



Ti6Al4V/Xenogeneic bone composite promotes osseointegration by recapitulating the osteogenic microenvironment

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Abstract

Ti6Al4V implants are used to repair critical-sized bone defects, but their bioinert surfaces often cause insufficient early osteogenic stimulation, potentially leading to compromised long-term fixation. The osteogenic microenvironment is vital for osseointegration between the host bone and the implant and influences long-term clinical outcomes. Consequently, researchers have attempted to mimic the natural osteogenic microenvironment using microporous design, physical and chemical modification, and loading of bioactive factors. The present study proposes a strategy for fully recapitulating the natural osteogenic microenvironment within microporous Ti6Al4V implants. First, Ti6Al4V scaffolds were implanted into rat femoral condyle defects to establish osseointegration. The resulting construct was then demineralized and decellularized to produce an implantable Ti6Al4V/xenogeneic bone composite. Pull-out tests demonstrated that the bone tissue remained well-integrated with the scaffold after processing. Nucleic acid detection and galactose- α -1,3-galactose (α -Gal) analysis verified the elimination of immunogenicity in the composite. Immunofluorescence staining for bone morphogenetic protein-2 and transforming growth factor- β confirmed the preservation of key bioactive factors. In vitro cell experiments revealed favorable biocompatibility and osteoinductive activity. When implanted into rabbit femoral condyle defects, the composite significantly enhanced bone ingrowth into the microporous Ti6Al4V implant and strengthened osseointegration. Histological and quantitative analyses further showed that the composite shifted the osteogenic pattern from the osteoconduction-dominant mode characteristic of Ti6Al4V implants to an osteoinduction-dominant pattern of bone formation. In summary, we developed a novel Ti6Al4V/xenogeneic bone composite that fully recreated the natural osteogenic microenvironment. This composite effectively improved the osseointegration capacity of conventional scaffolds and demonstrated considerable potential for clinical application.

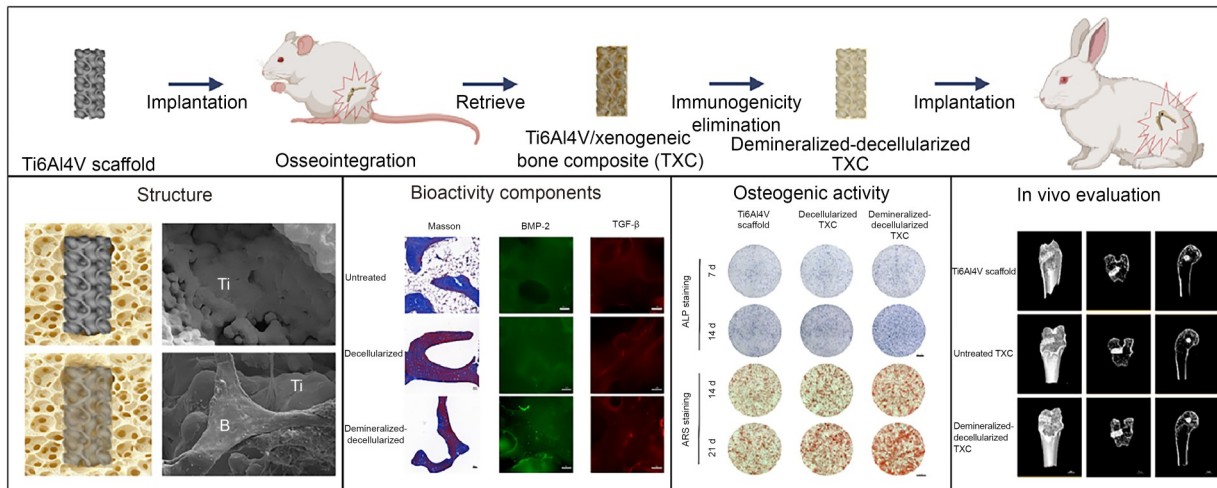
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Graphical abstract



Keywords Bone tissue engineering · Osseointegration · Demineralized-decellularized bone matrix · Ti6Al4V scaffold · Composite · Osteogenic microenvironment

1 Introduction

Bone defects are commonly caused by congenital anomalies, trauma, tumors, inflammation, and other factors. While bone tissue possesses a unique intrinsic healing capacity that enables spontaneous healing of small defects, large bone defects are generally incapable of self-repair [1]. Bone tissue engineering has emerged as a highly promising technological strategy for promoting bone regeneration at defect sites. Given the specific biological requirements of bone, scaffolds must possess sufficient mechanical strength, good biocompatibility, and excellent osteoconductivity [2]. Ti6Al4V scaffolds are therefore widely used in bone tissue engineering. However, the inherent bioinertness of the titanium alloy scaffold surface may hinder the interaction between the biological environment and the implant, limiting the effectiveness of its microporous structure in facilitating osteogenesis [3, 4]. Studies have shown that if new bone formation on the surface is not promptly stimulated, the implant is prone to fibrous encapsulation, ultimately leading to implant failure due to poor osseointegration [5].

In recent decades, researchers have focused on developing various strategies to recreate an osteogenic microenvironment within implants, aiming to actively guide and accelerate bone regeneration and achieve stable osseointegration. Commonly employed methods include optimizing pore topology, surface modification, and biofunctionalization [6–8]. For instance, Yang et al. optimized a titanium alloy implant by mimicking the trabecular bone structure, which promoted cell proliferation, osteogenic differentiation, and bone regeneration within the scaffold [9]. While this approach achieves structural biomimicry, it still lacks the osteogenic microenvironment,

limiting regenerative efficiency. To better simulate the natural osteogenic microenvironment, Silva de Almeida et al. modified the implant surface by increasing roughness and coating it with type I collagen, thereby introducing key components of bone tissue [10]. Going beyond this, Sheng et al. constructed a biomimetic mineralized collagen coating on the implant surface, mimicking both the composition and morphological structure of the natural osteogenic environment [11]. Their results demonstrated improved biocompatibility and significantly enhanced osseointegration. Nevertheless, existing technologies still struggle to precisely replicate the complex three-dimensional (3D) hierarchical structure of natural bone and its rich biological cues, resulting in limited control over the osteogenic process [12]. A key limitation of these strategies is their inability to incorporate the bioactive factors essential for bone repair. Lyu and Lee attempted to enhance the bioactivity of the scaffold by loading recombinant human bone morphogenetic protein-2 (BMP-2) onto the implant surface [13]. Li et al. biofunctionalized the scaffold with a vascular endothelial growth factor coating to promote vascularization and osseointegration [14]. However, the loaded bioactive factors are relatively singular, and the method faces challenges such as low loading efficiency, insufficient long-term stability, high cost, and risks of dose-dependent side effects.

In our previous study, we implanted a Ti6Al4V porous scaffold into the cancellous bone of a rabbit femoral condyle to construct a Ti6Al4V/autogenous bone composite (i.e., a robust “autogenous bone coating” formed through osseointegration between the surrounding bone and the implant). This composite successfully endowed the porous titanium alloy scaffold with the structural and functional properties of natural

bone. When explanted and transplanted into a defect in the contralateral femoral condyle, it significantly enhanced bone regeneration and implant integration [15]. This study confirmed that the retrieved integrated prostheses from patients with periprosthetic fractures [16] could be reused for autologous applications and facilitate new osseointegration. However, the utility of this approach appears to be limited to this specific scenario, with little potential for broader application.

Inspired by the proven clinical success and widespread application of xenogeneic bone [17], we proposed a novel strategy involving pre-implanting a porous implant into the cancellous bone of a xenogeneic donor and leveraging the *in vivo* microenvironment to facilitate the formation of a stable composite structure through osseointegration between the scaffold and the host bone. This composite was then harvested to fabricate a Ti6Al4V/xenogeneic bone composite (TXC), which, after appropriate processing, was transplanted into the bone defect site. The key innovation of this strategy was the complete recapitulation of the natural osteogenic microenvironment within a microporous titanium alloy implant. We compared the effects of several immunogenicity removal strategies, including deproteinization, decellularization, and demineralization–decellularization, on the composite. Although these approaches are widely adopted for processing xenogeneic grafts, their suitability for TXC and their effects on the integrity of the pre-integrated bone–metal interface remain unclear. We used pull-out testing to evaluate the bonding strength between the bone matrix and the Ti6Al4V scaffold within the processed composites. Nucleic acid assays and galactose- α -1,3-galactose (α -Gal) analysis were employed to assess the residual immunogenicity following the different treatments. Immunofluorescence staining for BMP-2 and transforming growth factor- β (TGF- β) was used to characterize the retention of growth factors. *In vitro* cell experiments were conducted to evaluate the biocompatibility and osteoinductive activity. Finally, a xenogeneic implantation animal experiment was performed to validate the composite's ability to enhance implant osseointegration.

2 Materials and methods

2.1 Materials

Ti6Al4V metal powder was provided by AK Medical Co., Ltd. (Beijing, China). Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) and penicillin–streptomycin dual antibiotic solution were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and serum replacement were sourced from Xrbio (Hangzhou, China). Formaldehyde solution and phosphate-buffered saline (PBS) were obtained from Servicebio (Wuhan, China). Hydrogen peroxide, chloroform, methanol, and ether solution were supplied

by Guangzhou Chemical Reagent Factory (Guangzhou, China). Triton X-100, sodium dodecyl sulfate (SDS), and DNase I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA)-2Na and an α -Gal quantification kit were provided by Sangon Biotech (Shanghai, China). The PicoGreen dsDNA quantification kit and RiboGreen RNA quantification kit were acquired from Solarbio (Beijing, China). The enhanced cell counting kit-8 (CCK-8) for cell viability assay and calcein acetoxymethyl ester (calcein-AM)/propidium iodide (PI) were sourced from Bioss (Beijing, China). Osteogenic induction medium for alkaline phosphatase (ALP) and Alizarin Red S (ARS) staining was obtained from Procell (Wuhan, China). Hematoxylin and eosin (H&E) staining reagents, a Masson's trichrome staining kit, and Van Gieson (VG) staining reagents for histological analysis were purchased from Thermo Fisher Scientific (Shanghai, China). Fluorescein isothiocyanate (FITC)-phalloidin for cytoskeleton staining and 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining were also supplied by Thermo Fisher Scientific. The ultrapure water used in the experiments was prepared using a Milli-Q[®] A10 water purification system (Millipore, Billerica, MA, USA). Primary antibodies against BMP-2, α -Gal, and TGF- β for immunofluorescence experiments were obtained from Abcam (Cambridge, UK). A BMP-2 enzyme-linked immunosorbent assay (ELISA) kit and a TGF- β ELISA kit were purchased from Liankebio (Hangzhou, China). A SureScript[™] First-Strand cDNA synthesis kit and a BlazeTaq[™] SYBR[®] Green quantitative polymerase chain reaction (qPCR) mix 2.0 kit were supplied by GeneCopoeia (Maryland, USA). Other reagents are specified in the article.

2.2 Fabrication of TXC

2.2.1 Manufacturing of Ti6Al4V scaffolds

The 3D-printed triply periodic minimal surface (TPMS) Ti6Al4V microporous scaffold used in this study was fabricated according to a previously reported method [18]. We selected a TPMS-based micro-unit because its continuously curved surface and high interconnectivity provided favorable permeability and mass transport, thereby facilitating tissue ingrowth and stable fixation [18]. Accordingly, the scaffold was designed as a 3D cylindrical model with a diameter of 3 mm and a height of 6 mm. A pore size of 800 μ m was chosen to support vascular infiltration and cancellous bone ingrowth, which are critical for early osseointegration in porous metallic scaffolds [19]. The porosity was set to 70% to provide sufficient space and permeability for tissue formation while maintaining adequate structural integrity for load bearing [20]. The scaffold was manufactured using biomedical-grade Ti6Al4V powder via an electron beam melting (EBM) printer (Arcam Q10, Mölndal, Sweden). Following EBM printing, the scaffolds were routinely depowdered using

compressed air and subsequently ultrasonically cleaned in ethanol and deionized water to remove residual loose powder particles from the surface and porous structure. The cleaned scaffolds were then dried prior to further use.

2.2.2 Preparation of TXC

The study protocol was approved by the Animal Ethics Committee of the College of Basic Medical Sciences, Jilin University (No. 2024-555). Twelve adult male Sprague–Dawley rats were used. Following induction of general anesthesia via inhalation of isoflurane, the bilateral lateral femoral condyles were prepared as the surgical implantation sites. The fur around the knee joint was shaved preoperatively before performing routine aseptic preparation. Following local infiltration anesthesia with lidocaine, a 1–2 cm incision was made laterally over the distal femur using a surgical scalpel. The lateral condyle was exposed by blunt dissection of the subcutaneous muscle, ligaments, and blood vessels. Subsequently, a bone defect cavity measuring 3 mm in diameter and 6 mm in depth was created in the distal femur using an orthopedic drill bit, with dimensions precisely matching those of the scaffold to be implanted. Bone debris within the defect was carefully removed, and the area was irrigated with physiological saline. The scaffold was then press-fitted into the femoral defect cavity. The incision was closed layer by layer. For pain relief, intramuscular injections of meloxicam were administered postoperatively for 3 d.

Twelve weeks after scaffold implantation, the rats were euthanized by CO₂ asphyxiation. A hollow trephine drill with an internal diameter of 5 mm was used to extract 6-mm-high cylindrical Ti6Al4V/bone composite samples, with the metal scaffold at the center. This procedure ensured the inclusion of an approximately 1 mm margin of cancellous bone surrounding the implant for subsequent characterization. The harvested samples were stored at –80 °C.

2.2.3 Immunogenicity elimination treatments of TXC

Deproteinization treatment The deproteinization treatment was performed according to the method described by Wu et al. [21]. The specific procedures were as follows: the composite samples were immersed in 20% hydrogen peroxide (H₂O₂) solution for 72 h, with the solution replaced every 24 h. Subsequently, the samples were thoroughly rinsed under running water and transferred to a Soxhlet extractor containing 250 mL of ethyl ether, and then incubated in a constant-temperature water bath at 37 °C for 24 h. The samples were then placed in a fume hood to allow complete evaporation of the ether. Finally, each sample was cleaned three times with 75% ethanol and deionized water using an ultrasonic cleaner to entirely remove residual organic solvents from the bone blocks.

Decellularization treatment The composites were decellularized using the method described by Lin et al. [22]. The specific procedure was as follows: first, the composite underwent three freeze-thaw cycles, with each freezing and thawing phase lasting 30 min. Subsequently, it was treated sequentially with 1% Triton X-100 and 1% SDS for 24 h each. The composite was then incubated in a solution of DNase I (200 U/mL) for 12 h. Finally, it was thoroughly rinsed with PBS and stored at –80 °C for future use.

Demineralization–decellularization treatment The demineralization–decellularization treatment was performed following the method described by Hu et al. [23]. The materials were first immersed in 12% EDTA-2Na solution (15 mL per sample) and demineralized for 120 h in an ultrasonic decalcifier (Servicebio) maintained at 15 °C, with the demineralization solution replaced every 24 h. Subsequently, they were decellularized using 1% Triton X-100 for 48 h, followed by delipidation in a chloroform/methanol solution for 24 h. After thorough washing, the samples were incubated in 200 U/mL DNase I solution at 37 °C for 2 h, and then washed under constant agitation in PBS at room temperature. Finally, the materials were immersed in absolute ethanol for 4 h to remove cellular residues, rinsed extensively with deionized water for 2 h, and stored at –80 °C for future use.

We selected this “demineralization–first, decellularization–second” sequence because detergents used during decellularization (e.g., SDS and Triton X-100) may disrupt tissue structure and bioactive matrix components. Given the density of mineralized bone with osteocytes embedded within lacunae, demineralization transforms bone into a more compliant matrix and improves solution permeability, thereby enabling a shorter and less aggressive decellularization step while better preserving extracellular matrix (ECM) structure and bioactivity.

2.3 Characterization of TXC

2.3.1 Characterization of Ti6Al4V scaffolds

To ensure that the 3D-printed scaffolds conformed to the design specifications, a post-fabrication visual inspection was performed, and the surface morphology of the micropores was examined using scanning electron microscopy (SEM, ZEISS, Oberkochen, Germany). Qualitative analysis of the elemental composition was conducted using energy-dispersive X-ray spectroscopy (EDS; Oxford Instruments, Aztec software, Abingdon, UK) to confirm the absence of impurities or contamination during manufacturing. Furthermore, to evaluate the surface hydrophilicity of the scaffolds, the water contact angle was measured using a contact angle goniometer (Kyowa DM-500, Kyowa Interface Science, Saitama, Japan). The cleanliness and dryness of the Ti6Al4V scaffolds were ensured prior to testing. A droplet of deionized

water was deposited on the material surface, and its morphology was recorded using the instrument's integrated camera. ImageJ (National Institutes of Health, MD, USA) was used to analyze the images and measure contact angles. Measurements were repeated three times per sample across three independent samples to obtain reliable average contact angle data.

2.3.2 Characterization of Ti6Al4V/bone composites

Similarly, the prepared Ti6Al4V/bone composites were characterized using SEM, EDS, and contact angle measurements to examine the interlocking state between the xenogeneic bone and the Ti6Al4V implant, the elemental composition of the composite, and its surface hydrophilicity. To further evaluate osseointegration status and mechanism at the interface between the bone and the Ti6Al4V microporous scaffold within the composite, the harvested samples were fixed in 4% paraformaldehyde for one day, followed by micro-computed tomography (micro-CT) scanning (SkyScan 1076 scanner, Bruker, Kontich, Belgium). The scanning parameters were set as follows: X-ray voltage of 70 kVp, anode current of 200 μ A, exposure time of 300 ms, and a resolution of 30 μ m, with the sample rotated 360°. After micro-CT scanning, hard sections of the implant region were prepared and stained with Masson's trichrome staining. The stained sections were then observed and imaged under an optical microscope.

2.3.3 Evaluating the osseointegration in TXC after immunogenicity elimination treatments

Bonding strength testing of TXC The bonding strength between the xenogeneic bone and the Ti6Al4V porous scaffold following various immunogenicity-removal treatments was evaluated using a universal testing machine (WDW10 kN; Sinter, Changchun, China). Standard connectors were designed using Rhino software, with a contact area of 2 mm $\times$ 2 mm between the connector and the composite, and fabricated via a resin printer. The test specimens were adhesively bonded to the standard connectors using E-7 epoxy resin. The composite and the connectors were then securely clamped to the universal testing machine, and a tensile test was conducted at a crosshead speed of 2 mm/min.

SEM and EDS analysis of interface Samples were sectioned perpendicular to the long axis of the composite using a hard tissue microtome (Leica, Wetzlar, Germany). The cross-sectional surfaces were then analyzed using SEM and EDS to examine the surface morphology and chemical composition of the prepared specimens.

2.3.4 Characterization of immunogenicity in TXC after immunogenicity elimination treatments

Bone tissue from the TXC subjected to different treatments was extracted for analysis. The samples from each group

were fixed in 4% paraformaldehyde for 24 h. Subsequently, demineralization was performed using 10% EDTA. Conventional sectioning was performed, and the sections were stained with H&E and DAPI to identify residual nuclei. DNA and RNA contents were quantified using the PicoGreen dsDNA and RiboGreen RNA assay kits.

Immunofluorescence staining was performed to assess α -Gal in the bone tissue of the composites following the various treatments. Decalcified paraffin sections (5 μ m) were deparaffinized, rehydrated, and subjected to antigen retrieval. Sections were then permeabilized with 0.1% Triton X-100 and blocked with 5% serum for 1 h at room temperature, followed by incubation overnight at 4 °C with an anti- α -Gal primary antibody. After washing, sections were incubated with the corresponding fluorescent secondary antibody (1:1000) for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI. ELISA was used for quantitative measurement.

2.3.5 Evaluating the bioactivity of TXC after immunogenicity elimination treatments

Bone tissue within the composite was sectioned using the same method described in Sect. 2.3.4. Masson's trichrome staining was performed to evaluate the effect of various immunogenicity removal treatments on collagen in the bone tissue, and a hydroxyproline (HYP) assay kit was used to quantify collagen content quantitatively.

Bone tissue from composites subjected to various immunogenicity removal treatments was processed for immunofluorescence staining to assess the distribution of BMP-2 and TGF- β . The procedures were as follows: the bone tissue was first sectioned along a single plane using a hard-tissue microtome (Leica) and then ground to an appropriate thickness using a grinding/polishing machine (Leica). After PBS rinses, sections were permeabilized with 0.1% Triton X-100 and blocked with 5% goat serum for 1 h at room temperature, followed by incubation overnight at 4 °C with anti-BMP-2 or anti-TGF- β primary antibodies. Subsequently, the sections were incubated for 2 h at room temperature with FITC- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Abcam) diluted 1:1000 in blocking buffer. Stained sections were observed under a fluorescence microscope (Nikon, Tokyo, Japan). BMP-2 and TGF- β concentrations were quantitatively assessed using an ELISA kit.

2.4 In vitro cell experiments

2.4.1 Isolation and culture of bone marrow-derived mesenchymal stem cells (BMSCs)

BMSCs were isolated from 1-week-old New Zealand rabbits. The long bones of the limbs were harvested, and bone marrow was flushed out using a sterile 5 mL syringe and sterile PBS.

The flushed cells were then cultured in DMEM/F-12 medium supplemented with 10% FBS and 1% penicillin–streptomycin. The culture dishes were placed in a constant-temperature incubator for cell cultivation. On the second day of culture, a partial medium change was performed; thereafter, the medium was replaced every three days. When the cells reached 80%–90% confluence, they were digested with trypsin and subcultured. All BMSCs used in this study were at the third passage.

2.4.2 Biocompatibility assessment

The cytotoxicity of the composite materials was evaluated using the CCK-8 assay. The specific procedure was as follows: (1) Ti6Al4V scaffolds, decellularized TXC, and demineralized-decellularized TXC were sterilized and placed in a 24-well plate. DMEM/F-12 medium containing 10% FBS and 1% penicillin–streptomycin was added. The plate was incubated in a CO₂ incubator (BB 150, Thermo, MA, USA) at 37 °C for 72 h, after which the culture medium was aspirated to obtain the extracts. (2) In a 96-well plate, 5×10^3 BMSCs were seeded per well for each group, with three parallel replicates set per group. (3) After 24 h of seeding, when the cells were fully adherent, the DMEM/F-12 medium in the wells was aspirated and replaced with the respective extracts. On Days 1, 4, and 7, 10 µL of CCK-8 solution was added to each sample, which was incubated in the CO₂ incubator for 2 h. The optical density (OD) values of the samples at 450 nm were measured using a microplate reader.

Cells on the Ti6Al4V scaffolds, decellularized TXC, and demineralized-decellularized TXC were stained using a Live/Dead fluorescent staining kit. Briefly, BMSCs were seeded onto scaffolds in a 48-well plate at a density of 5×10^4 cells per well for each group. After culturing for 3 d, the materials from each group were transferred to a new 48-well culture plate. According to the manufacturer's protocol, calcein-AM and PI were used to stain live and dead cells, respectively. The scaffolds were then removed from the wells and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Immunofluorescence staining of the cytoskeleton was performed to assess the effect of each material group on cell morphology. The specific protocol was as follows: in a 48-well plate, BMSCs were seeded onto the scaffolds of each group at a density of 5×10^4 cells per well. After culturing for 3 d, the materials from each group were collected to evaluate cell morphology. Filamentous actin (F-actin) was stained with FITC-phalloidin, and the cell nuclei were counterstained with DAPI.

2.4.3 Osteogenic evaluation

The materials from each group were placed in a 24-well plate and immersed in osteogenic induction medium (DMEM/F-12

supplemented with 50 µg/mL ascorbic acid, 10 mmol/L β-glycerol phosphate disodium, and 10 nmol/L dexamethasone). The plate was incubated at 37 °C for 72 h. Following incubation, the medium was centrifuged and filtered to obtain the osteogenic differentiation extract. BMSCs were seeded at a density of 1×10^4 cells per well in a 48-well culture plate for the assessment of osteogenic activity. When cell confluence reached approximately 80%, the culture medium was replaced with the corresponding osteogenic differentiation extract from each group. The cells were cultured in a humidified incubator at 37 °C with 5% CO₂, and the medium was changed every two days. Osteogenic evaluation was performed after 7 and 14 d of induction.

ALP staining and quantitative analysis: ALP staining was performed using the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium chloride (NBT) alkaline phosphatase color development kit (Beyotime). Samples were washed with PBS and fixed with 4% paraformaldehyde before incubation with the staining working solution in the dark at room temperature according to the manufacturer's instructions. Staining results were observed and recorded under a stereo microscope (Olympus BX51TF, Olympus). For quantitative ALP analysis, the samples were lysed with cell lysis buffer (Beyotime). The lysates were transferred to centrifuge tubes and centrifuged at 14,000g for 5 min. The supernatant was collected, and p-nitrophenyl phosphate substrate was added. Absorbance was measured at 405 nm. To account for sample variability, the total protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime). ALP activity data were normalized to the total protein content.

ARS staining and quantitative analysis: ARS staining (Beyotime) was used to detect calcium nodule formation. The specific steps were as follows: after washing with PBS, samples were fixed with 4% paraformaldehyde and then incubated with ARS staining solution in the dark at 37 °C for 30 min. After staining, the samples were thoroughly rinsed with distilled water, and calcium nodule morphology was observed under a stereo microscope. Semi-quantitative analysis was performed using 10% cetylpyridinium chloride (CPC; Aladdin, Shanghai, China) solution (prepared in ultrapure water). Specifically, CPC solution was added to each well, and the culture plate was placed on a shaker and agitated at room temperature for 30 min. The absorbance (OD) of each experimental group at 562 nm was measured using a microplate reader. The OD value reflected the amount of ARS released from the calcium nodules, enabling relative quantification of calcium deposition.

Reverse transcription qPCR (RT-qPCR) for osteogenic genes: Total RNA was isolated from rabbit BMSCs co-cultured on different samples at Day 7 using an RNA extraction kit following the manufacturer's protocol. RNA concentration and purity were assessed by NanoDrop (Thermo

Scientific, MA, USA), and equal amounts of RNA were reverse-transcribed into cDNA using a first-strand cDNA synthesis kit. qPCR was performed using a BlazeTaq™ SYBR® Green qPCR Mix 2.0 kit on the LightCycler 480 system (Roche Co., Ltd., Basel, Switzerland). The expression levels of *ALP*, *BMP-2*, and runt-related transcription factor 2 (*RUNX2*) were quantified, with β -actin as the internal reference gene. Primer sequences are provided in Table S1 (supplementary information). Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_T}$ method.

2.5 Xenograft experiment of TXC

A xenograft experiment was performed using rabbits. The experimental group was implanted with demineralized-decellularized TXC, control group 1 received Ti6Al4V scaffolds, and control group 2 received untreated TXC. Nine adult male New Zealand rabbits (18 femoral condyles) were randomized into three groups (Ti6Al4V scaffold, untreated TXC, and demineralized-decellularized TXC; $n=6$ condyles per group). For imaging and histological analyses, three condyles from each group were scanned using micro-CT and were subsequently processed for histology. The remaining three condyles per group were reserved for push-out testing ($n=3$ per group). During surgery, a cylindrical bone defect with a diameter of 3 mm and a depth of 6 mm was created in the lateral femoral condyle using a 3-mm drill bit. In the experimental group, the demineralized-decellularized TXC (processed as described in Sect. 2.2.3) was implanted into the bone defect. The Ti6Al4V scaffold was directly implanted in control group 1, and the untreated TXC (obtained as described in Sect. 2.2.2) was implanted in control group 2. The incision was closed layer by layer. Postoperatively, penicillin was administered for 3 d to prevent infection, and the rabbits were closely monitored for one week. Twelve weeks after implantation, the rabbits were euthanized by CO₂ asphyxiation, and bilateral femoral condyles were harvested for further evaluation.

2.5.1 Micro-CT analysis

Micro-CT scanning was performed to evaluate the osseointegration between the host bone and the composites at the implantation site. The scanning was conducted following the same method described in Sect. 2.3.2, and the acquired data were reconstructed three-dimensionally. A cylindrical region of interest (ROI) with a diameter of 3 mm and a height of 6 mm was selected for the analysis of bone morphometric parameters. Specific parameters included the bone volume/total volume ratio (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), and trabecular number (Tb.N, mm⁻¹).

2.5.2 Push-out test

A standardized push-out test was performed using a universal mechanical testing machine to evaluate the interfacial bonding strength between the host bone and the composites. The specific procedure was as follows: the specimen was securely fixed on the testing platform, and a push-out load was applied at a constant displacement rate of 1.0 mm/min. The load exerted on the scaffold was monitored in real time via the accompanying software until complete separation between the scaffold and the surrounding bone tissue occurred (test endpoint). The interfacial bonding strength was characterized as the maximum push-out force recorded during the entire loading process.

2.5.3 Hard tissue sectioning and staining

Following micro-CT scanning, hard-tissue sections of the scaffold implantation area were prepared. These sections were subsequently stained with H&E, Masson's trichrome, and VG. The stained sections were examined under an optical microscope, and corresponding images were acquired for documentation and analysis.

2.5.4 Quantitative analysis of sections

Section images were processed in ImageJ and segmented into approximately 1500 ROIs. ROIs containing the implant, those showing new bone ingrowth within the implant, osteoinduction ROIs, and osteoconduction ROIs were then quantified. In the Masson's trichrome staining, red areas represent immature and incompletely mineralized osteoid, while blue-stained tissue corresponds to mature and mineralized bone (Fig. S1 in the supplementary information). The pattern of bone ingrowth was determined according to the relative position of the red- and blue-stained areas in the Masson's trichrome staining with respect to the metallic scaffold. The osteogenic pattern was defined as osteoinduction when mature bone (blue) predominated at the scaffold–tissue interface, whereas osteoid (red) was primarily distributed in regions farther from the scaffold surface. Conversely, when the bone tissue did not directly contact the metallic scaffold and the red-stained osteoid was located closer to the scaffold than the blue-stained mature bone, the pattern was defined as osteoconduction [24]. The osteogenic pattern within each ROI inside the Ti6Al4V scaffold was identified, and a corresponding heatmap was generated. The bone ingrowth rate (r_{BI}) was calculated as follows:

$$r_{BI} = N_{ROI} / N_{TROI},$$

where N_{ROI} represents the number of ROIs showing new bone ingrowth within the implant and N_{TROI} represents the total number of ROIs containing the implant.

2.6 Statistical analysis

All experimental data were presented as mean±standard deviation and were obtained from a minimum of three independent experiments. Normality was assessed using the Shapiro–Wilk test and homogeneity of variance using Levene’s test. Student’s *t*-test was used for two-group comparisons. For multiple-group comparisons, one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was applied when assumptions were met; otherwise, a Kruskal–Wallis test with Dunn’s correction was used. Statistical analyses were performed using SPSS (version 19.0; SPSS Inc., Chicago, USA). A *p*-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3 Results

3.1 TXC was successfully constructed 12 weeks after implantation

The TPMS Ti6Al4V porous scaffold fabricated via 3D printing is shown in Fig. 1c. The scaffold exhibited a cylindrical porous structure with a 3-mm diameter and a 6-mm height, consistent with the design specifications. Subsequently, the lateral femoral condyle of a rat was exposed, and the Ti6Al4V porous scaffold was implanted into the cancellous bone of the femoral condyle. The surgical procedure was illustrated in Fig. 1a. Figure 1b presents anteroposterior and lateral knee radiographs of the rat at 12 weeks post-implantation, showing that the Ti6Al4V scaffold remained well-positioned within the cancellous bone of the femoral condyle, closely integrated with the surrounding bone tissue without displacement or extrusion. At 12 weeks post-implantation, a 5-mm inner-diameter trephine was used to harvest the Ti6Al4V scaffold along with the surrounding bone tissue, centered on the scaffold, successfully constructing the Ti6Al4V/xenogeneic bone composite (Fig. 1e).

Figures 1d and 1f show SEM images of the Ti6Al4V and TXC scaffolds, respectively. The Ti6Al4V scaffold exhibited a microscopically rough surface with partially fused powder particles of varying sizes. Such microscale roughness was associated with improved cell adhesion and enhanced osseointegration. In contrast, SEM results of the TXC revealed that the surface of the porous Ti6Al4V scaffold exhibited both Ti6Al4V particles and bone tissue that had grown into the pores, forming an interpenetrating network structure through mechanical interlocking. EDS analysis showed that Ti, Al, and V were the main components of the Ti6Al4V scaffold, and no other impurity elements were detected (Fig. 1g). In addition to the metallic components of Ti6Al4V, TXC contained elements related to bone tissue, including C, N, O, Ca, and P (Fig. 1h).

Figures 1i and 1j show the water contact angle measurements for the Ti6Al4V material and TXC, respectively. The results indicated that the water contact angle on the surface of the 3D-printed TPMS Ti6Al4V porous scaffold was $74.60^\circ \pm 3.27^\circ$, while that of TXC decreased significantly to $54.01^\circ \pm 5.31^\circ$, demonstrating improved hydrophilicity of the composite.

Micro-CT results showed that bone tissue had grown into the pores of the Ti6Al4V scaffold within the composite (Fig. 1k). Meanwhile, hard-tissue sections combined with Masson’s trichrome staining further confirmed effective osseointegration between Ti6Al4V and the xenogeneic bone within the composite (Fig. 1l).

3.2 The osseointegration structure of TXC was damaged by deproteinization treatment

The external appearance of TXC subjected to deproteinization, decellularization, and demineralization–decellularization treatments was depicted in Fig. 2a. Macroscopic observation revealed that decellularization and demineralization–decellularization treatments had minimal impact on the bone tissue within the composites, whereas deproteinization resulted in partial detachment of the bone tissue. Micro-CT results indicated that, following the demineralized–decellularized treatment, the bone matrix within the pores of the composite was radiolucent, and substantial bone matrix was visible in the composites treated with deproteinization and decellularization (Fig. 2a). Analysis of bone tissue parameters revealed that decellularization treatment exerted the least influence on the bone tissue within the Ti6Al4V scaffolds. Deproteinization treatment led to partial bone loss, whereas the bone matrix was not visible on radiographic imaging after demineralization–decellularization treatment (Figs. 2b–2e).

Figure 2f illustrates the interfacial bonding strength between the xenogeneic bone and the Ti6Al4V porous scaffold in untreated, deproteinized, decellularized, and demineralized–decellularized TXC, with the statistical analysis results presented in Fig. 2g. The results showed that the bonding strength of the decellularized group (maximum pull-out force: (38.13 ± 3.03) N) was comparable to that of the untreated group (maximum pull-out force: (40.67 ± 2.43) N), with no statistically significant difference observed. In contrast, the bonding strength was significantly reduced after the demineralized–decellularized treatment (maximum pull-out force: (15.67 ± 3.53) N). The weakest bonding strength was observed in the deproteinized group (maximum pull-out force: (10.27 ± 1.64) N).

SEM and EDS analyses were further performed to evaluate the effects of the three immunogenicity removal treatments on the osseointegration interface within the composites (Fig. 2h). SEM observation showed that in both the decellularized and

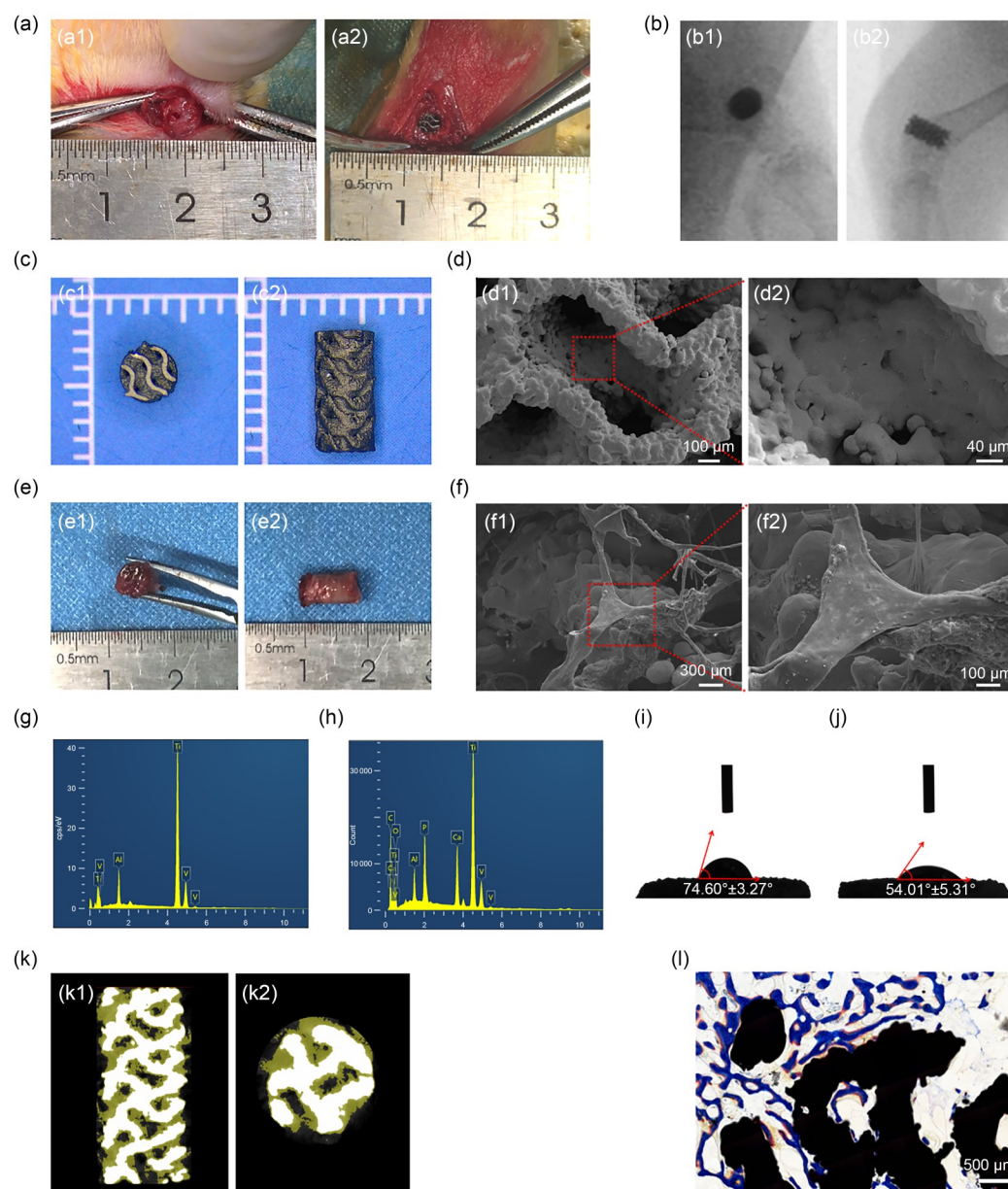


Fig. 1 Fabrication of Ti6Al4V/xenogeneic bone composite (TXC). (a) Surgical procedure for Ti6Al4V scaffold implantation: (a1) surgical exposure of the lateral femoral condyle in a rat; (a2) macroscopic view of the Ti6Al4V scaffold implanted into the rat lateral femoral condyle. (b) X-ray views of the knee joint at 12 weeks post-implantation of the Ti6Al4V scaffold into the rat femoral condyle: (b1) anteroposterior X-ray views; (b2) lateral X-ray views. (c) Macroscopic appearance of a 3D-printed triply periodic minimal surface (TPMS) Ti6Al4V microporous scaffold. (d) Scanning electron microscopy (SEM) images of the Ti6Al4V scaffold at different magnifications. (e) Macroscopic appearance of the TXC. (f) SEM images of the composite at different magnifications. (g) Elemental composition of the Ti6Al4V scaffold. (h) Elemental composition of the TXC. (i) Water contact angle measurement of the Ti6Al4V scaffold. (j) Water contact angle measurement of the TXC. (k) Micro-computed tomography (micro-CT) scans of the TXC. (l) Hard-tissue section and Masson's trichrome staining of the TXC

demineralized-decellularized groups, the xenogeneic bone and the Ti6Al4V scaffold surface remained in close contact without significant gaps, indicating that the osseointegration interface was preserved following decellularization or demineralized-decellularized treatment. However, significant gaps were observed at the interface in the deproteinized group, suggesting severe disruption of osseointegration. EDS further revealed the elemental distribution characteristics at the

osseointegration interface. In the decellularized group, carbon (C, indicating organic components such as collagen) and calcium (Ca, derived from the mineral phase of bone, hydroxyapatite) were in direct contact with titanium (Ti, from the Ti6Al4V scaffold). In the demineralized-decellularized group, due to demineralization, Ca was absent, and C was directly adjacent to Ti. In the deproteinized group, the C content was very low, and significant gaps were observed at the interface.

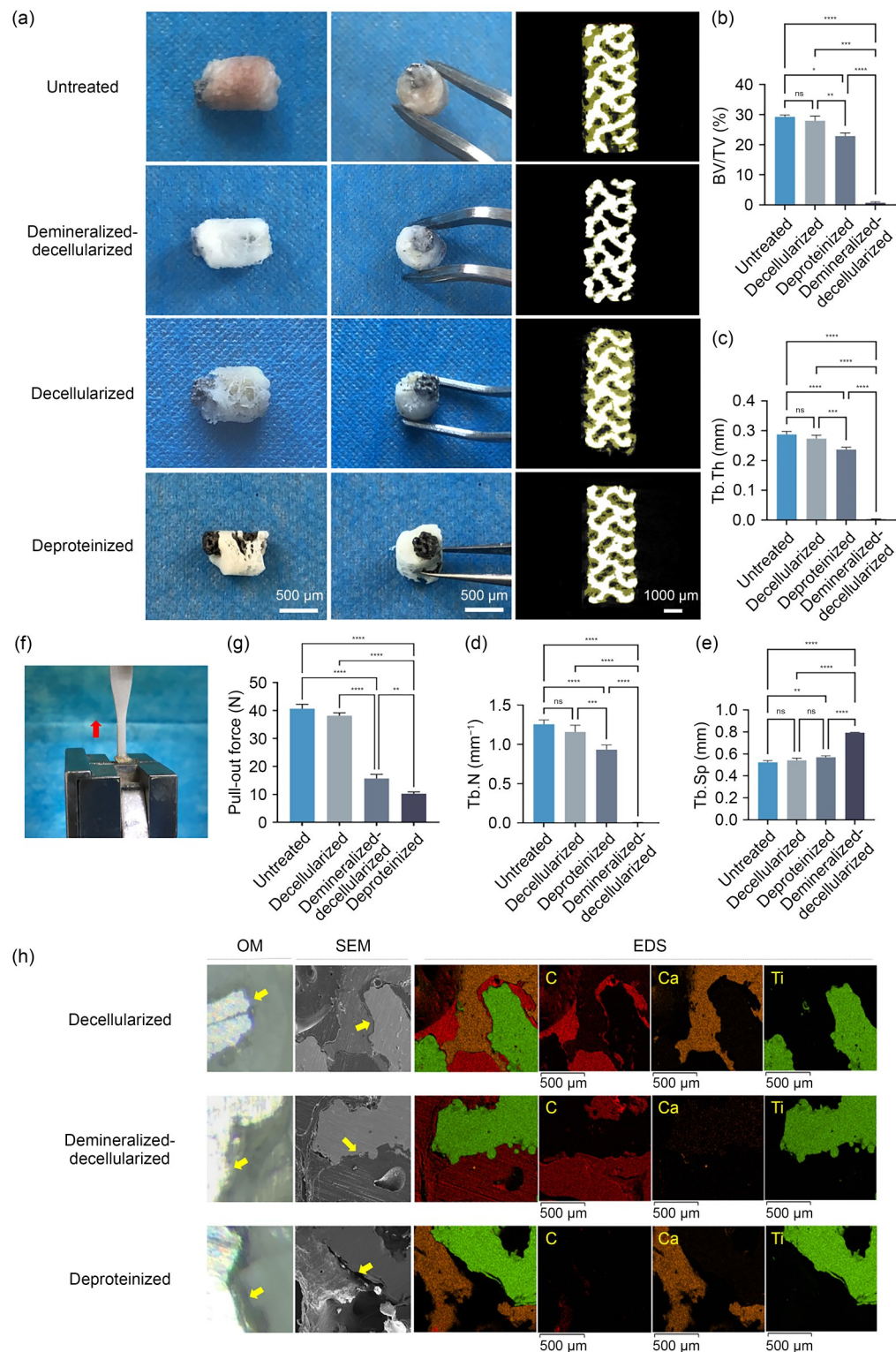


Fig. 2 Effects of different immunogenicity removal treatments on the osseointegration within the Ti6Al4V/xenogeneic bone composite (TXC). (a) Macroscopic appearance and micro-computed tomography (micro-CT) scans of untreated TXC, demineralized-decellularized TXC, decellularized TXC, and deproteinized TXC. Quantitative analysis of BV/TV (b), Tb.Th (c), Tb.N (d), and Tb.Sp (e) for the four groups ($n=3$). (f) Biomechanical test of the interfacial bonding strength between bone tissue and Ti6Al4V scaffold in the composite before and after three immunogenicity removal treatments. (g) Quantitative analysis of the maximum pull-out force from the bonding strength test ($n=3$). (h) Optical microscopy (OM), scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDS) images of the interface between the bone tissue and the Ti6Al4V scaffold in the composite after three immunogenicity removal treatments. Data are presented as mean $\pm$ standard deviation. “ns” indicates no significant difference between groups, while * indicates a significant difference between groups. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$

The core of the TXC lay in osseointegration between bone tissue and porous scaffold. Since the deproteinization treatment severely compromised this interface and led to a loss of structural integrity, the protocol was excluded from subsequent studies.

3.3 Different immunogenicity removal strategies eliminated the immunogenicity of TXC

Figure 3a shows the results of H&E, DAPI, and α -Gal immunofluorescence staining of the bone tissue within the composites following decellularization or demineralization–decellularization treatment. H&E staining revealed that untreated bone tissue contained abundant adipose tissue, with numerous cellular components visible within the bone

matrix. In contrast, after decellularization or demineralization–decellularization treatment, the bone matrix was free of surrounding tissues, and the internal cellular components were effectively removed. DAPI fluorescence staining confirmed these findings: untreated bone tissue sections exhibited dense nuclear fluorescence signals, whereas no nuclear staining was observed in the bone matrix after decellularization or demineralization–decellularization treatment. α -Gal immunofluorescence staining demonstrated widespread expression of α -Gal antigen in the untreated bone matrix, whereas no significant residual expression was detected after decellularization or demineralization–decellularization treatment. Additionally, DNA, RNA and α -Gal contents in the bone matrix were quantified (Figs. 3b–3d). After decellularization treatment, the DNA content in the bone matrix of the

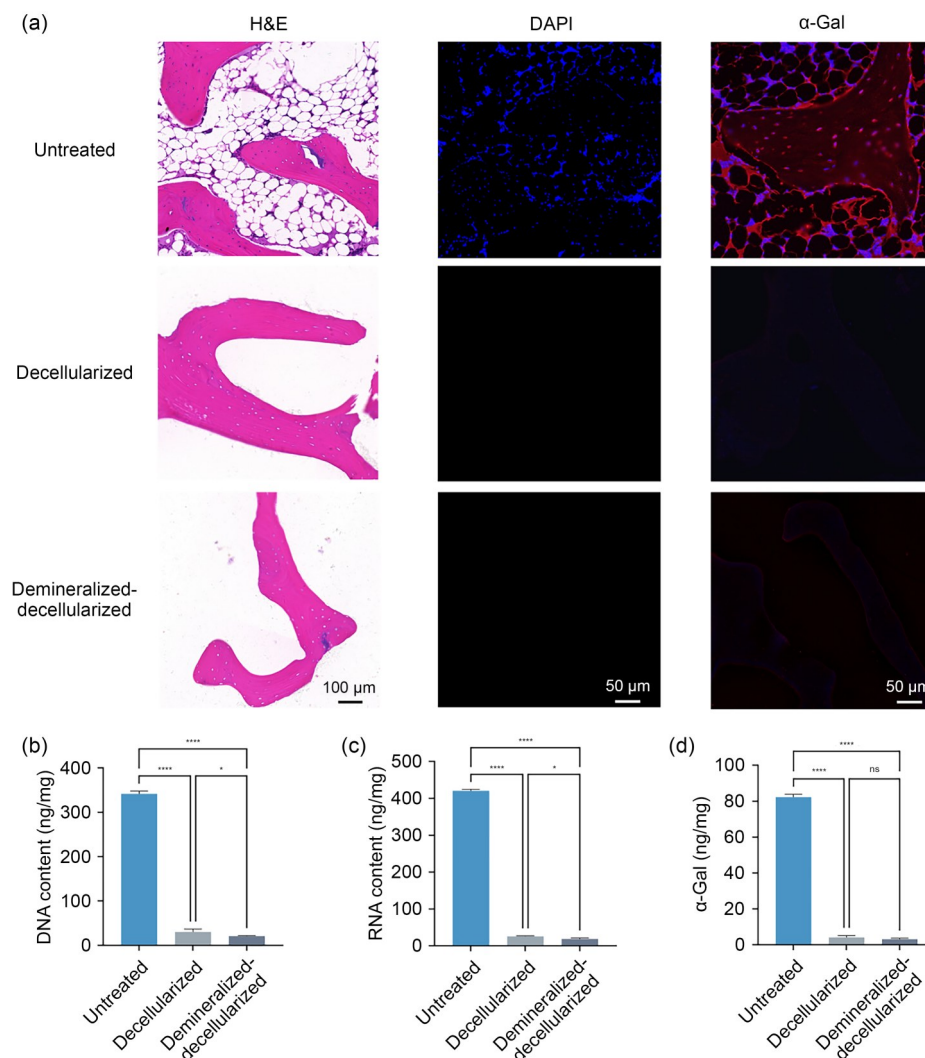


Fig. 3 Immunogenicity of the Ti6Al4V/xenogeneic bone composite (TXC) after different immunogenicity removal treatments. (a) Hematoxylin and eosin (H&E), 4',6-diamidino-2-phenylindole (DAPI), and galactose- α -1,3-galactose (α -Gal) immunofluorescence staining of the bone tissue in the composite following no treatment, decellularization treatment, and demineralization–decellularization treatment. (b) Quantitative analysis of DNA content in the bone tissue of the composite before and after treatments ($n=3$). (c) Quantitative analysis of RNA content in the bone tissue of the composite ($n=3$). (d) Quantitative analysis of α -Gal content in the bone tissue of the composite ($n=3$). Data are presented as mean $\pm$ standard deviation. “ns” indicates no significant difference between groups, while * indicates a significant difference between groups. * $p<0.05$ and **** $p<0.0001$

composites decreased from (341.40 ± 6.15) ng/mg to (30.07 ± 6.65) ng/mg, RNA content decreased from (420.47 ± 4.07) ng/mg to (25.81 ± 1.32) ng/mg, and α -Gal content decreased from (82.25 ± 1.56) ng/mg to (4.14 ± 1.02) ng/mg. Following demineralization–decellularization treatment, the DNA, RNA, and α -Gal contents were reduced to (20.81 ± 1.10) ng/mg, (19.02 ± 2.44) ng/mg, and (3.14 ± 0.52) ng/mg, respectively.

3.4 Demineralized-decellularized TXC retained and exposed the bioactive components

Figure 4a displays the results of Masson's trichrome staining, BMP-2, and TGF- β immunofluorescence staining of the bone tissue in untreated, decellularized, and demineralized-decellularized composite groups. Masson's trichrome staining showed that both decellularization and demineralization–decellularization treatments preserved the structural integrity of the bone matrix with minimal collagen disruption. Quantitative analysis of collagen content (Fig. 4b) further confirmed that the untreated group contained (76.74 ± 4.83) μ g/mg of collagen, the decellularized group contained (55.83 ± 8.44) μ g/mg, and the demineralized-decellularized group contained (86.36 ± 6.28) μ g/mg, indicating that collagen was well preserved following the treatments. BMP-2 immunofluorescence staining revealed that demineralized-decellularized treatment significantly exposed BMP-2 that was otherwise obscured by the inorganic components, such as hydroxyapatite, whereas negligible BMP-2 was detected in the decellularized and untreated groups. ELISA quantification results were consistent with this observation (Fig. 4c): the BMP-2 content in the demineralized-decellularized group was (18.11 ± 1.33) pg/mg, significantly higher than that in the decellularized $((4.73 \pm 0.62)$ pg/mg) and untreated $((3.01 \pm 0.54)$ pg/mg) groups. Furthermore, TGF- β immunofluorescence staining demonstrated abundant TGF- β distribution in the trabecular bone areas of the demineralized-decellularized composites, while only scant TGF- β was observed in the decellularized and untreated groups. ELISA assays further corroborated this finding (Fig. 4d): the TGF- β content was (16.33 ± 0.89) pg/mg in the demineralized-decellularized group, (3.01 ± 0.65) pg/mg in the decellularized group, and (1.56 ± 0.36) pg/mg in the untreated group.

3.5 Demineralized-decellularized TXC exhibited excellent biocompatibility and osteogenic activity

To evaluate the biocompatibility of TXC following decellularization and demineralization–decellularization treatments, the scaffolds were co-cultured with rabbit BMSCs, and their effects on BMSC growth were systematically assessed by measuring cell viability and morphology. Live/Dead staining results (Fig. 5a) showed that, compared to the

biocompatible Ti6Al4V scaffold, neither the decellularized nor the demineralized-decellularized TXC significantly inhibited BMSC viability. Notably, within the composite systems, cells tended to aggregate preferentially on the decellularized or demineralized-decellularized bone matrix regions.

Phalloidin staining was used to visualize the cytoskeleton of BMSCs cultured on the scaffolds (Fig. 5b). Cells on the Ti6Al4V scaffold exhibited an elongated spindle shape, while those located on the bone matrix of the decellularized or demineralized-decellularized composites displayed a distinct spindle-shaped morphology with extended pseudopodia, indicating improved cell spreading.

The proliferation of BMSCs co-cultured with the three scaffold groups on Days 1, 4, and 7 was assessed using the CCK-8 assay (Fig. 5e). The number of BMSCs increased over time in all groups. By Day 7, the cell proliferation rate in the demineralized-decellularized TXC group was significantly higher than that in both the decellularized TXC group and the Ti6Al4V scaffold group.

The osteogenic potential of BMSCs on the composites was analyzed using ALP staining, ARS staining, and RT-qPCR. Figure 5c presents the results of ALP staining, indicating strong positive ALP expression in the demineralized-decellularized TXC group, while little difference was observed between the decellularized TXC group and the Ti6Al4V scaffold group. ALP quantitative analysis confirmed the staining results (Fig. 5f). As shown in Fig. 5d, ARS staining revealed that only a small number of calcium nodules had formed in the Ti6Al4V scaffold group by Days 14 and 21. In contrast, the demineralized-decellularized TXC group exhibited significant calcium nodule formation, which increased from Day 14 to Day 21. The decellularized TXC group showed an intermediate level of mineralization. These observations were further supported by quantitative analysis (Fig. 5g). RT-qPCR analysis showed that on Day 7 of BMSCs co-cultured with the samples, the demineralized-decellularized TXC group exhibited the highest expression of osteogenic genes, including *ALP*, *BMP-2*, and *RUNX2*, compared with the other groups (Figs. 5h–5j). In addition, the decellularized TXC group showed slightly higher *ALP*, *BMP-2*, and *RUNX2* expression than the Ti6Al4V scaffold group (Figs. 5h–5j).

Therefore, based on the comprehensive results, the demineralized-decellularized TXC, which exhibited an intact osseointegration interface, high osteoinductive activity, and low immunogenicity, was selected for subsequent xenograft experiments.

3.6 Demineralized-decellularized TXC promotes osseointegration

The implantation procedure of the demineralized-decellularized TXC was illustrated in Fig. 6a. Micro-CT images at 12 weeks

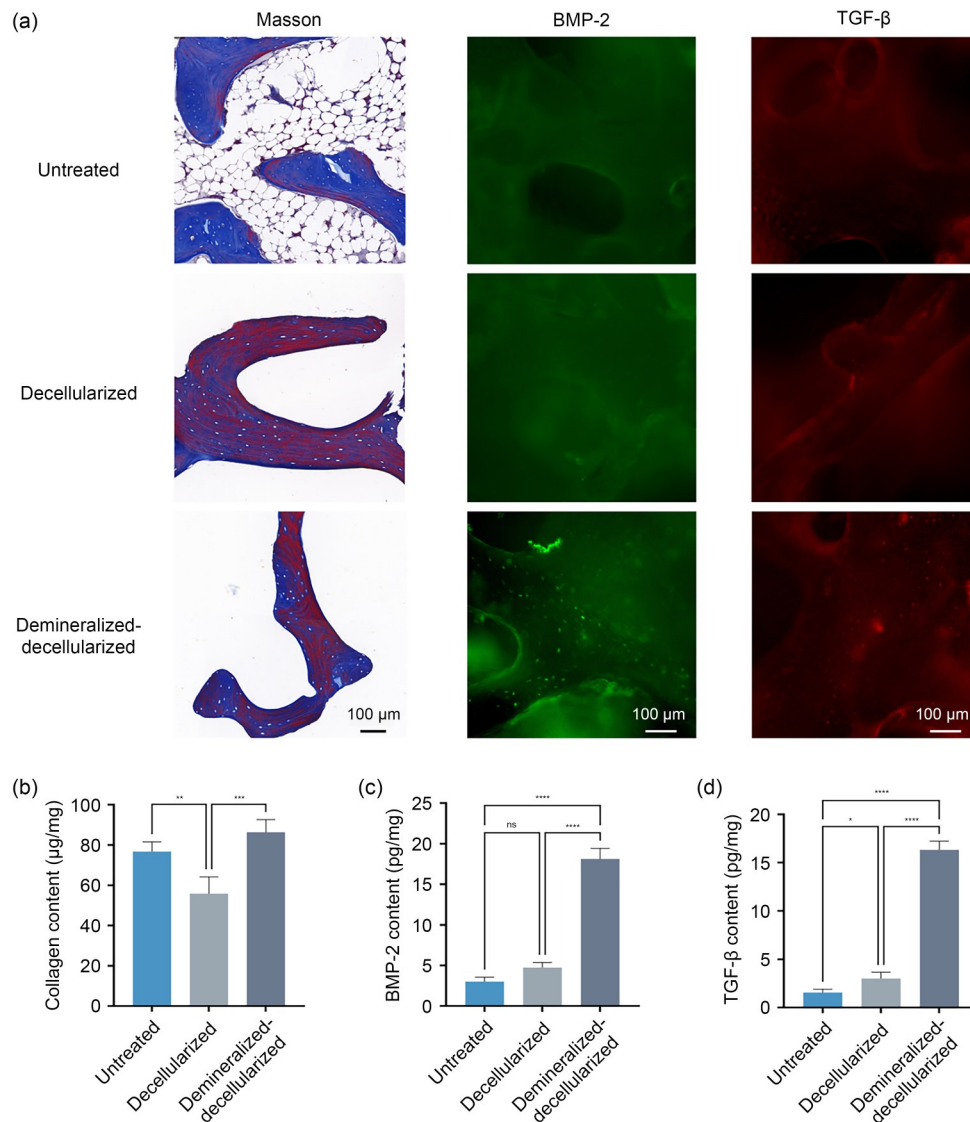


Fig. 4 Bioactivity of Ti6Al4V/xenogeneic bone composite (TXC) following different immunogenicity removal treatments. (a) Masson's trichrome staining, bone morphogenetic protein-2 (BMP-2) and transforming growth factor- β (TGF- β) immunofluorescence staining of the bone tissue in the composite following no treatment, decellularization treatment, and demineralization-decellularization treatment. (b) Quantitative analysis of collagen content in the bone tissue of the composite before and after treatments ($n=3$). (c) Quantitative analysis of BMP-2 content in the bone tissue of the composite ($n=3$). (d) Quantitative analysis of TGF- β content in the bone tissue of the composite ($n=3$). Data are presented as mean $\pm$ standard deviation. "ns" indicates no significant difference between groups, while * indicates a significant difference between groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

post-implantation (Fig. 6b) revealed excellent integration with the surrounding host bone tissue in both the demineralized-decellularized TXC group and the Ti6Al4V scaffold group. In contrast, a distinct gap was observed between the implant and the host bone in the untreated composite group. Analysis of bone morphometric parameters indicated that the demineralized-decellularized composite group exhibited significantly greater bone ingrowth than the Ti6Al4V scaffold group (Figs. 6c–6f).

Furthermore, the osseointegration strength between the composite and the surrounding bone tissue was validated by a mechanical push-out test (Figs. 6g and 6h). The results

demonstrated superior osseointegration in the demineralized-decellularized TXC group, with a maximum push-out force of (231.11 ± 15.59) N, which was significantly higher than that of the Ti6Al4V porous scaffold group $((178.04 \pm 5.03)$ N). The untreated composite group showed the lowest value, at only (33.12 ± 8.80) N.

To further investigate osseointegration at the histological level, hard-tissue sections were prepared from the harvested samples. H&E, Masson's trichrome (as shown in Fig. 7), and VG staining results (as shown in Fig. S2 in the supplementary information) demonstrated excellent bone fusion between the host bone and the composite in the

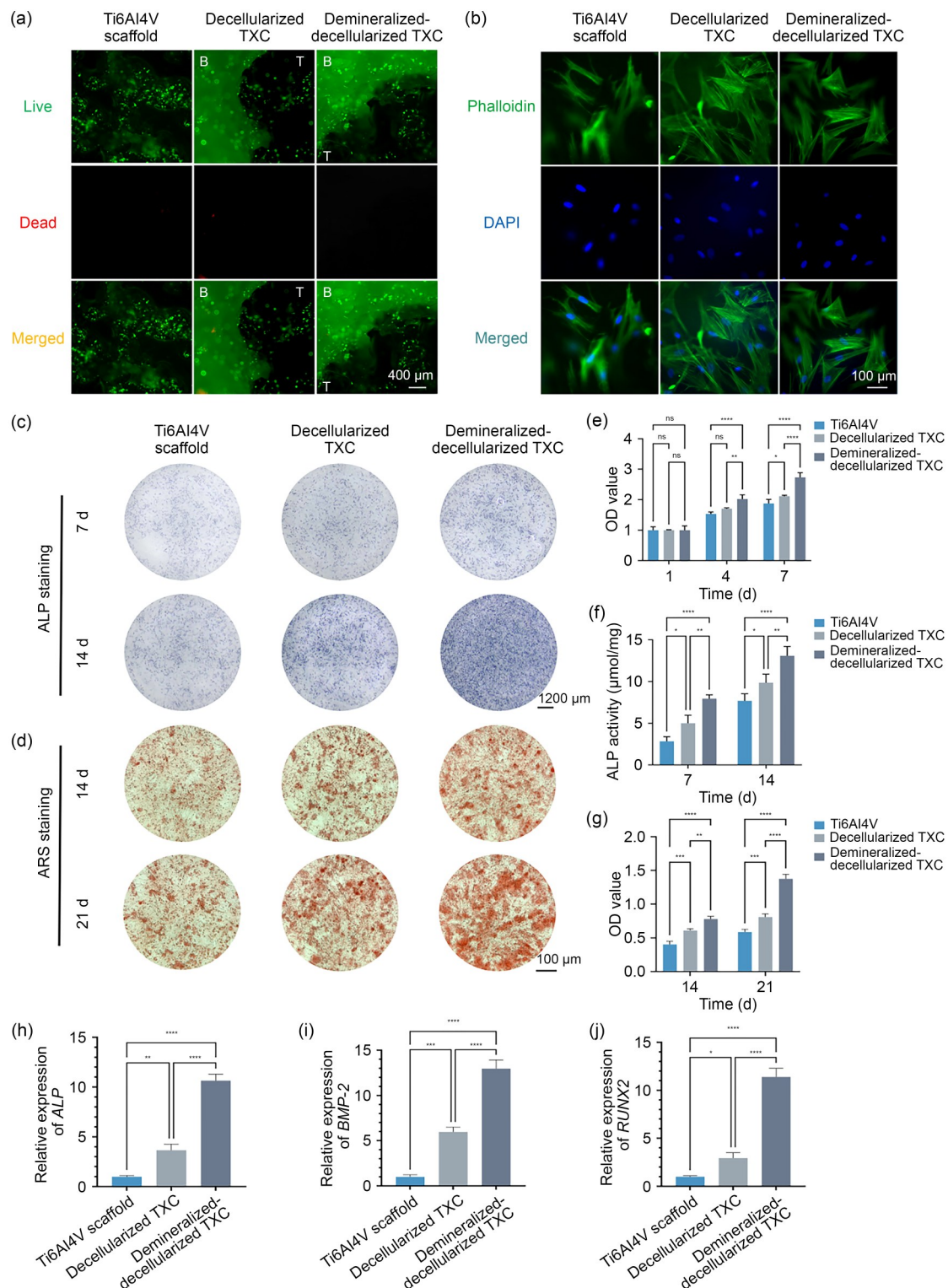


Fig. 5 Biocompatibility and osteogenesis induction activity of Ti6Al4V/xenogeneic bone composite (TXC) following different immunogenicity removal treatments. (a) Live/Dead staining of the Ti6Al4V scaffold, decellularized TXC, and demineralized-decellularized TXC. (b) Phalloidin and 4',6-diamidino-2-phenylindole (DAPI) staining of the Ti6Al4V scaffold, decellularized TXC, and demineralized-decellularized TXC. (c) Alkaline phosphatase (ALP) staining after 7 and 14 d of osteogenic induction. (d) Alizarin Red S (ARS) staining after 14 and 21 d of osteogenic induction. (e) Cell counting kit-8 (CCK-8) assay results after 1, 4, and 7 d of culture ($n=3$). (f) ALP activity of cells in the three groups ($n=3$). (g) Semi-quantitative analysis of ARS staining ($n=3$). Expression of osteogenesis-related genes *ALP* (h), *BMP-2* (i), and *RUNX2* (j) at 7 d ($n=3$). B: bone matrix in TXC; T: Ti6Al4V scaffold in TXC. Data are presented as mean $\pm$ standard deviation. "ns" indicates no significant difference between groups, while * indicates a significant difference between groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

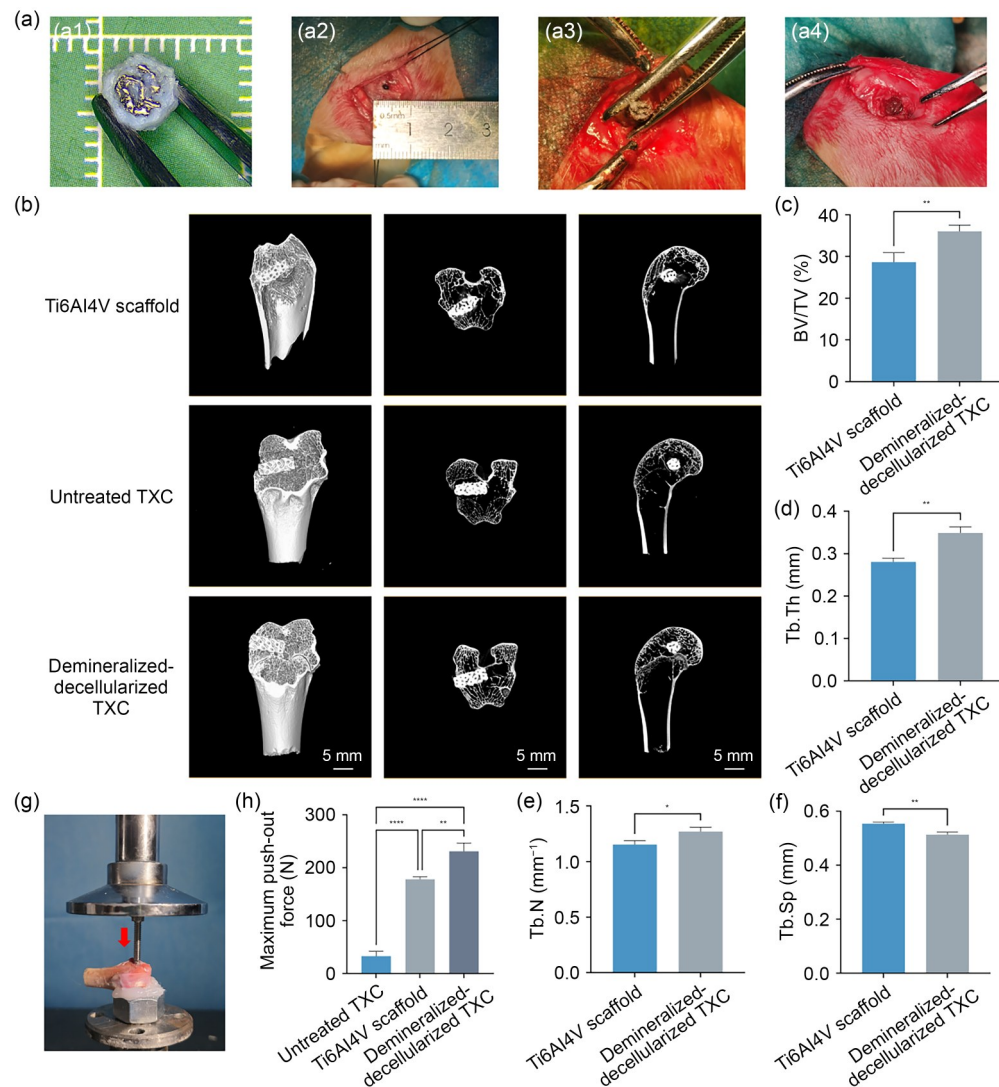


Fig. 6 Xenotransplantation procedure and radiographic/biomechanical evaluation of demineralized-decellularized Ti6Al4V/xenogeneic bone composite (TXC). (a) Surgical procedure for the xenotransplantation of demineralized-decellularized TXC. (b) Micro-computed tomography (micro-CT) results of the Ti6Al4V scaffold, untreated TXC, and demineralized-decellularized TXC 12 weeks after implantation in rabbits. Quantitative analysis of BV/TV (c), Tb.Th (d), Tb.N (e), and Tb.Sp (f) for the two groups at 12 weeks post-implantation ($n=3$). (g) Schematic diagram of the push-out test. (h) Quantitative analysis of the maximum push-out force from the biomechanical test ($n=3$). Data are presented as mean $\pm$ standard deviation. * indicates a significant difference between groups. * $p<0.05$, ** $p<0.01$, and **** $p<0.0001$

demineralized-decellularized group, with host bone growing into the pores of the Ti6Al4V implant via the “bridge” formed by the xenogeneic bone. The Ti6Al4V scaffold group also achieved osseointegration, but the amount of bone tissue within the pores was noticeably less than that in the demineralized-decellularized TXC group (Figs. 7a1-1, 7a1-2, 7a3-1, 7a3-2, 7b1-1, 7b1-2, 7b3-1, and 7b3-2). In the demineralized-decellularized TXC group, incompletely resorbed demineralized-decellularized xenogeneic bone matrix was observed on the surface of the titanium scaffold. The surface of new bone within the composite was lined with tall, morphologically active osteoblasts. In contrast, in the Ti6Al4V scaffold group, no morphologically active osteoblasts were observed, and only osteoblast–osteocyte transitional cells

were present (Figs. 7a1-3, 7a1-4, 7a3-3, and 7a3-4). In the untreated TXC group, a distinct gap filled with fibrous tissue was observed between the host bone and the composite, indicating failure of bony fusion. Within this composite, residual untreated xenogeneic bone contained numerous retained cell nuclei, accompanied by marked inflammatory cell infiltration (Figs. 7a2-1–7a2-4 and 7b2-1–7b2-4). These findings were consistent with the micro-CT and mechanical testing results.

Quantitative analysis of the section images showed that, in the demineralized-decellularized TXC group, (802.33 ± 18.50) ROIs were identified within the implant, among which (309.00 ± 7.00) ROIs contained bone ingrowth. Correspondingly, in the Ti6Al4V scaffold group, (757.00 ± 17.35) ROIs

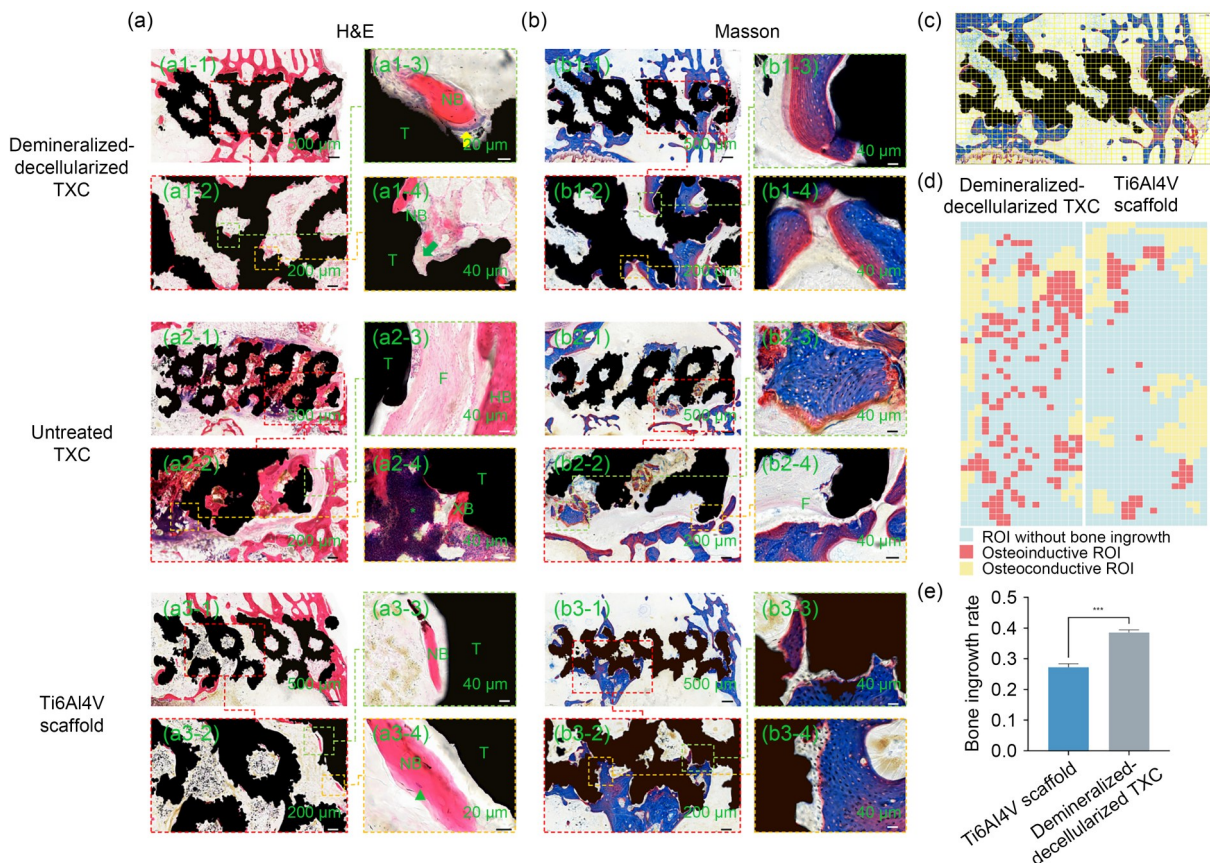


Fig. 7 Histological analysis of bone regeneration around the scaffolds. (a) Hematoxylin and eosin (H&E) staining and higher-magnification views of rabbit femoral condyles 12 weeks after implantation of Ti6Al4V scaffolds, untreated Ti6Al4V/xenogeneic bone composite (TXC), and demineralized-decellularized TXC. (b) Masson's trichrome staining and higher-magnification views of rabbit femoral condyles 12 weeks after implantation of Ti6Al4V scaffolds, untreated TXC, and demineralized-decellularized TXC. (c) Grid partitioning on the Masson's trichrome-stained sections (scale bar: 500 μ m). (d) Heatmap of bone ingrowth patterns within Ti6Al4V scaffolds. (e) Bone ingrowth rate between demineralized-decellularized TXC and Ti6Al4V scaffold groups ($n=3$). The yellow arrow indicates osteoblasts lining the surface of new bone. The green arrow points to residual demineralized-decellularized xenogeneic bone matrix within the composite. The green triangle indicates osteoblast–osteocyte transitional cells. The asterisk indicates the foci of inflammatory cell infiltration. T: Ti6Al4V scaffold; F: fibrous tissue; NB: new bone; HB: host bone; XB: xenogeneic bone. Data are presented as mean $\pm$ standard deviation. * indicates a significant difference between groups. *** $p < 0.001$

were identified within the implant, of which (206.00 ± 7.00) ROIs showed bone ingrowth. In the demineralized-decellularized TXC group, the bone ingrowth rate was 0.3852 ± 0.0089 , and the process of bone ingrowth was predominantly osteoinductive (Figs. 7d and 7e). In the Ti6Al4V scaffold group, the bone ingrowth rate was 0.2724 ± 0.0110 , and the process of bone ingrowth was predominantly osteoconductive (Figs. 7d and 7e).

4 Discussion

This study aimed to develop a TXC that recreated the natural osteogenic microenvironment within microporous Ti6Al4V scaffolds to address the clinical issue of poor osseointegration caused by insufficient bioactivity. We found that the demineralization–decellularization treatment was an ideal processing method for the TXC. The treated composite not

only maintained the original osseointegration but also exhibited high osteoinductive activity and low immunogenicity. The pre-integrated demineralized-decellularized bone matrix served as a “bridge” between the implant and the host bone, facilitating significant bone ingrowth and leading to robust osseointegration.

TXC was successfully constructed 12 weeks after implantation into the cancellous bone of rat femoral condyles. Previous studies have established that bone possesses a certain regenerative capacity, allowing it to grow into the micropores of metal implants and achieve osseointegration [25–27]. Micro-CT analysis showed that bone tissue had grown into the scaffold's microporous structure, forming mechanical interlocking, thereby verifying successful TXC construction (further confirmed by SEM results shown in Fig. 1f). Histological sections further indicated extensive contact between the host bone and the scaffold's microporous surface, with new bone tissue integrated into pores. The mechanisms of

osseointegration primarily include (1) osteoconduction and (2) osteoinduction. Osteoconduction is the process by which host bone tissue grows directly into the micropores of the implant, forming mechanical interlocking through new bone deposition. The microporous titanium alloy implant primarily functions through osteoconduction, as shown in Figs. 5c and 5d, which demonstrate that the scaffold was unable to promote osteogenic differentiation of BMSCs [28]. Osteoinduction is the process by which undifferentiated mesenchymal stem cells migrate and colonize the microporous surface, differentiate into osteoblasts under the stimulation of the local microenvironment, and ultimately form new bone at the implant interface [29, 30]. This process primarily relies on bioactive factors, as demonstrated by Urist's confirmation of the osteoinductive activity in decalcified bone matrix [31]. We therefore aimed to confer osteoinductive activity to the porous Ti6Al4V scaffold by harnessing natural bone. However, because xenogeneic bone is inherently immunogenic, it must be appropriately processed before use [32]. The current mainstream technical approach for preparing clinically applicable xenogeneic bone grafts involves removing the immunogenicity of xenogeneic bone through processes such as deproteinization (e.g., Bio-Oss[®]), decellularization (e.g., ABCcolla[®]), or demineralization/partial demineralization–decellularization (e.g., Decell Matrix[™]), and their clinical effectiveness has been validated to some extent [33–35]. In the present study, we systematically compared the effects of these treatments on TXC.

The deproteinization treatment compromised the bone–titanium integration interface of the TXC, leading to the failure of the composites. The composite is an integrated structure formed by xenogeneic bone and Ti6Al4V implants through osseointegration. Consequently, understanding how to completely retain the original osseointegration interface in the composite while eliminating the immunogenicity of xenogeneic bone has become a core issue requiring research focus, which this study addresses. To evaluate the impact of different antigen-removal treatments on the structure of the osseointegration interface, we performed SEM and EDS analyses on the interface between the treated xenogeneic bone and the Ti6Al4V scaffold. It was found that deproteinization damaged the interface connection between the xenogeneic bone and the implant. In contrast, after decellularization, the osseointegration interface remained intact and was primarily composed of collagen and hydroxyapatite. Similarly, after combined demineralization–decellularization treatment, the osseointegration interface also remained intact, with the bone and implant connected via collagen. The contemporary academic explanation of the osseointegration process is as follows: fibrin first adheres to the implant surface, and then osteoblasts migrate along this interface, secrete non-collagenous proteins, and guide the deposition of hydroxyapatite crystals (forming a cement line). Subsequently, osteoblasts

secrete additional collagen and complete mineralization, ultimately forming a stable bone–implant interface [36, 37]. Thus, collagen and hydroxyapatite are the fundamental material basis of this connection [38]. Given that collagen bundles are closely apposed to the implant surface and directly contact its oxide layer, once deproteinization has removed collagen at the bone–implant interface, it will disrupt the structural basis of osseointegration [39]. Therefore, deproteinization is not suitable for processing TXC.

The decellularization-based treatment substantially reduced the immunogenicity of the composites. It has been demonstrated that the immunogenicity of xenogeneic grafts arises primarily from donor cells, including nucleic acids in nuclear and membrane-bound components such as α -Gal epitopes and major histocompatibility complex (MHC) molecules [40, 41]. Decellularization removes these immunogenic components while preserving the 3D architecture of the native bone ECM and its intrinsic bioactive constituents [41]. The histological and biochemical analyses performed in this study collectively confirmed that both decellularization protocols efficiently removed cellular and immunogenic components from the composites. H&E and DAPI staining revealed abundant adipose tissue in the native bone and cellular nuclei within the matrix, whereas after either treatment, almost no residual cells or nuclei were detectable, indicating effective decellularization of the constructs. α -Gal immunofluorescence staining consistently revealed a widespread distribution of α -Gal epitopes in untreated bone, while only a minimal or no detectable signal remained following processing, suggesting a marked reduction in xenogeneic carbohydrate antigens. However, merely removing the composite's immunogenicity is insufficient to achieve rapid bone ingrowth—the material must also be rendered bioactive [42, 43].

The demineralization–decellularization treatment imparts osteoinductive activity to the composites by exposing their bioactive sites. The bioactivity of the bone matrix coating in the Ti6Al4V/xenogeneic bone composite is the core link between the “implant and the host bone.” By producing osteogenic signals, it promotes the chemotaxis, proliferation, and osteogenic differentiation of host stem cells at the implantation site, thereby directly determining the speed, quality, and long-term stability of osseointegration [44, 45]. Therefore, when preparing the TXC, under the premise of effectively removing the immunogenicity of the xenogeneic bone, processing techniques that can effectively preserve and optimize the composite's bioactivity should be prioritized to ensure efficient and reliable osseointegration. Unlike other tissues, bone contains hydroxyapatite, and mere decellularization exposes limited active sites, making it difficult to achieve the required bioactivity [46]. To address this limitation, we adopted a comprehensive strategy of demineralization followed by decellularization. On the one hand, removing the mineralized components transforms dense hard tissue into

soft tissue, significantly improving the clearance efficiency of cells and other immunogenic components (Fig. 3). On the other hand, multiple growth factors (such as BMP-2 and TGF- β) secreted by osteoblasts and osteocytes are deposited within the organic matrix of trabecular bone and on the surface of the mineral phase, where they are stored in bone tissue as a “matrix reservoir.” When hydroxyapatite crystals dissolve, or the bone matrix undergoes remodeling, these bioactive factors, which are bound to type I collagen fibers and the hydroxyapatite interface, are gradually released and participate in the coupled processes of bone resorption and bone formation [47, 48]. Demineralization effectively exposes bioactive factors (such as BMP-2 and TGF- β) that are sequestered within bone tissue, thereby significantly enhancing the osteoinductive and bone regeneration potential of the bone matrix coating (Fig. 4) [46, 49]. Importantly, TXC does not involve exogenous growth factor loading. Demineralization mainly exposes endogenous, matrix-bound osteogenic cues within the pre-integrated bone matrix on the implant surface. Given the reservoir effect of the dense ECM and growth factor-binding components (e.g., glycosaminoglycans (GAGs)), these cues are expected to become progressively available through osteoclast-mediated remodeling rather than via uncontrolled burst release [50]. The results of in vitro experiments also indicated that a porous Ti6Al4V scaffold alone provides limited stimulation of BMSC osteogenic differentiation, whereas the incorporation of demineralized-decellularized TXC markedly enhances ALP activity and matrix mineralization (Figs. 5c, 5d, 5f, and 5g). Consistent with these phenotypic results, RT-qPCR at Day 7 showed that osteogenesis-related genes (*ALP*, *BMP-2*, and *RUNX2*) were upregulated in BMSCs co-cultured with TXC-based samples, with the highest expression observed in the demineralized-decellularized TXC group (Figs. 5h–5j). The superior performance of this group suggests that additional demineralization following decellularization more effectively exposes matrix-bound osteoinductive cues. In contrast, the decellularized TXC group elicited only intermediate osteogenic responses, highlighting the importance of appropriately processed xenogeneic bone in converting an osteoconductive metallic scaffold into a composite with pronounced osteoinductive capacity.

In vivo experiments are essential for evaluating the role of demineralized-decellularized TXC in promoting bone regeneration and osseointegration. Histological analysis revealed clear differences among the groups. In the demineralized-decellularized TXC group, H&E, Masson’s trichrome, and VG staining showed continuous bony bridging from the host bone into the pores of the Ti6Al4V scaffold via the xenogeneic matrix, resulting in intimate bone–implant contact. Conversely, in the Ti6Al4V scaffold group, osseointegration was primarily limited to the interface, with less bone tissue and more residual voids inside the pores (Figs. 7a, 7b, and 7e). The untreated TXC group, which exhibited a persistent fibrous

gap, retained cell nuclei within xenogeneic bone, and marked inflammatory infiltration, clearly represents a failed osseointegration response. From another perspective, this further demonstrated that the demineralization–decellularization treatment effectively eliminated the immunogenicity of the TXC (Fig. 7a). The cellular morphology observed at the bone–material interface provides additional clues about the underlying mechanism. In the demineralized-decellularized TXC group, tall, morphologically active osteoblasts lining the surface of newly formed bone suggest ongoing, robust osteogenic activity at 12 weeks. In contrast, the Ti6Al4V scaffold group lacked such morphologically active osteoblasts and predominantly showed osteoblast–osteocyte transitional cells, consistent with limited or prematurely attenuated osteogenic stimulation within the scaffold pores. The quantitative analysis of Masson’s trichrome-stained sections further supports these qualitative observations. Compared with the Ti6Al4V scaffold group, the demineralized-decellularized TXC group featured a higher number of ROIs containing bone tissue and a markedly increased bone ingrowth rate (0.3852 ± 0.0089 vs. 0.2724 ± 0.0110), corresponding to an increase of roughly 40%. More importantly, the number of osteoconductive ROIs was of a similar order of magnitude in both groups, whereas osteoinductive ROIs were substantially enriched in the demineralized-decellularized TXC group. This indicates that the composite converts the osteogenic pattern from predominantly osteoconduction to an osteoinduction-dominant mode of bone formation (Fig. 7d). However, histological sections only provide two-dimensional information on bone ingrowth and integration. Micro-CT 3D reconstruction enables comprehensive quantitative analysis of bone volume and morphological structure within the composite. The results indicated that the demineralized-decellularized TXC significantly promoted bone tissue ingrowth into the scaffold (Figs. 6b–6f). Push-out mechanical tests further assessed the osseointegration strength of different groups. The results showed that the bonding strength between this composite and the host bone was significantly higher than that of the Ti6Al4V scaffold, indicating superior osseointegration performance (Figs. 6g and 6h).

The demineralized-decellularized TXC plays a dual role at the bone defect site. On the one hand, the demineralized-decellularized bone matrix in the composite acts as a bioactive “coating” with a natural bone structure, significantly enhancing cell adhesion and spreading on the implant surface (Fig. 5b). This “coating” not only improves the surface hydrophilicity of the metal implant (Figs. 1i and 1j), but, more importantly, it likely retains adhesive proteins (e.g., collagen and fibronectin) present in the original bone matrix. These proteins can be recognized and bound by integrin receptors on cell surfaces, further promoting cell adhesion and spreading on the composite [51]. On the other hand, the demineralized-decellularized bone matrix, pre-integrated with

the implant through a pre-integration strategy, serves as a “bridge” between the host bone and the implant. This structure acts as a placeholder at the osseointegration interface, effectively preventing the ingrowth of fibrous tissue during host bone creeping substitution. It ensures that the host bone can reach the implant surface directly via this “bridge” and, through creeping substitution, gradually replaces the “old osseointegration” on the implant surface, ultimately forming a stronger “new osseointegration” interface. It is worth emphasizing that this “bridge” we constructed between the host bone and the implant is, in fact, a bioactive “highway.” These assays indicated enrichment of BMP-2 and TGF- β within the matrix (Fig. 4). Among these, BMP-2, as the most critical osteoinductive factor, can directly activate the osteogenic differentiation program of stem cells, thereby driving new bone formation. Simultaneously, the demineralized-decellularized bone matrix, as a natural carrier, protects BMP-2 activity and enables its sustained release [52, 53]. TGF- β upregulates integrin expression, promoting cell adhesion and proliferation; it also stimulates collagen and proteoglycan synthesis, thereby regulating ECM synthesis and remodeling [54, 55]. Therefore, following implantation of this composite, mesenchymal stem cells may rapidly proliferate and colonize the composite surface, and differentiate into osteoblasts under the synergistic action of its surface-active components, rather than relying on the slow process of creeping

substitution. This improves the osteogenic microenvironment within the implant and accelerates bone defect repair.

In summary, this study demonstrates that demineralized-decellularized TXC effectively repairs bone defects and achieves robust, reliable implant osseointegration. As a pre-integration strategy that combines an implant with grafted bone, the TXC offers advantages such as wide availability of sources and prevention of fibrous tissue ingrowth. For future applications, clinically required implants can first be pre-implanted into large animals (e.g., pigs or cattle). Following the completion of osseointegration between the implant and the animal’s bone, the implant is harvested, along with the surrounding attached bone tissue. Subsequently, the bone tissue surrounding the composite is precisely sculpted using 3D computer-aided design/computer-aided manufacturing (CAD/CAM) technology to exactly match the patient’s bone defect morphology. Finally, following demineralization–decellularization treatment, this personalized composite is implanted into the patient’s defect site, thereby achieving the dual goals of repairing the bone defect while simultaneously promoting osseointegration at the implant interface (Fig. 8). However, clinical translation of this multi-step, xenogeneic-based manufacturing process will require scalable, good manufacturing practice (GMP)-compliant manufacturing with batch-to-batch consistency and validated biosafety, and may face additional regulatory scrutiny of manufacturing

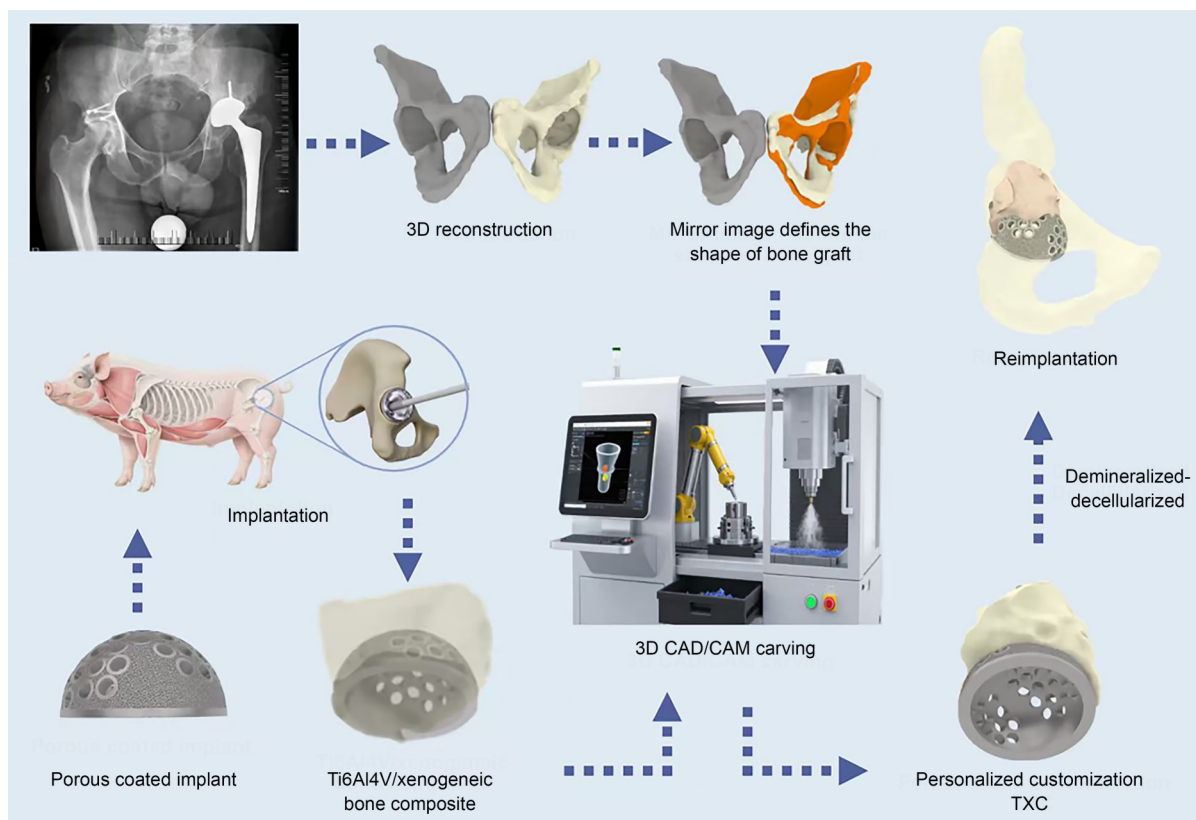


Fig. 8 Potential demineralized-decellularized Ti6Al4V/xenogeneic bone composite (TXC) application scenarios

controls and long-term safety as a tissue–metal combination product.

5 Conclusions

We developed a Ti6Al4V/xenogeneic bone composite (TXC) to address the clinical challenge of poor osseointegration caused by the limited bioactivity of metal implants. After comparing the interfacial connection strength, microstructure, immunogenicity, and bioactivity of the composite under different treatment methods, the demineralized-decellularized TXC was ultimately identified as the optimal choice because it exhibits high osteoinductive activity and low immunogenicity while preserving the integrity of the osseointegration interface. In vivo experiments demonstrated that the demineralized-decellularized TXC effectively repaired bone defects and enhanced osseointegration.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval We strictly followed the guidelines of the Institutional Animal Care and Use Committee of China. Animal experiments were approved by the Animal Ethics Committee of the College of Basic Medical Sciences of Jilin University (No. 2024-555).

Data availability Data will be made available on request.

Use of generative AI tools During the preparation of this work, the authors used DeepSeek in order to improve language and check for grammatical errors. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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