. Accordingly, the absence of micro-computed tomography, immunohistochemical analysis, or biomechanical testing may have constrained the overall scope of the results.

Third, the study was limited to a single healing interval of four weeks. Given that bone regeneration is a dynamic and time-dependent process, the lack of longer follow-up periods restricted the evaluation of late-stage remodeling.

Finally, the analysis was confined to allogeneic bone grafts derived from the tibia and femur, and the exclusion of grafts from other anatomical sites limited a broader biological comparison of graft origin. Despite these limitations, this study contributes

meaningful experimental data by providing a comparative histological evaluation of allogeneic bone grafts from different anatomical sources within a standardized tibia fracture model.

CONCLUSIONS

In this study, new bone formation was histologically evaluated by applying allogeneic bone grafts obtained from different anatomical regions (tibia and femur) to fracture models created in rat tibias. Based on the findings, it was concluded that both allogeneic graft types positively biologically affect the bone healing process, although this effect may vary depending on the structural properties of the bone from which the grafts were obtained.

The numerical differences observed between the groups suggest that microstructural density and mineralization characteristics of the graft material may influence the bone regeneration process. In the present study, both tibia- and femur-derived allogeneic graft groups exhibited higher levels of new bone formation compared with the control group, accompanied by more organized callus architecture and increased osteoblastic activity.

However, healing was observed in all groups, and histological confirmation confirmed the potential of allogeneic grafts to support the regenerative process. These results indicate that the biological behavior of allogeneic bones obtained from different anatomical sources should be taken into account in bone repair and that further experimental and clinical studies are needed to demonstrate this effect more comprehensively.

Conflict of interests

The authors of this study declare that there is no conflict of interest with the publication of this manuscript.

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