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Research Paper

Homologous Osteoblast Transplantation Combined With Commercially Available HA/β-TCP Scaffolds Enhances Bone Regeneration

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ABSTRACT

Background: Critical-sized bone defects represent a major clinical challenge due to their limited capacity for spontaneous healing. Autologous bone grafting, while effective, is associated with donor-site morbidity and limited tissue availability. Tissue engineering approaches using osteoblasts combined with biocompatible scaffolds offer promising alternatives. We evaluated the effectiveness of homologous osteoblast transplantation on commercially available hydroxyapatite/beta-tricalcium phosphate (HA/β-TCP) scaffolds in promoting the repair of critical-sized tibial defects in a rabbit model.

Methods: Critical-sized defects (3 mm²) were surgically created in the tibia of 12 male New Zealand white rabbits. Animals were randomly assigned to receive either osteoblast-loaded HA/β-TCP scaffolds or acellular scaffolds. Contra-lateral limbs served as untreated controls. Bone regeneration was assessed 6 weeks post-implantation via histology, alkaline phosphatase (ALP) staining, and quantitative analysis of bone thickness and cellularity.

Results: Osteoblast-seeded scaffolds significantly improved bone healing compared to controls and acellular scaffold groups, demonstrated by increased new bone formation, enhanced tissue thickness, and higher osteoblast counts ($P<0.05$). Histological analyses revealed abundant collagen matrix and mineralized bone within the scaffold pores in the osteoblast group.

Conclusion: Homologous osteoblast transplantation utilizing HA/β-TCP scaffolds significantly promotes bone regeneration in critical-sized tibial defects in rabbits, demonstrating superior efficacy compared to acellular scaffold treatment. This strategy represents a promising approach for advancing clinical bone repair therapies.

Keywords: Bone tissue engineering (BTE), Osteoblasts, hydroxyapatite/beta-tricalcium phosphate (HA/β-TCP) scaffold, Bone regeneration

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Highlights

- Critical-sized bone defects represent a major clinical challenge due to their limited capacity for spontaneous healing.
- Tissue engineering approaches using osteoblasts combined with biocompatible scaffolds are promising alternatives to bone grafting.
- Homologous osteoblast transplantation utilizing HA/β-TCP scaffolds significantly improved bone regeneration in critical-sized tibial defects in rabbits.

Plain Language Summary

Critical-sized bone defects typically fail to heal without intervention. Bone tissue engineering (BTE) offers a promising multidisciplinary approach that combines cells, biocompatible scaffolds, and growth factors to promote bone regeneration. This study investigates the efficacy of homologous osteoblast transplantation combined with commercially available HA/β-TCP scaffolds for repairing critically sized tibial defects in rabbits, to develop advanced BTE solutions. The results showed that osteoblast-seeded scaffolds significantly improved bone healing compared to controls and acellular scaffold groups. It improved new bone formation, enhanced tissue thickness, and increased osteoblast counts. The results suggest superior efficacy of the used method compared to acellular scaffold treatment. This strategy represents a promising approach for advancing clinical bone repair therapies.

Introduction

Large bone defects, arising from trauma, infection, or disease, represent a major orthopedic challenge globally, often leading to substantial morbidity and requiring complex reconstructive procedures [1]. Critical-sized defects typically fail to heal without intervention [2]. Annually, millions of orthopedic procedures are performed worldwide to address bone loss, with a substantial portion dedicated to managing non-unions, segmental defects, and bone tumor resections [3].

Both autografts and allografts, despite their current utility, have challenges such as secondary surgical sites, insufficient graft volume, and risks of disease transmission or immune rejection [4], creating a demand for synthetic bone graft substitutes that are safe, effective, and readily available. The high incidence of donor-site morbidity, reported to be as high as 20% in some series, necessitates the urgent development of effective alternative strategies for bone repair that can overcome these limitations [2, 5].

Bone tissue engineering (BTE) offers a promising multidisciplinary approach that combines cells, biocompatible scaffolds, and growth factors to promote the regeneration of bone tissue. This field aims to develop functional bone substitutes that can recapitulate the complex biological and mechanical properties of native bone. The ideal bone substitute should be osteoconductive, osteoinductive, and ideally osteogenic. Artificial bone substitutes are frequently synthesized from materials such as hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP), which are calcium phosphate ceramics closely resembling the mineral phase of natural bone [6]. Their high chemical similarity to natural bone and teeth, along with their excellent biocompatibility, non-toxicity, and osteoconductive properties, make them desirable biomaterials [7, 8]. HA/β-TCP composites offer a balance between HA's high mechanical strength and slow degradation, and β-TCP's faster biodegradability and enhanced osteoconductivity, leading to improved bone regeneration outcomes [9, 10]. Scaffold architecture, porosity, graded pore structure, and interconnectivity critically influence bone ingrowth. For example, graded porosity β-TCP scaffolds have been shown to promote significantly greater bone volume in animal models [11].

The appropriate selection of cell sources is critical for successful BTE strategies. Among cell types, osteoblasts—the primary bone-forming cells—are especially appealing due to their direct osteogenic capacity. These cells can be isolated and expanded ex vivo, and then seeded on scaffolds to enhance bone formation [10, 12]. While mesenchymal stem cells (MSCs) from sources such as bone marrow, adipose tissue, and dental pulp are also widely studied for their multipotency and osteogenic differentiation potential [13], the transplanta-

tion of mature osteoblasts offers a more straightforward approach to rapid bone formation. Recent animal studies show promising results with osteoblast transplantation combined with scaffold materials.

Despite advances in orthopedic surgery, critical-sized bone defects often do not heal spontaneously and represent a major challenge due to the limitations of autologous grafts and variable outcomes of alternative strategies [14]. Successful repair could reduce morbidity, improve recovery, and decrease healthcare costs.

This study investigates the efficacy of homologous osteoblast transplantation combined with commercially available HA/β-TCP scaffolds for repairing critically sized tibial defects in rabbits, aiming to develop advanced BTE solutions for clinical application.

Materials and Methods

Ceramic scaffold

A commercially available HA/β-TCP scaffold, which combines HA with β-TCP to optimize biodegradability and mechanical properties, was employed.

Cell isolation and culture

Trabecular bone was harvested aseptically from male New Zealand white rabbits' calvaria (weight: 500–700 g). The bone fragments were placed in culture dishes containing DMEM F-12 (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cultures were maintained at 37 ˚C in a humidified atmosphere of 5% CO_2, with medium changes every 3 days. Once the osteoblast cultures reached approximately 70% confluency after 10 days, cells were washed twice with sterile phosphate-buffered saline (PBS) and detached using 2 mL of 0.25% trypsin-Ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, USA). Detached cells were centrifuged and resuspended in osteoblastic culture medium.

Seeding and culture of osteoblasts on ceramic scaffold

Approximately 5×10^5 osteoblasts were seeded onto each HA/β-TCP scaffold. After 4 hours of incubation to allow cell attachment, the scaffolds were cultured in minimum essential medium (MEM) for two weeks, with medium renewal performed three times daily. Cell adherence and morphology were monitored microscopically.

Scaffold transplantation into bone defects

Twelve adult male New Zealand white rabbits were anesthetized, and critical-sized defects (3 mm^2 area, 1 mm depth) were created on the proximal medial tibial surface. Animals were randomly assigned to receive either osteoblast-seeded scaffolds (group A, n=6) or acellular scaffolds (group B, n=6); the contralateral limbs served as controls. Cyclosporin A was administered for immunosuppression.

Von Kossa stain

For mineralization assessment, 5×10^3 osteoblast cells were seeded onto sterile Petri dishes and cultured. Cells were fixed with 10% neutral buffered formalin (Sigma-Aldrich, USA) for 15- 30 minutes. After fixation, cells were rinsed thoroughly with distilled water. Then, the cultures were incubated with 5% silver nitrate solution (Sigma-Aldrich, USA) and exposed to ultraviolet (UV) light for 30 minutes. Following this, the dishes were washed with PBS. Negative silver ions were then removed by incubating the samples in 0.3% sodium thiosulfate solution (Sigma-Aldrich, USA) for 5 minutes. The presence of black or brown calcium phosphate deposits was visualized using a light microscope (Zeiss, Germany), confirming the formation of mineralized nodules [15].

Histological evaluation

Animals were sacrificed at the 4th and 6th weeks post-implantation to assess bone graft integration and tissue response. Following euthanasia, bone graft specimens were carefully harvested and immediately fixed in 10% neutral buffered formalin. Subsequently, the fixed bone samples underwent decalcification in a 10% nitric acid solution, followed by washing with distilled water. After graded alcohols are processed and cleared with xylene. The decalcified specimens were embedded in paraffin wax. Serial sections of 5 µm thickness were cut and stained with hematoxylin and eosin (H&E) for examination.

Alkaline phosphatase (ALP) staining for osteoblast identification

To confirm the osteoblastic nature of cultured cells, ALP staining was performed. Initially, the culture medium was removed, and cells were fixed in 4% paraformaldehyde prepared in PBS for 1 to 2 minutes at room temperature. Following fixation, cells were rinsed three times with 1x Rinse Buffer (e.g. TBST: 20 mM Tris-HCl, pH 7.4; 0.15 M NaCl; 0.05% Tween-20). The

staining solution was freshly prepared by dissolving 0.8 g/L fast red violet (FRV) and 4 mg/mL naphthol AS-BI phosphate in an AMPD buffer (2 mol/L). This staining solution was then applied to the cells in an adequate amount and incubated to allow for the enzymatic reaction. After incubation, the cells were washed again with 1x rinse buffer [16].

Statistical analysis

Data were analyzed using one-way ANOVA with Tukey's post hoc test; $P<0.05$ was considered significant.

Results

Macroscopic evaluation

Six weeks after implantation, all scaffolds in both experimental groups maintained their original shape and size, displaying smooth surfaces and clearly visible porous structures. Bone union and osseous integration at the implant-host bone interface were distinctly evident.

Osteoblast isolation and proliferation in culture

During early culture days, no osteoblast migration from bone fragments was observed. By day 7, osteoblasts began to emerge from the bone tissue, exhibiting characteristic star-shaped and spindle morphologies. Cell proliferation accelerated notably by day 12, reaching suitable confluence by day 20, allowing for further passaging (Figure 1).

Osteoblast characterization

Von Kossa staining confirmed isolated osteoblasts' capacity to deposit calcium, visualized by black mineralized matrix patches, demonstrating active mineralization potential (Figure 2).

ALP staining and osteoblast activity

ALP staining confirmed the presence of active osteoblast populations capable of mineralization. Osteoblastic cells exhibiting ALP activity stained with a characteristic purple color, confirming their identity as bone-forming cells. This enzymatic staining method specifically highlights ALP, an early osteoblastic marker crucial in bone mineralization, making it a reliable tool for verifying osteoblast differentiation in vitro (Figure 3).

Histological bone healing

In the untreated control, periosteal coverage suggested an early reparative response. The defect area contained marrow and adipose tissue, with a thin immature bone layer connected to the periosteum. Osteoblasts and osteocytes, embedded within aligned collagen fibers, showed a reduced density peripherally, indicating incomplete repair (Figure 4a).

The scaffold-only group showed well-preserved porous scaffolds without periosteal invasion. Trabecular bone with irregular collagen bundles grew within scaffold pores, with round osteocytes and spindle-shaped osteoblasts present. Newly formed trabeculae were more abundant and active than those in the controls, supported by vascular infiltration. Enhanced bone repair was evident via increased trabecular complexity and cellularity (Figure 4b).

The osteoblast-seeded scaffold group revealed dense, organized trabeculae nearly filling the defect (Figure 4c). Scaffold remnants were minimal amid new bone. Marrow contained hematopoietic cells and adipose tissue. Areas of eosinophilic osteoid indicated high osteoblastic activity, and angiogenesis was present. Parallel collagen fibers suggested ongoing mineralization, facilitating the formation of HA crystals. This group exhibited greater

Figure 1. Morphology of osteoblasts isolated from bone fragments at different culture times

a) Day 7: Early migrating spindle- and star-shaped osteoblasts, b) Day 12: Actively proliferating osteoblasts, c) Day 20: Confluentosteoblast monolayer, indicating sufficient cell density for subsequent experimental procedures and scaffold seeding (×200 magnification)

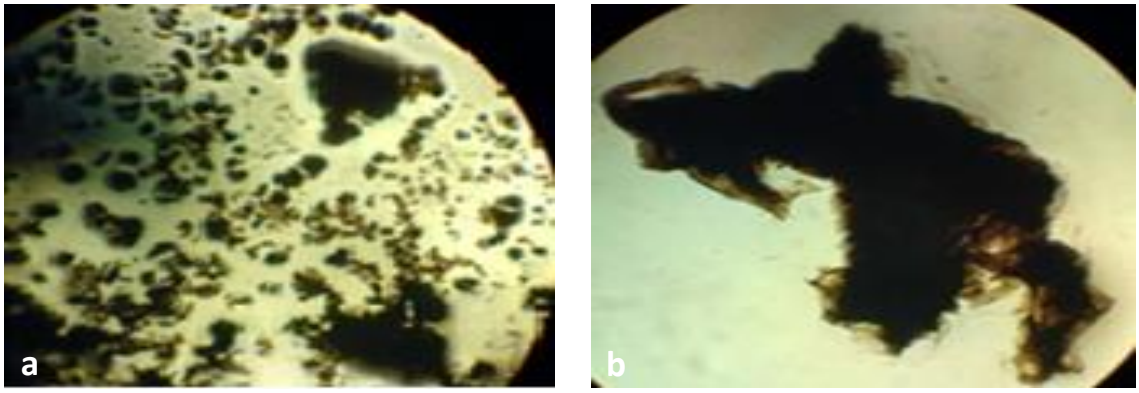

Figure 2. Von Kossa staining of isolated osteoblast cultures

a) Calcium deposition is clearly visible as dark areas, b) Black mineralized matrix deposits confirm the osteogenic potential of cultured osteoblasts (×200 magnification)

graft thickness, trabecular density, vascularization, and scaffold resorption compared to others. Bone union formation: Both graft groups showed histological bone continuity with the host tibia, with no significant differences (Figure 4d).

Statistical analysis

Bone repair in both graft groups was significantly better than controls ($P<0.01$). The osteoblast-seeded group trended higher than scaffold-only but without statistical significance. Osteoblast counts were significantly greater in the osteoblast-seeded group versus scaffold-only ($P<0.05$). Bone thickness was also significantly greater in both graft groups compared to controls ($P<0.01$) (Figure 5). These results indicate that scaffolds, particularly those seeded with osteoblasts, promote enhanced cellular proliferation and bone formation. Data are presented as Mean±SD.

Discussion

The present study provided compelling evidence that homologous osteoblast transplantation using a commercially available HA/β-TCP scaffold significantly enhances bone defect repair in a rabbit model, particularly when assessed through quantitative histological analysis and measurement of new bone thickness.

The repair of significant bone defects remains a formidable challenge in orthopedic surgery, often necessitating complex interventions [3]. While autologous bone grafting is the clinical gold standard [17], its inherent limitations, including donor site morbidity, restricted tissue availability, and prolonged recovery, underscore the critical need for alternative regenerative strategies [9].

Interestingly, we also observed a degree of bone repair in the acellular HA/β-TCP scaffold group, albeit to a lesser extent than in the osteoblast-seeded group. This phenomenon in small, critical-sized defects can be at-

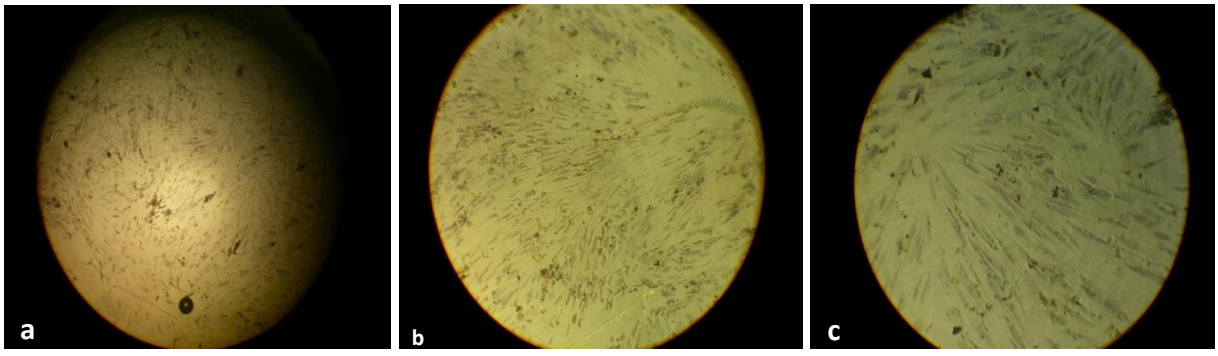

Figure 3. ALP staining of isolated osteoblasts staining of isolated osteoblasts used for seeding in the scaffold with osteoblasts group

Note: Magenta-stained cells indicate ALP enzymatic activity, confirming the osteoblastic phenotype and their potential for mineralization (a×40, b×100, c×200 magnifications).

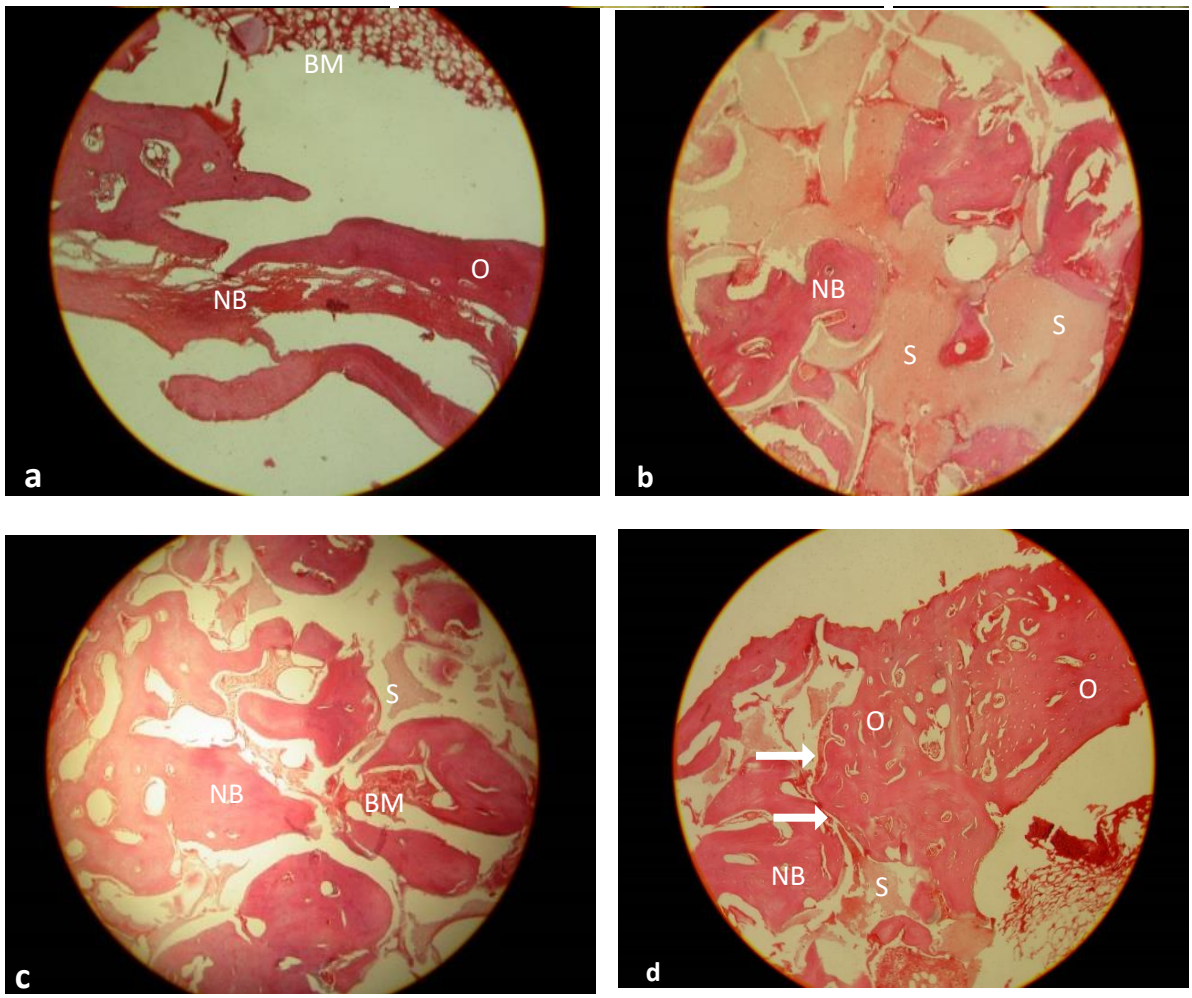

Figure 4. Histological evaluation of tibial defects in different experimental groups using H&E staining

a) Periosteal tissue covering the defect, early new bone formation; osteocytes within lacunae and spaces between parallel collagen strands are visible, b) Scaffold-only group: Porous scaffold integrated with bone trabeculae, showing parallel collagen strands and partial tissue ingrowth, c) Osteoblast-seeded scaffold group: Irregular new bone trabeculae with active osteoblasts; remnants of scaffold visible between trabeculae, d) Osteoblast-seeded scaffold group: Well-formed bone union with active osteoblasts and excellent integration with host bone (×100 magnification).

Abbreviations: NB: New bone; S: Scaffold; O: Osteoblast/Osteocyte; BM: Bone marrow.

tributed to the natural regenerative capacity of the host and the osteoconductive properties of the HA/β-TCP scaffold [17-19].

The porous structure of HA/β-TCP provides an ideal framework for the ingrowth of host-derived cells, including MSCs and osteoprogenitors from the adjacent periosteum, bone marrow, and surrounding connective tissues [7, 11, 20]. These endogenous cells can migrate to the defect site and differentiate into osteoblasts, contributing to new bone formation [21].

Studies using genetically labeled host cells have confirmed that a significant portion of new bone formation in acellular scaffolds can originate from host-derived cells, underscoring the importance of intrinsic healing mechanisms. Furthermore, the debate regarding the contribution of vascular-derived cells, such as pericytes, to osteogenesis within defects is ongoing, with some studies suggesting that they differentiate into bone-forming cells following injury [22].

However, the limited repair observed in the acellular group, particularly when considering the significant enhancement with cell seeding, underscores a critical principle in BTE. While osteoconductive scaffolds facilitate natural healing, they are often insufficient for achieving robust regeneration in larger or more challenging de-

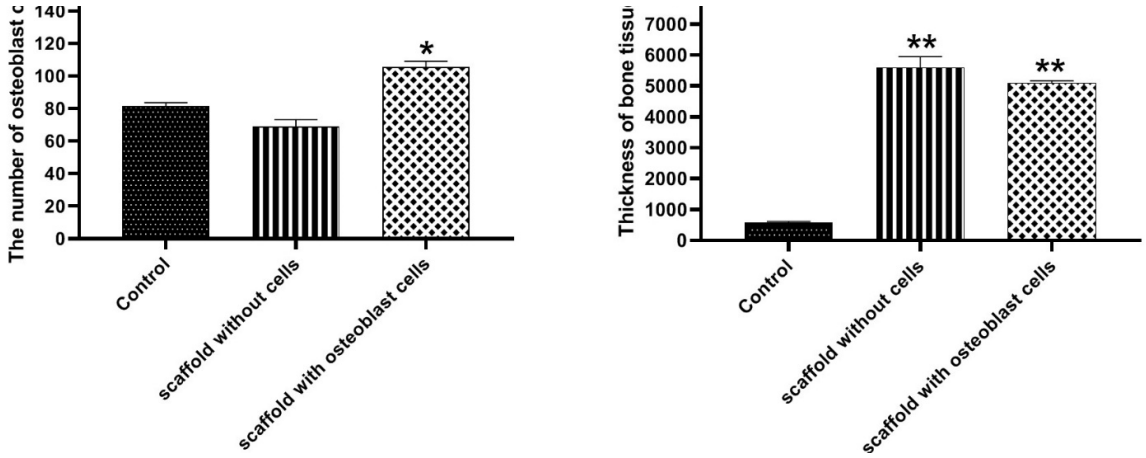

Figure 5. a) The number of osteoblast cells in the scaffold with osteoblasts was significantly higher than in the other groups (*P<0.05); b) Both scaffold groups with and without osteoblast cells, showed significantly greater bone tissue thickness than the control group (**P<0.01).

Note: Data are presented as Mean±SD.

fects. Our findings corroborate evidence from studies on larger defects, where acellular scaffolds alone often lead to fibrous tissue infiltration or incomplete healing. For example, in critical-sized defects that exceed the body's natural regenerative capacity, such as those used in large animal models, scaffolds require either cellular components or osteoinductive factors to achieve successful repair [23]. This finding highlights that osteoblasts play a crucial role in contributing to bone volume and structural integrity, surpassing what the native healing cascade alone can achieve.

The choice of HA/β-TCP as a scaffold material proved to be highly effective. Its biocompatibility, osteoconductivity, and controlled biodegradability are crucial for providing a stable environment for cell proliferation and subsequent bone remodeling [7]. The observed maintenance of scaffold integrity throughout the six weeks allowed for sustained cellular activity and matrix deposition, suggesting that it provides a durable template for bone regeneration, which is then gradually resorbed. This finding aligns with the understanding that the optimal scaffold design strikes a balance between mechanical support and gradual degradation, matching the rate of new bone formation [24].

Although our study focused on direct osteoblast transplantation, it is important to acknowledge alternative strategies involving osteoinductive growth factors. For instance, recombinant human bone morphogenetic protein-2 (rhBMP-2) has shown remarkable efficacy in inducing bone formation, even in challenging defect scenarios [25]. Future studies could explore the synergistic effects of combining osteoblast transplantation with growth factor delivery to potentially accelerate and enhance bone repair further, especially in complex defect morphologies [26].

The results of this research have significant clinical implications for treating challenging bone defects. The ability to achieve robust bone regeneration using ex vivo expanded osteoblasts on a readily available and biocompatible HA/β-TCP scaffold offers a promising alternative to autologous bone grafting, potentially reducing donor site morbidity, surgical complexity, and patient recovery time [27]. This approach could be particularly beneficial for patients with limited autograft sites or those requiring extensive bone reconstruction. Furthermore, the use of homologous cells reduces the risks associated with allogeneic tissue transplantation, such as immune rejection [28], making it a potentially safer and more accessible option for a broader patient population [17].

Despite its promising findings, our study had several limitations. The use of a rabbit model may not fully translate to human physiology and bone healing mechanisms [29]. The 6-week follow-up period, while sufficient to observe initial bone formation, did not capture long-term graft integration, remodeling, and biomechanical strength. Furthermore, the defect size (3 mm^2) was relatively small, which, as discussed, allows for some intrinsic host healing. Larger, critical-sized defects might further highlight the necessity and superiority of cell-seeded constructs [30]. Finally, although we confirmed the presence and function of osteoblasts, a detailed analysis of vascularization within the defect site was not fully explored [31].

Conclusion

This study demonstrated that homologous osteoblast transplantation using HA/β-TCP scaffolds significantly enhances bone repair in rabbits with critical-sized tibial defects. The superior histological outcomes, characterized by robust new bone formation and increased osteoblast activity, highlight the considerable potential of this cell-based tissue engineering strategy. Despite the observed intrinsic healing in acellular scaffold groups, the present results affirm that osteoblast-seeded constructs offer a more reliable and effective solution for promoting comprehensive bone regeneration.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization, study design, data acquisition, interpretation and analysis: All authors; Supervision and resources: Alireza Shams; Experiments: Mohammad amin Shams; Writing: Alireza Shams and Maryam Amirinejad.

Conflict of interest

The authors declared no conflict of interest.

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