



Original Article

Keratin-Incorporated Gradient Hydroxyapatite-Reinforced GelMA/OCMC Hydrogel Scaffolds for Rotator Cuff Tendon-to-Bone Repair

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Abstract

Background: Suboptimal tendon-to-bone interface (TBI) healing after rotator cuff repair is associated with fibrous scar formation and limited restoration of the graded tendon–fibrocartilage–bone transition. This study developed a keratin-incorporated, gradient hydroxyapatite (gHAP)-reinforced gelatin methacryloyl (GelMA)/oxidized carboxymethyl cellulose (OCMC) hydrogel scaffold, designated K+gHAP@GC, to provide spatial mineral cues, keratin-associated bioactivity, and local mechanical support for TBI repair. **Methods:** K+gHAP@GC comprised a GelMA/OCMC/keratin matrix with a layer-by-layer assembled HAP gradient and was stabilized by Schiff-base reaction and ultraviolet photocrosslinking. Scaffold morphology, chemical composition, HAP distribution, mechanical and rheological properties, swelling, degradation, and *in vivo* retention were characterized. Its effects on bone marrow mesenchymal stem cell (BMSC) viability, osteogenic differentiation, fibrocartilage-like matrix deposition, endothelial angiogenic activity, and *in vivo* tendon-to-bone healing were evaluated. **Results:** The scaffold exhibited an interconnected porous architecture, an scanning electron microscopy–energy-dispersive X-ray spectroscopy (SEM-EDS)-verified HAP gradient, improved gel stability, and enhanced compressive, rheological, and cyclic compression performance. *In vitro*, the scaffold sustained BMSC viability and proliferation, promoted osteogenic differentiation, and enhanced COL2A1-related matrix expression, with enhanced YAP1 nuclear localization in HAP-containing groups. Keratin-containing hydrogels further enhanced endothelial cell migration, tube formation, and angiogenesis-related gene expression. *In vivo*, K+gHAP@GC improved bone-side reconstruction, Safranin O-Fast Green (SO-FG)-positive fibrocartilage-like matrix formation, collagen organization, local VEGF-positive staining, and biomechanical performance. **Conclusions:** K+gHAP@GC provides an integrated mineral–protein gradient hydrogel strategy that combines spatial mineral cues, keratin-associated bioactivity, and local mechanical support to improve rotator cuff TBI healing. These findings support its potential for TBI repair, although long-term efficacy and causal mechanisms require further validation.

Keywords: Biomimetic scaffold, keratin, rotator cuff tear, gradient hydrogel, tendon-bone interface.

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Introduction

Rotator cuff tears (RCTs) are among the most prevalent degenerative conditions in sports medicine and orthopedics, significantly impairing shoulder function and diminishing patient quality of life. With the rapid aging of the global population, the incidence of RCTs has risen markedly, affecting 50%–62% of individuals aged 80 and above [1–4]. Despite substantial advancements in minimally invasive arthroscopic suture techniques, challenges persist in the form of suboptimal postoperative functional recovery and high re-tear rates, which reportedly range between 20% and 90%, particularly in cases of massive RCTs. These issues constitute significant clinical challenges that necessitate urgent advancements [5–7]. At a fundamental level, the native tendon-to-bone interface (TBI) involves four primary layers: tendon, uncalcified fibrocartilage, calcified fibrocartilage, and bone [8]. This native TBI serves as a sophisticated “soft-to-hard” transitional interface [9], facilitating a gradual transition in mechanical modulus from tendon (~200 MPa) to bone (~20 GPa), thereby effectively mitigating stress concentration at the interface [10,11]. The gradient structure effectively links the resilient tendon to the rigid bone mass, thereby endowing the tendon-bone interface TBI with superior biomechanical properties [12]. Nonetheless, conventional surgical techniques are limited to restoring anatomical continuity. In the absence of an appropriate biological microenvironment, the regenerated tissue is often supplanted by fibrovascular scar tissue, which possesses suboptimal mechanical properties. The lack of a normal fibrocartilage layer renders the interface highly vulnerable to fatigue failure under cyclic loading [10,13].

To address this challenge, tissue-engineered hydrogels have emerged as promising candidates for TBI repair, given their high water content, tunable physicochemical properties, and suitability for biological modification. Current scaffold design strategies mainly include structural biomimicry and functional bioactivation [14]. Structurally biomimetic scaffolds attempt to reproduce the graded organization of the native insertion site through monophasic, biphasic, or multiphasic designs, thereby providing spatial guidance for region-specific tissue regeneration. Representative studies have demonstrated the potential of gradient HAP/collagen scaffolds, ECM-based aligned composites, and multilayer fibrous or hydrogel systems to support fibrocartilage and bone formation at the repaired interface [8,15,16]. In parallel, functionally bioactive scaffolds seek to regulate the local regenerative microenvironment by incorporating immunomodulatory, angiogenic, or osteoinductive cues to enhance repair efficacy [14]. Overall, these studies have established the importance of anatomical stratification and compositional gradation in scaffold design. Nevertheless, most existing scaffolds still focus predominantly on reproducing structural organization or compositional layering, while their ability to simultaneously provide mechanical continuity, biological stimulation, and

site-specific regenerative cues remains insufficient [17].

Indeed, an ideal scaffold for TBI repair should satisfy at least two additional requirements beyond structural mimicry. First, it should provide localized micromechanical cues and compliant viscoelastic support within the soft-to-hard transition zone, thereby effectively attenuating mechanical stress concentration under physiological cyclic loading [18]. Second, it should actively regulate the regenerative microenvironment in a site-specific manner, promoting osteogenesis and vascular ingrowth on the bone side while facilitating fibroblast adhesion, migration, and matrix deposition on the tendon side, and supporting continuous fibrocartilage-like matrix deposition at the transitional interface [19,20]. Therefore, the key challenge in scaffold design is not merely to reproduce the morphology of the TBI, but to integrate material chemistry, structural architecture, and biological functionality into a coordinated regenerative system.

Among the biological factors involved in tendon-to-bone healing, early angiogenesis has increasingly been recognized as a critical determinant of repair quality. The repaired interface is relatively hypovascular, and insufficient vascularization may restrict oxygen and nutrient supply, delay extracellular matrix remodeling, and impair tissue maturation [21]. This issue is particularly important during the early healing stage, when reparative cell survival, migration, and functional activation depend heavily on local microenvironmental support. Emerging evidence has shown that promoting neovascularization around the repaired footprint can enhance biological integration and improve the mechanical competence of the healing interface. For example, bioactive exosomes, ion-releasing materials, and vascularization-promoting biomaterials have all demonstrated beneficial effects on tendon-to-bone repair by improving local angiogenesis and regenerative signaling [22,23]. These findings collectively suggest that angiogenic regulation is not merely an auxiliary event, but an essential component of functional TBI reconstruction [24–27].

In this context, keratin is a highly viable biomaterial, given its inherent biochemical activity and abundant reactive groups for network construction. Previous studies have shown that keratin-based biomaterials can promote endothelial adhesion, migration, and proliferation, suggesting their potential to improve the early vascular microenvironment at the repair site [28]. From a materials perspective, keratin contains abundant amino groups that can react with aldehyde-containing polysaccharides via dynamic Schiff base chemistry, thereby enabling the construction of mechanically reinforced and biologically active hydrogel systems. In other words, keratin is not only a bioactive component with potential pro-angiogenic value, but also a functional building block for enhancing network stability and scaffold integrity [29,30]. This dual role is particularly valuable for TBI repair, where both early biological activa-

tion and sustained structural support are required. Therefore, keratin may serve as an effective bridge between microenvironmental modulation and scaffold engineering.

Based on these considerations, an innovative biomimetic composite scaffold, designated as K+gHAP@GC, was developed in this study. In this nomenclature, K stands for keratin, gHAP indicates gradient hydroxyapatite, and GC represents the foundational gelatin methacryloyl (GelMA) and oxidized carboxymethyl cellulose (OCMC) matrix framework. This integrated monophasic scaffold system combines a chemically fortified design at the material level with the capacity to remodel the biological microenvironment, specifically aimed at TBI repair. The incorporated keratin, abundant in reactive amino groups, facilitates the formation of highly efficient dynamic Schiff base crosslinks with the aldehyde moieties of OCMC, while also establishing a dense interpenetrating double-network (DN) structure with GelMA through photocrosslinking. This design is intended to enhance the hydrogel's mechanical strength and fatigue resistance, thereby providing a more stable three-dimensional microenvironment conducive to cell adhesion and proliferation. At the structural biomimetic level, a spatially defined gHAP architecture was engineered across the hydrogel layers using a layer-by-layer assembly technique followed by sequential UV photocrosslinking. We hypothesized that this combined “bioactive-mechanical” strategy would exert a synergistic effect on tendon-to-bone healing: the localized mineral gradient provides physical cues associated with mechanosensitive responses, while the keratin network may promote pro-angiogenic endothelial responses. Therefore, this study sought to fabricate and characterize the K+gHAP@GC scaffold, and evaluate its cytocompatibility, osteogenic differentiation potential, fibrocartilage-like matrix-associated bioactivity, mechanosensitive cellular responses, and pro-angiogenic endothelial activity *in vitro*. Importantly, we aimed to investigate its therapeutic potential to promote fibrocartilage-like matrix deposition and restore rotator cuff biomechanics *in vivo*. The overall scaffold design, layer-by-layer assembly process, dual-crosslinked network formation, and proposed application in TBI repair are summarized in Scheme 1.

Materials and Methods

Preparation of GelMA, OCMC, and Precursor Solution

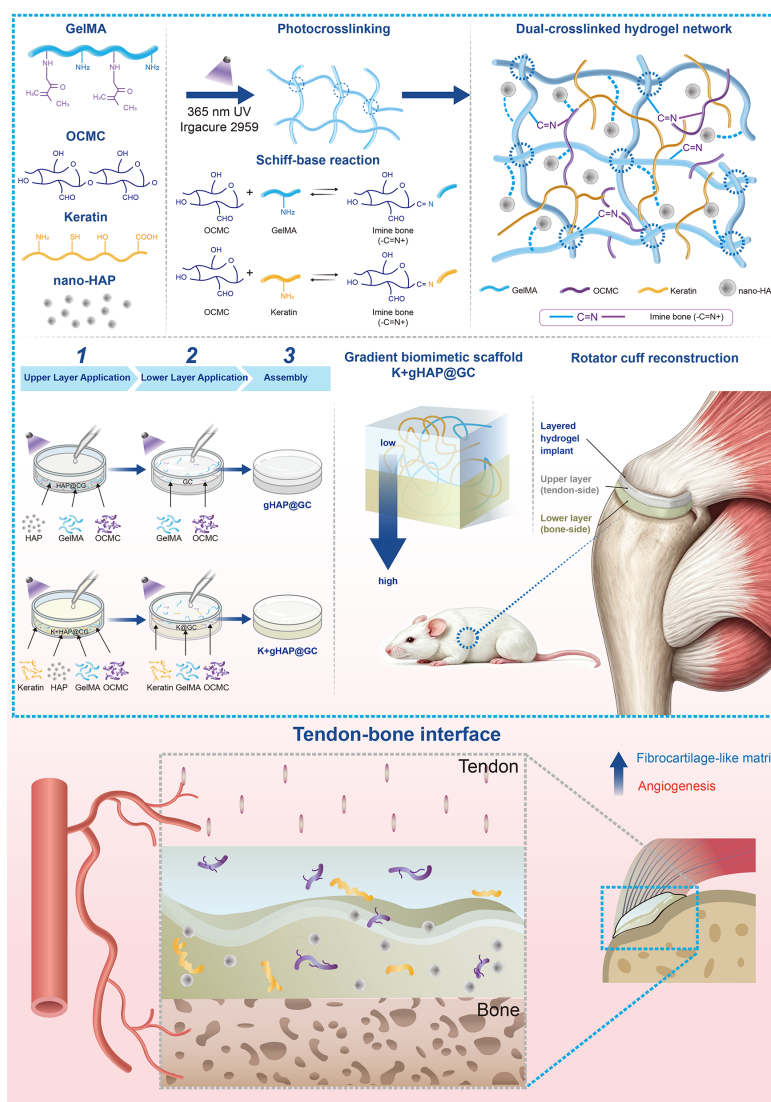
The preparation of GelMA and OCMC followed established protocols from previous research [31,32]. For GelMA: Briefly, 10 g of gelatin (Cat. No. G108396, Aladdin, China) was dissolved in 100 mL of phosphate-buffered saline (PBS, Cat. No. G4202, Servicebio, China) at 50 °C. Subsequently, 5 mL of methacrylic anhydride (MA, Cat. No. M812900, Macklin, China) was added gradually, allowing the reaction to proceed for 2 h at 50 °C in the absence of light. The reaction was terminated by adding

300–400 mL of pre-warmed PBS. The resulting solution was transferred into a dialysis bag (MW: 12–14 kDa, Solarbio, China) and dialyzed against deionized water for 4 days, with the water being refreshed every 4 h on the first day and every 8 h thereafter. The dialysate was then pre-frozen at –80 °C and lyophilized to obtain the final GelMA product, which was stored in a dark environment. For the synthesis of OCMC, 1 g of CMC (Cat. No. C804620, Macklin, China) was dissolved in 50 mL of deionized water at room temperature. Following the addition of 1 g of sodium periodate (Cat. No. S817518, Macklin, China), the pH was adjusted to approximately 4.0 using 50% acetic acid (Cat. No. A822379, Macklin, China). The oxidation reaction was conducted at 35 °C for 4 h in the dark. Unreacted sodium periodate was subsequently neutralized by the addition of 1 mL of ethylene glycol (Cat. No. E808738, Macklin, China), with continuous stirring for 1 h. The resulting mixture was precipitated using an excess volume of absolute ethanol (Cat. No. WHP0587, GHTECH, China). The collected precipitate was then redissolved in deionized water, subjected to sequential dialysis, and lyophilized to obtain purified OCMC.

To eliminate non-adherent cells and debris, a half-medium change was performed after 24 h, followed by full medium changes every other day. Upon reaching 80%–90% confluence, the primary cells (denoted as P0) were washed with PBS, detached using 0.25% trypsin-EDTA (25200072, Gibco, Thermo Fisher Scientific, USA), and centrifuged at 200 × g for 5 min. Finally, the cells were subcultured into new flasks at a split ratio of 1:2 to 1:4 for subsequent experiments. Human umbilical vein endothelial cells (HUVECs, SenBeJia Biological, China) were cultured in commercial endothelial cell complete medium (Cat. No. CM-ZY001, Procell, China). All cells were maintained in a incubator (HF240, Heal Force Bio-Meditech Holdings Limited, China) at 37 °C with 5% CO₂. The culture medium was refreshed every 2–3 days, and the cells were passaged using trypsin-EDTA upon reaching 80%–90% confluence. All cells used in subsequent experiments were routinely tested and verified to be negative for mycoplasma contamination.

Preparation of Biomimetic Scaffolds

To ensure compositional homogeneity and structural fidelity, hydrogel precursors were prepared by mixing pre-formulated high-concentration stock solutions. All procedures were performed under protection from light. Briefly, four stock solutions were prepared. Solution A was prepared by dissolving 0.025 g of the photoinitiator Irgacure 2959 (Cat. No. H823463, Macklin, China) in 3.5 mL of deionized (DI) water at 60 °C, after which 1 g of GelMA was added under continuous stirring. Solution B was prepared by dissolving 0.5 g of OCMC in 3.5 mL of DI water at 35 °C. Solution C was an 80 mg/mL nano-hydroxyapatite (HAP, Cat. No. H698836, Macklin, China)



Scheme 1. Schematic illustration of the K+gHAP@GC gradient hydrogel scaffold for rotator cuff tendon-to-bone interface reconstruction. The bionic composite scaffold, consisting of a crosslinked GelMA/OCMC network, was fabricated using a layer-by-layer assembly technique. The gradient design is intended to provide spatial mineral cues for bone-side reconstruction and fibrocartilage-like matrix deposition, while keratin incorporation provides angiogenesis-supportive bioactivity and microenvironmental support for interface healing. (This diagram was modeled and rendered in Cinema 4D (C4D) R23. The rendered outputs were further processed for image editing and figure layout assembly using Adobe Photoshop 2019 and Adobe Illustrator 2023.)

suspension (640 mg HAP in 8 mL DI water), which was probe-sonicated for 4 h to prevent nanoparticle agglomeration. Solution D was a 5% (w/v) keratin aqueous solution (Cat. No. K304415 Aladdin, China). To formulate 1 mL of the final hydrogel precursor, the stock solutions were sequentially mixed with rigorous vortexing between each addition: 350 μ L of Solution A, 350 μ L of Solution B, 100 μ L of Solution C (or equal volume of DI water for HAP-free layers), and 200 μ L of Solution D. The fully homogenized precursor was immediately injected into a customized mold and subjected to layer-by-layer UV crosslinking (365 nm, 15 s per layer) to prevent particle sedimentation and premature chemical gelation.

Based on the formulated compositions, the single-

layer homogeneous hydrogels were designated as GC (GelMA/OCMC), HAP@GC (HAP/GelMA/OCMC), K@GC (Keratin/GelMA/OCMC), and K+HAP@GC (Keratin/HAP/GelMA/OCMC). These single-layer homogeneous hydrogels were prepared from one uniform precursor formulation and therefore served as compositionally homogeneous, non-gradient controls without spatially varied HAP distribution. Furthermore, gradient biomimetic scaffolds constructed via the layer-by-layer assembly of GC and varied HAP@GC layers were termed gHAP@GC, whereas scaffolds assembled from K@GC and varied K+HAP@GC layers were denoted as K+gHAP@GC.

Characterization

The prepared hydrogel scaffolds were lyophilized and sectioned longitudinally. After sputter-coating with gold, the porous microstructures were observed using a scanning electron microscope (SEM, EVO MA 15, Carl Zeiss, Germany) at an accelerating voltage of 5 kV. The chemical compositions of the raw materials and the lyophilized scaffolds were characterized using a Fourier transform infrared spectrometer (FTIR, Nicolet iS50, Thermo Scientific, USA). Dried samples were ground, mixed with KBr, and pressed into pellets. Spectra were recorded from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1} to assess the functional groups and crosslinking networks.

The compressive properties of cylindrical hydrogel samples (8 mm in diameter and 3 mm in height) were evaluated using a universal testing machine (34SC-1, INSTRON, USA). Following an initial preload of 0.04 N, unconfined compression tests were performed at a crosshead speed of 10 mm/min up to a compressive strain of 80%, and the stress-strain curves were recorded. For cyclic compression testing, samples were placed on the lower platen of the universal testing machine. Both force and displacement were zeroed upon contact with the upper platen. The test was conducted for 100 continuous cycles with the compressive strain set between 0% and 40%, and the data were recorded to evaluate mechanical stability. The rheological properties were evaluated using a rheometer (Mars 40, Haake, Germany) equipped with an 8-mm rotor. Tests were conducted at a constant frequency of 1 Hz, utilizing defined strain conditions (1% for small strain and 70% for large strain) to assess the viscoelastic behavior of the hydrogels. To assess the spatial compositional gradient, elemental analysis was performed using an energy-dispersive X-ray spectrometer (EDS, X-Max 40, Oxford Instruments, UK) coupled with the SEM. Operating in line-scan mode along the longitudinal cross-section, the accelerating voltage was set to 15 kV with a working distance of 8–10 mm to map the distribution of calcium (Ca) and phosphorus (P) elements. The acquired elemental profiles were processed using the standard ZAF matrix correction method.

The initial weight (W_d) of the hydrogel scaffolds was recorded. The samples were then immersed in PBS and incubated in a thermostatic shaker (37 °C, 90 rpm). At predetermined time intervals (1, 2, 4, 8, 12, 24, and 48 h), the scaffolds were carefully removed, gently blotted with filter paper to remove excess surface water, and immediately weighed (W_w). The swelling ratio was calculated using the following Eqn. 1:

$$\text{Swelling ratio (\%)} = \frac{W_w - W_d}{W_d} \times 100\% \quad (1)$$

Cell Culture

Isolation and Culture of Primary Rat Bone Marrow Mesenchymal Stem Cells (BMSCs) [33]: Primary BMSCs were isolated from the femurs of 3-week-old healthy Sprague-Dawley (SD) rats. Euthanasia was performed via cervical dislocation, followed by surface sterilization with 75% ethanol for 25 min. Both femurs were aseptically excised and meticulously cleared of any attached soft tissues. The isolated femurs were immersed in PBS containing 10% (v/v) 100× penicillin-streptomycin (Cat. No. C0222, Beyotime, China) for 10 min. Subsequently, the epiphyses were removed, and the bone marrow cavities were flushed with complete Alpha-MEM (Cat. No. C12571500BT, Gibco, USA) using a 1-mL syringe until the bone shafts appeared pale. The collected marrow suspension was mechanically dissociated and centrifuged at $200 \times g$ for 5 min. The cell pellet was resuspended in complete Alpha-MEM containing 15% (v/v) fetal bovine serum (FBS, Cat. No. F0193, Sigma-Aldrich, USA) and 1% (v/v) penicillin-streptomycin, then seeded in T25 culture flasks and incubated at 37 °C in a humidified atmosphere with 5% CO_2 .

In Vitro Cytocompatibility Evaluation

The hydrogel scaffolds were placed into untreated 24-well plates. BMSCs were directly seeded onto the surface of the scaffolds at a density of 1×10^5 cells/mL. The cell-seeded scaffolds were then cultured in complete Alpha-MEM at 37 °C with 5% CO_2 , and the medium was replaced daily. Cytocompatibility was evaluated on days 1 and 7 using Live/Dead staining and a cell counting kit-8 (CCK-8) assay. Live/Dead Staining: Cell viability on the hydrogel surfaces was assessed using a Calcein-AM/PI Live/Dead staining kit (Cat. No. CA1630, Solarbio, China). Briefly, the samples were washed with pre-warmed PBS and incubated with a working solution (2 μM Calcein-AM and 3 μM PI) for 15 min at 37 °C in the dark. Fluorescence images were subsequently captured using a fluorescence microscope. CCK-8 Assay: Cell proliferation was quantified using a CCK-8 (Cat. No. CA1210, Solarbio, China). The samples were gently washed with PBS and incubated with 350 μL of fresh medium containing 10% CCK-8 reagent per well at 37 °C for 2 h. After incubation, 100 μL of the supernatant from each well was transferred to a new 96-well plate to avoid interference from the underlying scaffolds. The absorbance at 450 nm was measured using a microplate reader.

Alkaline Phosphatase Staining

After cell seeding as above, hydrogels were cultured in uncoated 24-well plates with complete medium (changed every 48 h). Alkaline phosphatase (ALP) staining was performed on day 7 of culture using a commercial ALP staining kit (Cat. No. G1480, Solarbio, China). Briefly, samples were washed with PBS and fixed in 4% paraformaldehyde (PFA) (500 μL /well) for 20 min at room temperature. Af-

ter two PBS washes, the samples were incubated with 300 μL /well freshly prepared ALP staining solution for 10 min at room temperature in the dark. The reaction was terminated with ddH₂O, and stained hydrogels were observed and imaged under a light microscope.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells cultured on different hydrogels using RNAiso Plus reagent (Cat. No. 9109, Takara, Japan). DNase treatment was applied to remove genomic DNA contamination. The isolated RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) by using a cDNA reverse transcription kit (Cat. No. R223-01, Vazyme, China). The synthesized cDNA templates were then subjected to qRT-PCR on a Real-Time PCR System (StepOnePlus, Applied Biosystems, USA), with quantification performed using a SYBR Green-based qRT-PCR kit (Cat. No. Q341-02, Vazyme, China). Data collection and analysis were carried out using the matched qRT-PCR instrument software (StepOne™ Software, Applied Biosystems, USA), and relative quantification was performed using the $2^{-\Delta\Delta C_T}$ method. The primer sequences used in this study are listed in Table 1.

Table 1. Primer sequences.

Primer name	Primer sequences (5' → 3')
<i>GAPDH</i> -F	GGCACAGTCAAGGCTGAGAATG
<i>GAPDH</i> -R	ATGGTGGTGAAGACGCCAGTA
<i>Alp</i> -F	CCTTTGTGGCTCTCTCCAAG
<i>Alp</i> -R	CGATGTCCTTGATGTTGTGC
<i>Col2a1</i> -F	GGCTCCCAGAACATCACCTA
<i>Col2a1</i> -R	ATCCTTCAGGGCAGTGACG
<i>NOS3</i> -F	GCCACCTGATCCTAACTTGCCTTG
<i>NOS3</i> -R	TCGTGTAATCGGTCTTGCCAGAATC
<i>Runx2</i> -F	CACAAGTGCGGTGCAAACTT
<i>Runx2</i> -R	CTCAGAGCACTCACTGACTCG
<i>Sox9</i> -F	CTCCTACTACAGCCACGCAG
<i>Sox9</i> -R	GCTGTGTGTAGACGGTTGT
<i>VEGFA</i> -F	AAAGCCCATGAAGTGGTGAA
<i>VEGFA</i> -R	TCTGCATAGTGAACGTTGCTC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *Sox9*, SRY-box transcription factor 9; *NOS3*, nitric oxide synthase 3; *Col2a1*, collagen type II alpha 1 chain; *VEGFA*, vascular endothelial growth factor A; *Alp*, alkaline phosphatase; *Runx2*, runt-related transcription factor 2.

Immunofluorescence

BMSCs were seeded onto hydrogels in 24-well plates (1×10^4 cells/mL, 200 μL /well) and incubated for 2 h for initial attachment. Subsequently, 1 mL of the corresponding medium was added to each well: basal culture medium for osteogenic-marker evaluation and YAP1 local-

ization, and chondrogenic induction medium for cartilage matrix evaluation. For osteogenic-marker and cartilage matrix evaluation, cells were cultured for 7 days, with half of the medium replaced every 2 days. For YAP1 localization, cells were cultured for 48 h before fixation. Chondrogenic induction was performed using Alpha-MEM supplemented with 50 $\mu\text{g/mL}$ vitamin C, 100 nM dexamethasone (Cat. No. D137736, Aladdin, China), $1 \times$ Insulin-Transferrin-Selenium (ITS, Cat. No. 41400-045, Gibco, USA) and 10 ng/mL transforming growth factor- β_1 (TGF- β_1 , Cat. No. HY-P7118, MedChemExpress, USA). After induction, samples were rinsed with PBS, fixed with 4% PFA for 30 min, permeabilized with 0.3% Triton X-100 for 20 min, and blocked for 1 h at room temperature. The samples were then incubated overnight at 4 °C with specific primary antibodies against OPN (1:200, YT3467; ImmunoWay, USA), COL2A1 (1:200, 28459-1-AP; Proteintech, China), YAP1 (1:200, 13584-1-AP; Proteintech, China), and VEGF (1:500, EP1176Y; Abcam, UK). After being washed with PBS three times for 5 min each, the hydrogels were incubated with the corresponding fluorophore-conjugated secondary antibodies, including Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Cat. No. A-11008, Invitrogen, Thermo Fisher Scientific, USA) for OPN and COL2A1, and Alexa Fluor 594-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Cat. No. A-11012, Invitrogen, Thermo Fisher Scientific, USA) for YAP1 and VEGF, for 1 h at room temperature in the dark. F-actin was stained with phalloidin for 30 min (Cat. No. CA1610, Solarbio, China) and nuclei with DAPI for 10 min (Cat. No. DA0001, Leagene, China). Images were captured via confocal laser scanning microscope (LSM 880, Carl Zeiss Microscopy GmbH, Germany) at the corresponding excitation wavelengths, and fluorescence intensity was quantified using ImageJ (v1.54n6, Bethesda, Maryland, USA).

Endothelial Tube Formation Assay

HUVECs were cultured in commercial Endothelial Cell Medium (Cat. No. CM-ZY001, Procell, China), using the same incubation conditions as described for BMSCs. Matrigel (Cat. No. 356234, Corning, USA) was thawed overnight at 4 °C prior to use. 150 μL of liquid Matrigel was added to each well of a pre-chilled 48-well plate using pre-cooled pipette tips. The plate was left at 4 °C for 30 min to ensure even coating, then incubated at 37 °C for 30 min to allow full gelation. HUVECs were seeded onto the solidified Matrigel at 3×10^4 cells per well and incubated for 6 h.

Transwell Migration Assay

The migratory capacity of HUVECs was evaluated using Transwell chambers with 8.0 μm pore polycarbonate membranes (3464, Corning, USA). Briefly, hydrogel-conditioned media were prepared by immersing hydrogel

samples in endothelial cell complete medium at 37 °C for 48 h, followed by centrifugation to remove debris and sterile filtration through a 0.22 μm membrane. A volume of 600 μL of the conditioned medium was added to the lower chamber of each Transwell insert. HUVECs were suspended in serum-reduced endothelial cell medium, and 200 μL of the cell suspension was seeded into the upper chamber at a density of 2×10^4 cells per insert. After incubation for 24 h at 37 °C in a humidified 5% CO_2 atmosphere, the non-migrated cells on the upper surface of the membrane were gently removed with a cotton swab. The migrated cells on the lower surface were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet for 15 min. After washing with PBS, the stained cells were observed and imaged under a light microscope. The number of migrated cells was quantified in five randomly selected fields for each insert using ImageJ software.

Scratch Wound-Healing Assay

HUVEC migration was further assessed using a scratch wound-healing assay. HUVECs were seeded in 6-well plates and cultured until reaching near-complete confluence. A linear wound was then created in the cell monolayer using a sterile 200 μL pipette tip. The detached cells were removed by gentle washing with PBS, and the cells were subsequently cultured in hydrogel-conditioned media from each group. Images of the scratch area were acquired at 0 h and 24 h under an inverted optical microscope. The wound healing rate was calculated using the following formula: wound healing rate (%) = (initial wound area – remaining wound area at 24 h)/initial wound area $\times$ 100%. Quantitative analysis was performed using ImageJ software.

Establishment of a Rat Model of Rotator Cuff Injury

In this study, the individual rat was considered the experimental unit. A total of 40 8-week-old SD rats weighing 250–300 g were used for the 8-week repair evaluation and randomly divided into 4 groups using a computer-generated random number sequence: Blank group (suture-only repair), GC group, gHAP@GC group, and K+gHAP@GC group. In each group, six rats were used for micro-CT and histological analyses, and four rats were used for biomechanical testing. A longitudinal incision was made over the anterolateral shoulder. The deltoid was bluntly split to expose the supraspinatus tendon-greater tuberosity complex, and the tendon was transected from its bony insertion to establish a full-thickness rotator cuff tear model. Residual fibrocartilage at the tendon footprint was completely removed, and mild decortication was performed until punctate bleeding was achieved. Crossed transosseous micro-tunnels were drilled at the footprint using a 0.8 mm Kirschner wire. For the experimental groups, trimmed gradient hydrogel scaffolds were interposed between the tendon stump and bony footprint in a defined spatial orien-

tation: the HAP-containing layer was placed flush against the bony footprint, and the HAP-free layer faced the tendon stump. The tendon was then fixed to the greater tuberosity via transosseous sutures, with the scaffold secured tightly at the tendon-bone interface. The Blank group underwent the identical transosseous repair procedure without scaffold implantation. To minimize potential confounders, the surgeries were performed in a randomized order, and animal cages were randomly distributed on the housing racks.

Short-Term In Vivo Mass-Retention Assay

A short-term *in vivo* mass-retention assay was performed to estimate the early material stability of K+gHAP@GC after implantation. Freshly prepared sterile hydrated K+gHAP@GC scaffolds were implanted at the tendon-to-bone repair interface. For dry-mass normalization, parallel non-implanted scaffolds prepared from the same batch and with the same dimensions were lyophilized to constant weight and used to determine the equivalent initial dry mass reference (W_0). Additional rats were used for this assay, with three animals assigned to each retrieval time point. The implanted scaffolds were retrieved at 7, 14, and 21 days after implantation. After retrieval, visible surrounding soft tissues were carefully removed, and the recovered residual scaffold materials were rinsed with PBS, lyophilized to constant weight, and weighed to obtain the apparent remaining dry mass (W_t). The remaining mass was calculated as $W_t/W_0 \times 100\%$. This assay was used to evaluate short-term *in vivo* material retention rather than to establish a complete long-term degradation kinetic profile. Data are presented as mean $\pm$ SD, $n = 3$.

Micro-Computed Tomography (Micro-CT) Analysis

After the rats were euthanized with an overdose of isoflurane (Cat. No. R510-22, RWD Life Science, China), the supraspinatus-humerus complex was collected and fixed in 4% paraformaldehyde, and evaluated using a micro-CT system (μCT 45, SCANCO, Switzerland). For analysis, the region of interest (ROI) was defined as the bone-side repair region beneath and surrounding the repaired tendon footprint. Because this ROI represented an irregular repair-interface region rather than a standardized trabecular compartment, bone volume fraction (BV/TV) was selected as the primary quantitative micro-CT parameter for evaluating bone-side reconstruction within the repaired footprint. BV/TV was measured using CT Analyzer (v1.13, Bruker, Belgium). Accordingly, conventional trabecular morphometric indices, such as trabecular thickness (Tb.Th) and trabecular number (Tb.N), were not used as primary endpoints because their biological interpretation is limited in this irregular tendon-to-bone repair-interface ROI.

Histological Staining of Supraspinatus Tendon-Humerus Composite Tissues

Paraffin-embedded supraspinatus tendon-humerus composite tissues were mounted on a microtome and trimmed to expose the tissue edge. Serial 5 μm -thick sections were cut, and consecutive sections were collected once the TBI plane was reached, with sufficient sections prepared for all subsequent experiments. Sections were subjected to three histological staining assays to evaluate the repaired TBI: hematoxylin and eosin (H&E) for general tissue morphology, Safranin O-Fast Green (SO-FG) staining for glycosaminoglycan-rich fibrocartilage-like matrix deposition, and Masson's trichrome staining (commercial kit, Cat. No. G1006, Servicebio, China) for collagen deposition and microstructural organization. Images of all stained sections were acquired using an optical microscope (IX71, OLYMPUS, Japan). The area of Safranin O-positive staining and collagen continuity at the TBI were quantified using ImageJ software to evaluate fibrocartilage-like matrix formation and collagen organization at the repaired interface. Histological scoring was performed using the modified Ide scoring system as described in previous studies [34,35]. Scoring was independently performed by two pathologists who were blinded to group allocation.

Biomechanical Testing

During specimen harvest and processing, samples were wrapped in gauze moistened with 0.9% normal saline to prevent tissue dehydration, with continuous saline dripping maintained throughout testing. Biomechanical tests were conducted using a 34SC-1 universal testing system (34SC-1, Instron, USA). Specimens were preloaded with 0.1 N to mitigate errors from creep and relaxation, followed by 10 cycles of low-frequency preconditioning. Specimens were then loaded to failure via axial tension at a constant rate of 5 mm/min, with failure modes recorded as tendon midsubstance rupture or TBI avulsion fracture.

Pathological Staining of Major Organs

To evaluate the *in vivo* toxicity of the scaffold, major organs (heart, liver, spleen, lung, kidney) were harvested for histological analysis. After rinsing with PBS and blotting dry, the organs were fixed in 4% paraformaldehyde, processed into paraffin sections, and stained with H&E. Histological observation was performed to assess tissue integrity, inflammation and pathological changes.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were conducted using SPSS version 19.0 (IBM, USA), and graphical representations were created with GraphPad Prism version 9.0 (GraphPad Software, USA). Prior to selecting specific statistical tests, the assumptions of data normality and variance homogeneity were systematically assessed using the Shapiro-Wilk test

and Levene's test, respectively. For comparisons between two groups, an independent Student's *t*-test was employed when the data met the assumptions of normal distribution and equal variances, otherwise, Welch's *t*-test was utilized for data with unequal variances. Differences among three or more groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple pairwise comparisons, provided the assumptions of normality and variance homogeneity were satisfied. Two-way ANOVA with Dunnett's post hoc test was applied to analyze data involving multiple time points and interventions. Statistical significance was determined at $p < 0.05$. Quantitative analysis of histological and pathological images was performed using ImageJ software. While the surgeon could not be blinded to the scaffold allocation during the surgical procedure, the investigators performing the micro-CT evaluations, histological scoring, and biomechanical testing were completely blinded to the experimental group assignments.

Results

Fabrication and Characterization of the Gradient Biomimetic Scaffold

The biomimetic gradient hydrogel scaffolds were fabricated through precursor mixing and layer-by-layer assembly. As shown in Fig. 1A, all hydrogel groups maintained intact cylindrical morphology with smooth surfaces and no obvious collapse after gelation. The HAP-containing hydrogels exhibited slight opacity compared with the translucent GC hydrogel, while the gradient scaffolds retained structural integrity after multilayer construction. SEM observation of the lyophilized scaffolds revealed interconnected three-dimensional porous networks in all groups (Fig. 1B). Compared with GC and HAP@GC, the keratin-incorporated groups exhibited a comparatively denser pore structure, indicating that keratin incorporation altered the internal network architecture of the composite hydrogels. FTIR spectroscopy was further performed to confirm the chemical composition and crosslinking state of the hydrogels (Fig. 1C). The characteristic signals of GelMA, keratin, and OCMC were retained in the final K+gHAP@GC hydrogel. The aldehyde-associated peak of OCMC at approximately 1730 cm^{-1} was markedly attenuated, while an imine-related signal assigned to Schiff-base bonds (-N=CH-) appeared or overlapped with the Amide I band near 1640 cm^{-1} . In addition, a phosphate-related peak of HAP was observed at approximately 1030 cm^{-1} , confirming the incorporation of the inorganic HAP phase into the composite network.

The compressive mechanical properties of the hydrogels were then evaluated (Fig. 1D–G). GC showed the lowest compressive response, whereas the incorporation of keratin or HAP increased the stiffness and load-bearing capacity of the GelMA/OCMC matrix. Among the homogeneous hydrogels, K+HAP@GC exhibited the highest me-

chanical performance, with a compressive strength of approximately 100 kPa and an elastic modulus of approximately 150 kPa ($p < 0.0001$ vs. GC). The gradient scaffold groups, gHAP@GC and K+gHAP@GC, also exhibited improved compressive properties compared with GC, indicating that the layer-by-layer assembly process did not compromise the scaffold's mechanical integrity. Among all groups, K+gHAP@GC displayed the most favorable compressive behavior, suggesting that keratin incorporation and gradient HAP loading jointly enhanced the mechanical performance of the scaffold.

Rheological analysis further confirmed the gel-like viscoelastic behavior of the composite hydrogel networks. In both gHAP@GC and K+gHAP@GC, the storage modulus G' remained higher than the loss modulus G'' throughout the test period (Fig. 1H), indicating stable hydrogel network formation. Consistently, HAP- and keratin-containing hydrogels generally exhibited higher G' values than GC (Fig. 1I). The mechanical robustness of the hydrogels was then assessed by cyclic compression testing (Fig. 2A). GC exhibited the lowest cyclic stress response and showed a more evident decrease in peak stress during repeated loading. In contrast, HAP-containing hydrogels maintained higher stress amplitudes over successive cycles. K+HAP@GC showed the highest cyclic stress response among the homogeneous hydrogels, while gHAP@GC and K+gHAP@GC maintained stable loading–unloading profiles, indicating that the multilayer gradient construction did not compromise cyclic compression stability.

The compositional gradient of the multilayer scaffolds was next validated by SEM-EDS line scanning (Fig. 2B). Calcium and phosphorus signals were heterogeneously distributed along the longitudinal direction of the scaffold and gradually increased toward the HAP-rich region, confirming the successful establishment of an HAP gradient within the multilayer structure. Gel fraction analysis further showed that the UV-crosslinked GelMA/OCMC/keratin hydrogel exhibited a higher gel fraction than the non-UV-crosslinked group (Fig. 2C), indicating more complete network formation after dual crosslinking. All hydrogels exhibited rapid water uptake within the first 4 h, reached a dynamic swelling equilibrium between 12 and 24 h, and maintained relatively stable swelling ratios thereafter (Fig. 2D). In the *in vitro* degradation assay, all hydrogel groups showed progressive mass loss over 21 days (Fig. 2E). Compared with GC, the HAP- and keratin-containing composite hydrogels retained higher residual mass during degradation. Taken together, these results confirmed the successful construction of K+gHAP@GC with an interconnected porous morphology, an organic–inorganic composition, enhanced mechanical performance, an HAP gradient, and stable swelling/degradation behavior.

Cytocompatibility of Hydrogel Scaffolds

The cytocompatibility of the hydrogels was first evaluated using Live/Dead staining and CCK-8 assays. As shown in Fig. 3A, most BMSCs displayed green fluorescence in all hydrogel groups on days 1 and 7, whereas only a small number of dead cells were observed. Semi-quantitative analysis further confirmed that cell viability remained above 90% in all groups at both time points (Fig. 3B), indicating that the incorporation of HAP or keratin did not induce marked cytotoxicity. CCK-8 analysis showed a time-dependent increase in cell proliferation from day 1 to day 7 in all groups (Fig. 3C). The HAP@GC, K@GC, and K+HAP@GC groups showed comparable or slightly higher proliferative activity than the GC group. These results collectively demonstrate that all hydrogel formulations exhibited favorable cytocompatibility and supported BMSC survival and proliferation.

Osteogenic Differentiation of BMSCs on Hydrogel Scaffolds

Osteogenic differentiation was subsequently evaluated under basal culture conditions without exogenous osteogenic supplements. ALP staining showed weak and sparse positive staining in the GC and K@GC groups, whereas HAP-containing hydrogels exhibited more abundant ALP-positive deposits (Fig. 4A,B). Among all groups, K+HAP@GC showed the most intense ALP staining, a pattern consistent across macroscopic and microscopic images. Semi-quantitative analysis confirmed that ALP activity was highest in the K+HAP@GC group (Fig. 4C, $p < 0.05$).

At the transcriptional level, *Alp* expression was significantly higher in the K+HAP@GC group than in the GC ($p < 0.05$) and K@GC ($p < 0.01$) groups (Fig. 4D). *Runx2* expression also peaked in the K+HAP@GC group and was significantly increased compared with the GC ($p < 0.01$), K@GC ($p < 0.01$), and HAP@GC ($p < 0.05$) groups. Consistent with the *Alp* and qRT-PCR results, immunofluorescence staining showed increased OPN expression in HAP-containing hydrogels (Fig. 4E,F). The HAP@GC group exhibited stronger OPN fluorescence than the GC group, while the K+HAP@GC group showed the most pronounced OPN signal among all groups. Semi-quantitative fluorescence analysis further supported this trend. Taken together, these results demonstrated that the combined incorporation of keratin and HAP yielded the strongest osteogenic response in BMSCs.

Chondrogenic Matrix-Associated Responses and YAP1 Nuclear Localization in BMSCs

The chondrogenic potential of the hydrogels was subsequently evaluated by qRT-PCR and immunofluorescence staining. As shown in Fig. 5A, the relative mRNA expression of *Col2a1* was markedly increased in the keratin-containing groups, with significantly higher ex-

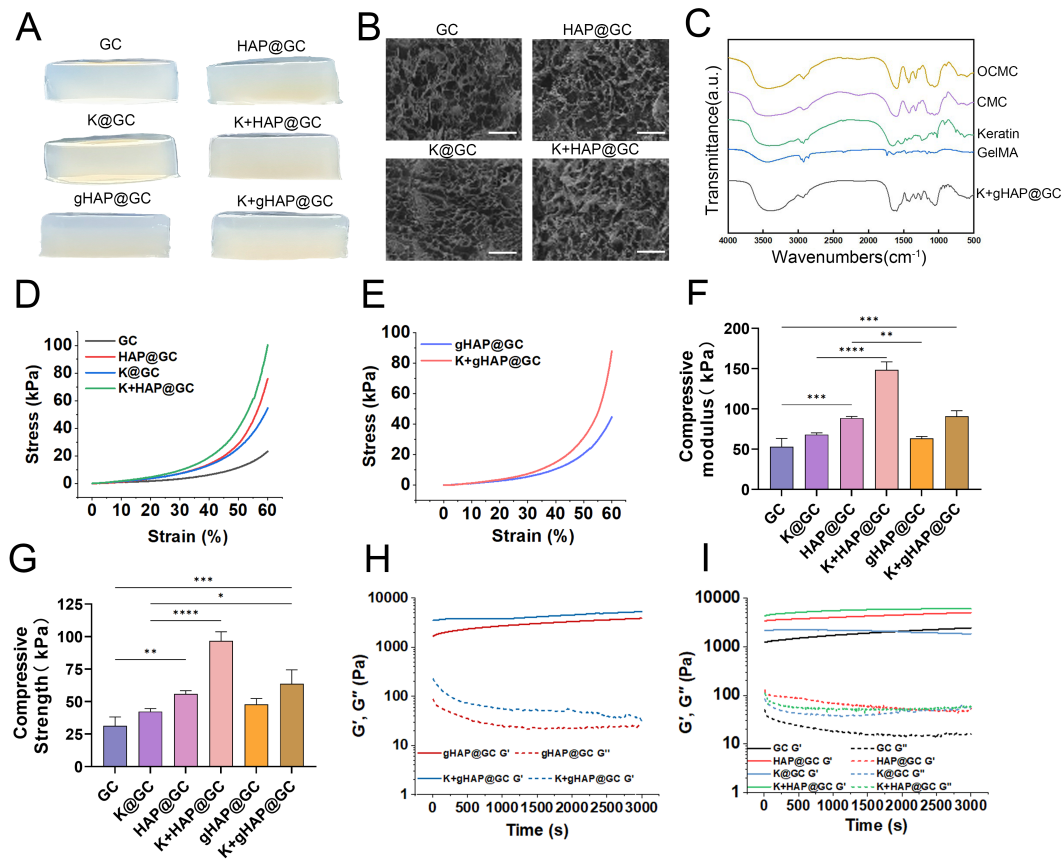


Fig. 1. Fabrication, morphology, chemical characterization, mechanical properties, and rheological behavior of the biomimetic hydrogels. (A) Representative macroscopic images of GC, HAP@GC, K@GC, K+HAP@GC, gHAP@GC, and K+gHAP@GC hydrogels (dimensions: 8 mm in diameter and 3 mm in height). (B) Representative SEM images showing the porous microstructures of GC, HAP@GC, K@GC, and K+HAP@GC hydrogels (Scale bars = 50 μ m). (C) FTIR spectra of OCMC, CMC, keratin, GelMA, and K+gHAP@GC hydrogel. (D) Representative compressive stress-strain curves of the homogeneous hydrogels (GC, HAP@GC, K@GC, and K+HAP@GC). (E) Representative compressive stress-strain curves of the gradient scaffold groups (gHAP@GC and K+gHAP@GC). (F) Compressive modulus of different hydrogel groups. (G) Compressive strength of different hydrogel groups. (H) Time-dependent rheological profiles of gHAP@GC and K+gHAP@GC hydrogels, showing storage modulus (G') and loss modulus (G''). (I) Rheological profiles of different hydrogel groups, showing storage modulus (G') and loss modulus (G'') over time. Data are presented as mean $\pm$ SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The images were plotted using GraphPad Prism 9.0.

pression in the K@GC and K+HAP@GC groups than in the GC and HAP@GC groups. In contrast, Sox9 expression did not exhibit a corresponding increase across the experimental groups. Semi-quantitative immunofluorescence analysis further confirmed enhanced COL2A1 deposition in the keratin-containing hydrogels (Fig. 5B,C). The K+HAP@GC group exhibited the strongest COL2A1 fluorescence signal among all groups, indicating increased chondrogenic matrix-associated protein expression.

YAP1 immunofluorescence staining was further performed to assess the subcellular localization of YAP1 in BMSCs cultured on different hydrogels (Fig. 5D). In the GC and K@GC groups, YAP1 signals were mainly distributed in the cytoplasmic region, with limited overlap with DAPI-stained nuclei. In contrast, YAP1 nuclear localization was more evident in HAP-containing hydrogels, espe-

cially the K+HAP@GC group, with greater YAP1-DAPI signal overlap in merged images. Collectively, these results demonstrated that the composite hydrogel enhanced COL2A1-related chondrogenic matrix expression and was accompanied by increased nuclear localization of YAP1 in BMSCs.

Pro-Angiogenic Activity of Keratin-Containing Hydrogel Scaffolds

The effects of the hydrogels on endothelial cell migration were evaluated using Transwell migration and scratch wound-healing assays in HUVECs. In the Transwell assay, more HUVECs migrated through the membrane in the K@GC and K+HAP@GC groups than in the GC and HAP@GC groups (Fig. 6A,C). Consistently, the scratch assay showed greater wound closure at 24 h in the keratin-

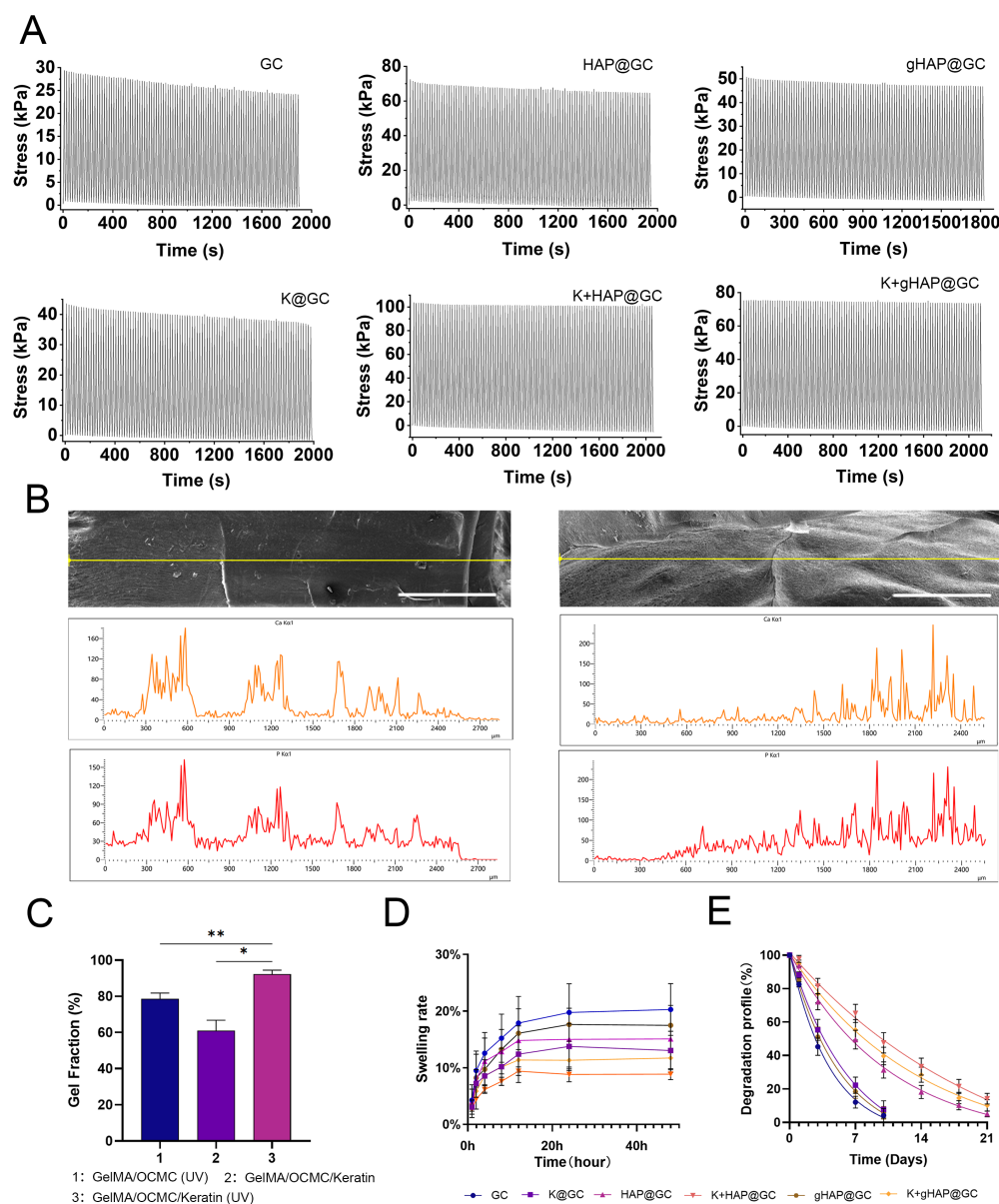


Fig. 2. Cyclic compression performance, compositional gradient validation, gel fraction, swelling behavior, and degradation profiles of the hydrogels. **(A)** Representative cyclic compression stress-time curves of GC, HAP@GC, gHAP@GC, K@GC, K+HAP@GC, and K+gHAP@GC hydrogels. **(B)** SEM images and corresponding EDS line-scan profiles showing the spatial distribution of calcium (Ca) and phosphorus (P) elements along the longitudinal direction of the gradient hydrogel scaffolds. Scale bars = 500 μm . **(C)** Gel fraction of GelMA/OCMC (UV), GelMA/OCMC/keratin, and GelMA/OCMC/keratin (UV) hydrogels. **(D)** Swelling behavior of different hydrogel groups over time. **(E)** *In vitro* degradation profiles of different hydrogel groups over 21 days. Data are presented as mean $\pm$ SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$. The images were plotted using GraphPad Prism 9.0.

containing hydrogel groups (Fig. 6B,D), indicating enhanced endothelial migratory activity.

Endothelial morphogenesis was further assessed using a tube formation assay (Fig. 6E). HUVECs in the GC and HAP@GC groups formed sparse and discontinuous tubular structures, whereas cells in the K@GC and K+HAP@GC groups formed denser and more interconnected capillary-like networks. Quantitative analysis (Fig. 6 F,G) revealed that both total tube length and junction

number were significantly higher in the keratin-containing groups than in the GC and HAP@GC groups ($p < 0.01$ to $p < 0.0001$). qRT-PCR analysis further showed that *VEGFA* and *NOS3* mRNA levels were significantly upregulated in the keratin-containing groups (Fig. 6H). Importantly, the K+HAP@GC group exhibited a 2.5-fold increase in *VEGFA* expression and a 2.3-fold increase in *NOS3* expression compared with the GC group (both $p < 0.01$).

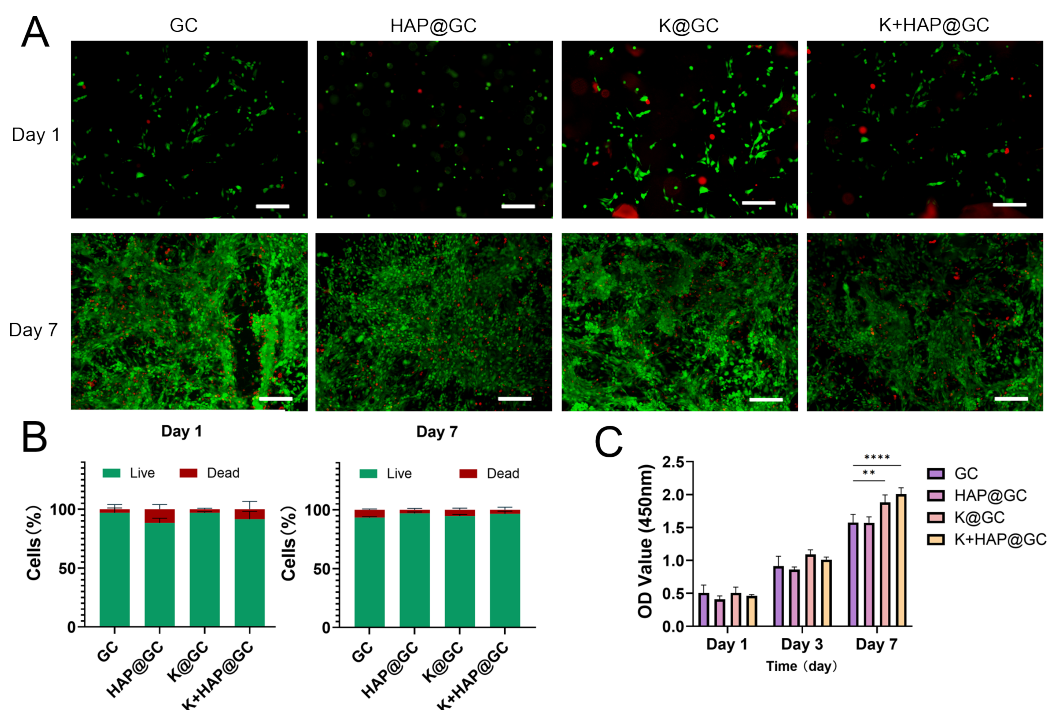


Fig. 3. *In vitro* cytocompatibility evaluation of the hydrogels: (A) Live/dead cell staining. Scale bars = 200 μ m. (B) Semi-quantitative analysis of cell viability based on the live/dead staining results in (A). (C) Cell proliferation analysis. Data are presented as mean $\pm$ SD, n = 3. ** p < 0.01, **** p < 0.0001. The images were plotted using GraphPad Prism 9.0.

In Vivo Tendon-to-Bone Healing, Biomechanical Restoration, and Biosafety

At 8 weeks after surgery, micro-CT, histological staining, and biomechanical testing were performed to evaluate tendon-to-bone healing (Fig. 7). Micro-CT images showed more mineralized tissue formation at the humeral footprint in the gradient scaffold groups than in the Blank and GC groups, with the K+gHAP@GC group showing the most pronounced bone-side reconstruction (Fig. 7A). Given the irregular repair-interface ROI, BV/TV was used as an index of bone-side mineralized tissue reconstruction, and BV/TV quantitative showed a similar trend (Fig. 7C).

Histological evaluation demonstrated distinct differences among groups (Fig. 7B). In this respect, H&E staining revealed disorganized scar tissue and a visible tendon-bone gap in the Blank and GC groups. The gHAP@GC group showed increased interfacial tissue density, while the K+gHAP@GC group exhibited greater interfacial cellularity and more spindle-shaped cells aligned along the tendon stress axis. Besides, SO-FG staining showed limited Safranin O-positive GAG deposition and no continuous fibrocartilage-like matrix formation in the Blank and GC groups. The gHAP@GC group showed partial GAG-positive regions, whereas the K+gHAP@GC group showed a more continuous fibrocartilage-like zone at the tendon-bone interface. Quantitative analysis showed that the relative SO-FG-positive fibrocartilage-like matrix areas were $13.2\% \pm 1.8\%$ in the Blank group and $17.8\% \pm 1.1\%$ in the GC group, with no significant difference

between these two groups. The gHAP@GC group increased the fibrocartilage-like area to $25.1\% \pm 2.7\%$, and the K+gHAP@GC group further increased it to $38.3\% \pm 2.2\%$ (p < 0.001 vs. all other groups; Fig. 7D).

Moreover, the histological scores were consistent with the staining results, with the K+gHAP@GC group showing significantly higher scores than the Blank and GC groups (p < 0.05; Fig. 7E). Masson staining showed sparse and disorganized collagen fibers at the tendon-bone interface in the Blank and GC groups, while the K+gHAP@GC group showed denser collagen deposition with more parallel alignment along the tendon stress axis. Biomechanical testing showed that the Blank group exhibited the lowest linear stiffness and ultimate failure load (Fig. 7F,G). The GC and gHAP@GC groups showed modest improvements, whereas the K+gHAP@GC group showed significantly higher stiffness and failure load than the Blank group (p < 0.05).

To further evaluate the structural stability during the repair process, the *in vivo* degradation profile of the K+gHAP@GC scaffold was quantitatively monitored. The hydrogel exhibited a steady and controlled mass loss *in vivo*, retaining approximately 62% of its original weight at 21 days post-implantation (Fig. 7H). This progressive degradation prevents premature collapse, providing sustained localized micromechanical support during early interface remodeling. To evaluate the local angiogenic microenvironment, VEGF expression at the repaired enthesis was assessed via immunofluorescence staining (Fig.

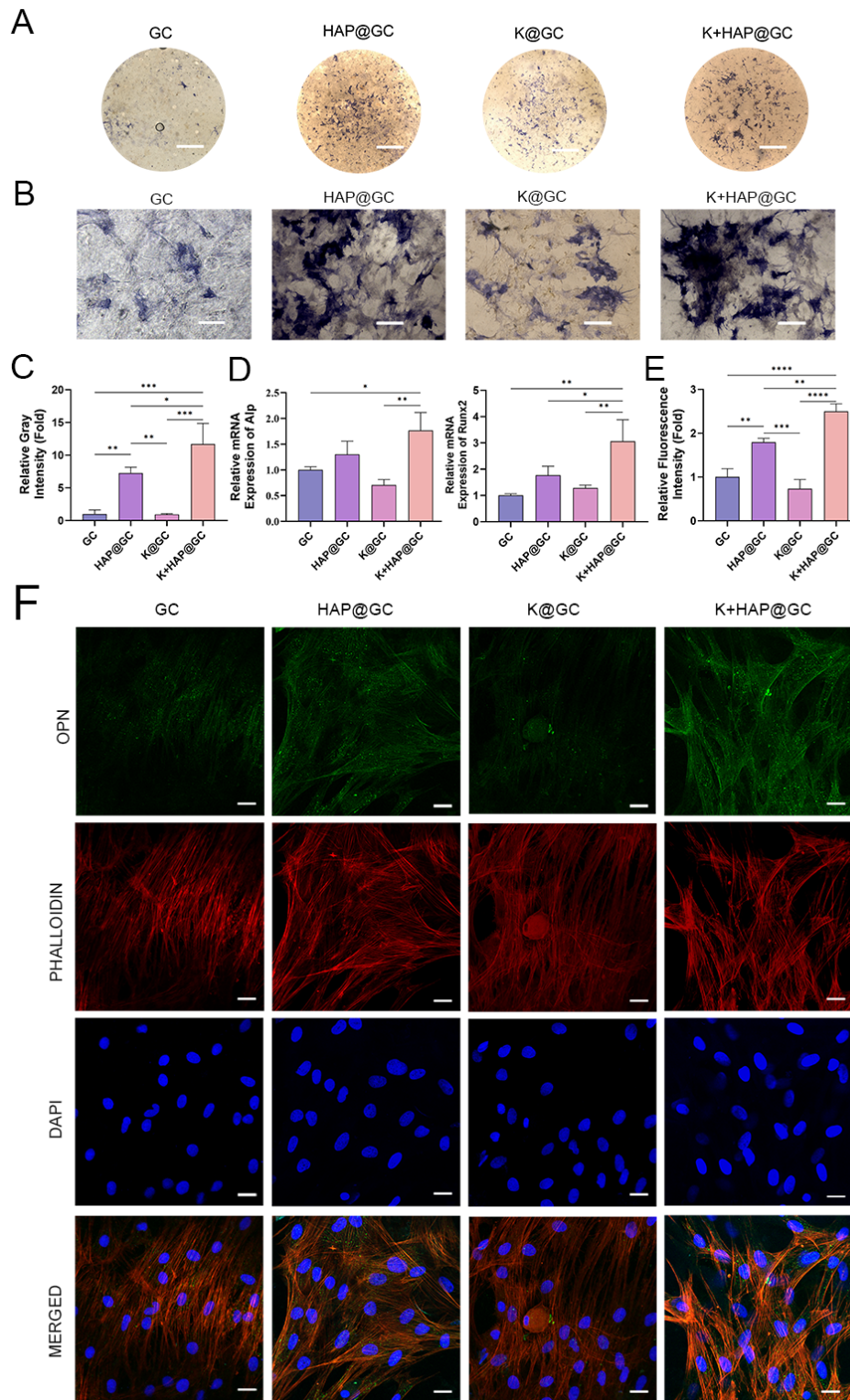


Fig. 4. *In vitro* osteogenic differentiation of BMSCs on different hydrogels. (A) Macro-images showing the overall morphology of the hydrogels (Scale bar = 1500 μ m). (B) Representative ALP staining images of the GC, HAP@GC, K@GC, and K+HAP@GC groups (Scale bar = 100 μ m). (C) Semi-quantitative analysis of ALP staining. (D) Relative mRNA expression of osteogenic-related genes (*Alp*, *Runx2*) in BMSCs cultured on different hydrogels. (E) Semi-quantitative analysis of the fluorescence intensity for (F). (F) Representative IF images of OPN expression in BMSCs cultured with each layer of hydrogels. The nuclei were counterstained with DAPI (blue), and the cytoskeleton was stained with Phalloidin (red). Scale bar = 20 μ m. Data are presented as mean $\pm$ SD, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. The images were plotted using GraphPad Prism 9.0.

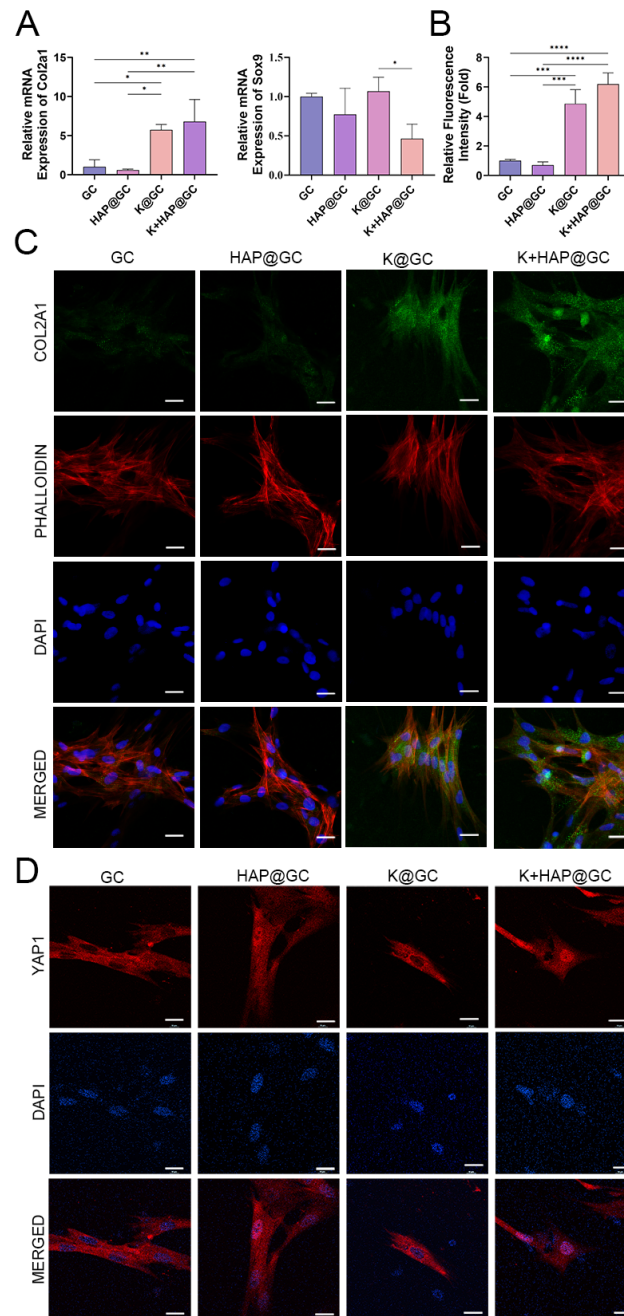


Fig. 5. *In vitro* chondrogenic matrix-associated responses and YAP1 nuclear localization. (A) Relative mRNA expression of chondrogenesis marker genes (*Col2a1*, *Sox9*) in BMSCs cultured on different hydrogel. (B) Semi-quantitative analysis of the fluorescence intensity for (C). (C) Representative IF images of COL2A1 expression in BMSCs cultured with each layer of hydrogels. The nuclei were counterstained with DAPI (blue), and the cytoskeleton was stained with Phalloidin (red). Scale bar = 20 μ m. (D) Representative IF images of YAP1 nuclear localization in BMSCs cultured with each layer of hydrogels. Scale bar = 20 μ m. Data are presented as mean $\pm$ SD, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. The images were plotted using GraphPad Prism 9.0.

7I). The Blank and GC groups exhibited minimal VEGF-positive signals. While the gHAP@GC group showed a slight increase in red fluorescence, the K+gHAP@GC group demonstrated the most robust and widespread VEGF expression among all groups.

The *in vivo* biosafety of the implanted scaffolds was assessed by H&E staining of the heart, liver, spleen, lung,

and kidney at 8 weeks after surgery (Fig. 8). No significant pathological abnormalities, inflammatory cell infiltration, tissue degeneration, or necrotic lesions were observed in the scaffold-treated groups compared with the Blank group. The normal histological architecture of the organs examined was preserved throughout the experimental period.

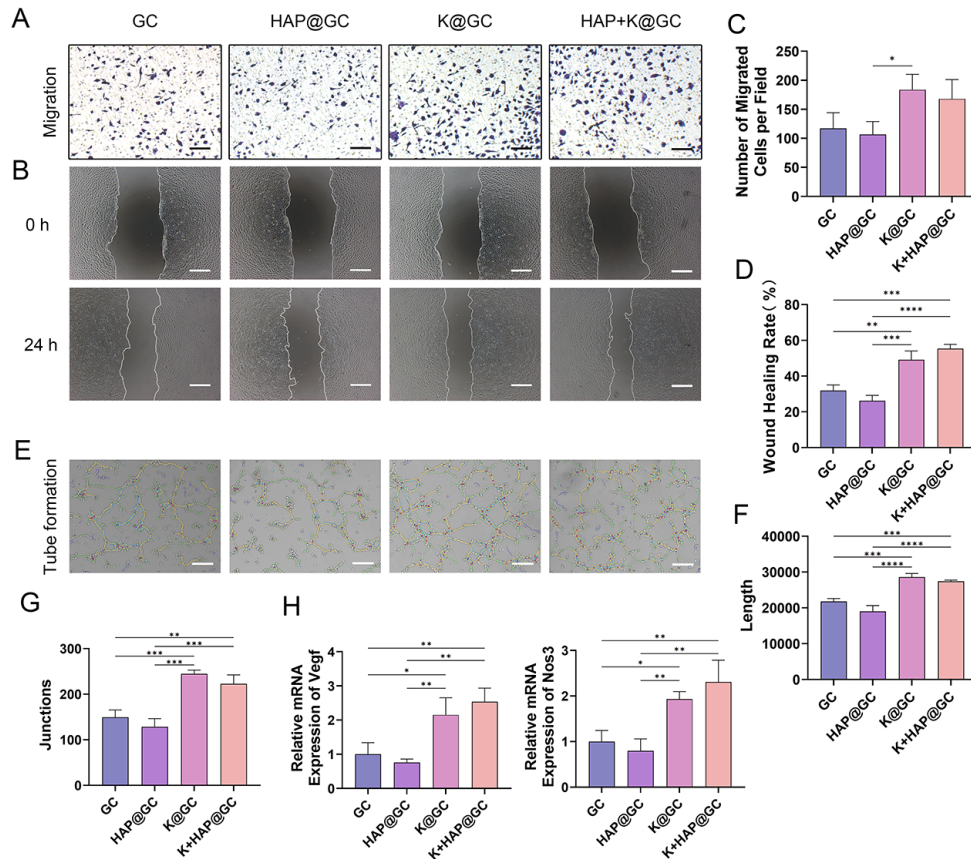


Fig. 6. In vitro pro-angiogenic evaluation of the hydrogel scaffolds. (A) Representative images of the migrated cells on the lower surface of the Transwell membrane stained with crystal violet after 24 h of incubation. Scale bar = 200 μ m. (B) Representative optical microscope images of the scratch wound healing assay at 0 h and 24 h post-treatment. Scale bar = 200 μ m. (C) Quantitative statistical analysis of the number of migrated cells per field. (D) Quantitative analysis of the wound healing rate (%) at 24 h. (E) Representative tube formation images of HUVECs after 6 h of co-culture with different hydrogel groups. Scale bar = 200 μ m. (F) and (G) Quantitative analysis of total tube length and number of junctions from the tube formation assay. (H) Relative mRNA expression of angiogenic marker genes (*VEGFA*, *NOS3*) in HUVECs cultured on different hydrogels. Data are presented as mean $\pm$ SD, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. The images were plotted using GraphPad Prism 9.0.

Discussion

The present study developed a keratin-incorporated, gradient HAP-reinforced GelMA/OCMC hydrogel scaffold for rotator cuff tendon-to-bone repair. Compared with blank repair, non-gradient hydrogel, and keratin-free gradient scaffold controls, K+gHAP@GC improved interface organization and biomechanical performance, while increasing fibrocartilage-like matrix deposition and local VEGF-positive staining. This finding is closely related to the structural nature of the native tendon-to-bone insertion. The native enthesis is not a homogeneous attachment site, but a graded transition composed of tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone. Previous studies have emphasized that this continuous structural transition is essential for mediating load transfer between mechanically dissimilar tissues, while failed repair commonly results in fibrovascu-

lar scar tissue rather than restoration of the native transitional interface [36]. Consequently, the scaffold's role extended beyond gap filling: it was designed to provide a hydrated, spatially organized material microenvironment that supports bone-side reconstruction, fibrocartilage-like matrix deposition, angiogenesis-supportive activity, and localized mechanical support during early tendon-to-bone interface healing.

Gradient scaffold design has been increasingly explored as a strategy to partially recapitulate the structural logic of the enthesis. Yea *et al.* [37] reported that a hydroxyapatite-gradient scaffold could induce mesenchymal stem cells to form tendon-, cartilage-, and bone-like matrices according to HA density and improve histological and biomechanical tendon-to-bone healing in a rat rotator cuff repair model. More recently, collagen/HAP-based bionic gradient scaffolds have also been investigated

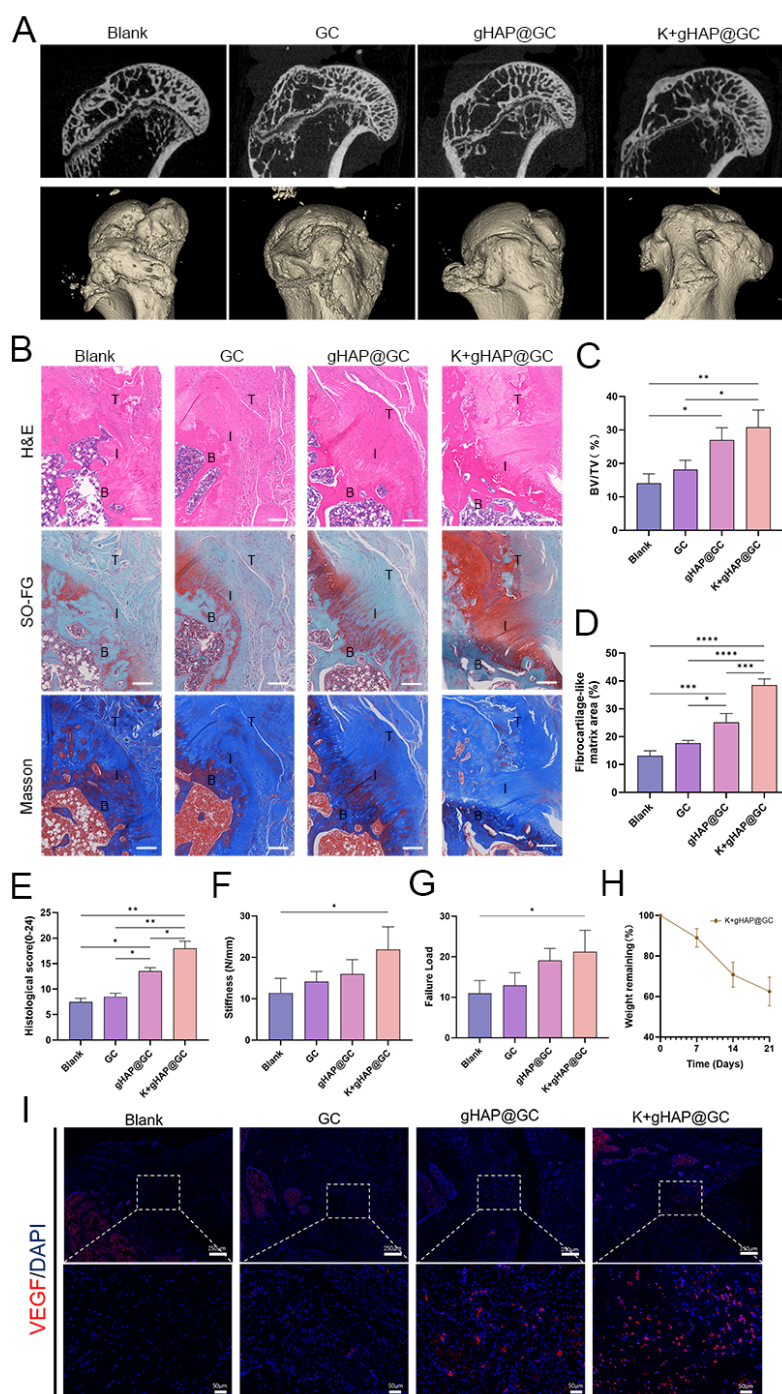


Fig. 7. *In vivo* evaluation of tendon-to-bone healing in a rat rotator cuff repair model. (A) Micro-CT images of the proximal humerus of rats at 8 weeks after surgery. **(B)** Histological evaluation of tendon-to-bone healing. Representative H&E, Safranin O-Fast Green (SO-FG), and Masson staining images showing repair morphology, fibrocartilage-like matrix formation, and collagen organization at the repaired rotator cuff tendon-to-bone interface. T, host tendon stump; B, host bone; I, tendon-to-bone interface. Scale bar = 200 μ m. **(C)** BV/TV. **(D)** Semi-quantitative analysis of the relative fibrocartilage-like matrix area based on SO-FG-stained sections. **(E)** Histological scores evaluated by two independent pathologists blinded to group allocation. **(F, G)** Biomechanical testing results, including stiffness and failure load. **(H)** Early *in vivo* mass retention of K+gHAP@GC within 21 days after implantation. **(I)** Representative VEGF immunofluorescence staining images at the repaired interface. VEGF is shown in red, and nuclei are counterstained with DAPI in blue. Scale bars = 250 μ m (main image), 50 μ m (inset). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. The images were plotted using GraphPad Prism 9.0 software.

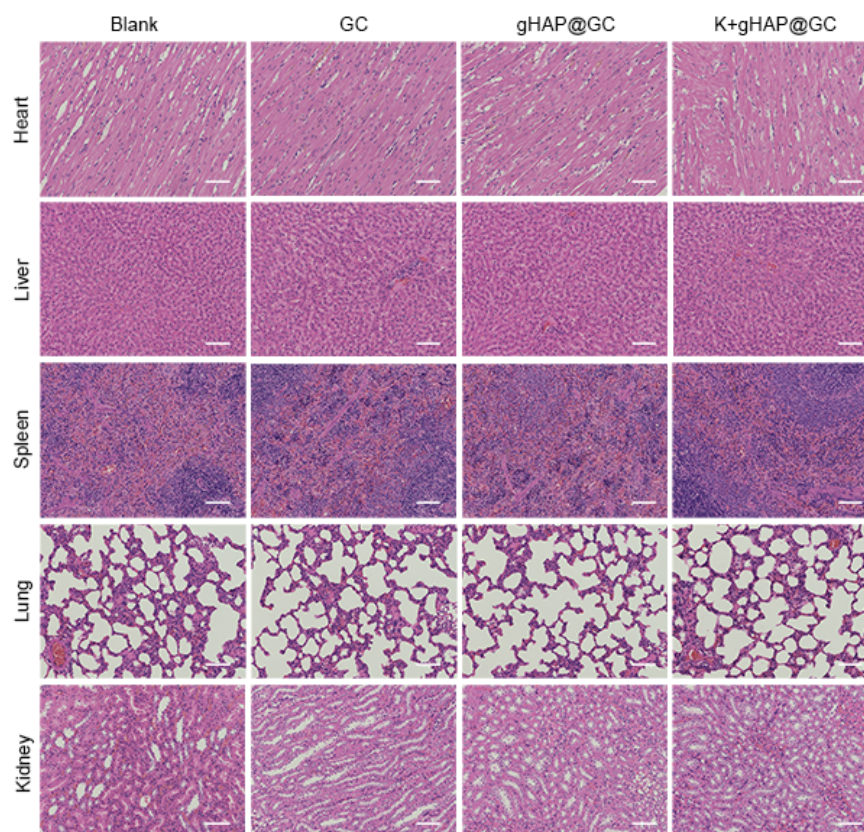


Fig. 8. *In vivo* biocompatibility evaluation. H&E staining of heart, liver, spleen, lung and kidney tissues. Scale bar = 100 μ m.

for rotator cuff tendon-bone interface regeneration [38]. These studies support the concept that spatially organized mineral cues are more relevant to tendon-to-bone repair than homogeneous material distribution. In contrast to cell-seeded or purely mineral-gradient systems, the present K+gHAP@GC scaffold was designed as a mineral–protein hybrid hydrogel, in which HAP provides spatial mineral and relative stiffness cues, while keratin contributes network participation and angiogenesis-supportive bioactivity. Thus, K+gHAP@GC should be regarded as a single, integrated gradient scaffold rather than a multiphasic construct that attempts to fully reproduce all four native enthesis zones. Its intended function is to introduce region-dependent material cues within one hydrogel system, rather than to anatomically recreate the complete native tendon–fibrocartilage–bone hierarchy.

The design rationale for K+gHAP@GC was based on integrating a dynamic hydrogel network, a spatially distributed mineral phase, and keratin-associated bioactivity. The GelMA/OCMC matrix provided a crosslinkable three-dimensional framework, while HAP and keratin were introduced to provide mineral-related and biological functions, respectively. FTIR analysis supported the incorporation of GelMA, OCMC, keratin, and HAP into the composite network. The attenuation of the aldehyde-associated signal of OCMC and the appearance of imine-related absorption

were consistent with Schiff-base formation between aldehyde groups in OCMC and amino-containing components from GelMA and keratin. Schiff-base hydrogels formed through amine–aldehyde reactions have been widely studied because dynamic imine bonds can contribute to network formation, injectability, self-adaptive behavior, and degradation regulation [39]. In this context, keratin may participate not only as a bioactive additive but also as a network-forming component through amino-mediated interactions.

Nevertheless, the chemical evidence should be interpreted within its methodological limits. Because reaction kinetics and crosslinking density were not directly quantified, the current data provide indirect evidence of network integration rather than definitive proof that Schiff-base crosslinking and photocrosslinking are independently controllable. Specifically, the gel fraction results further support this interpretation. The non-UV-crosslinked GelMA/OCMC/keratin hydrogel showed a relatively low gel fraction of approximately 60%, whereas the UV-crosslinked GelMA/OCMC/keratin hydrogel reached approximately 92%, indicating more complete network formation after combined Schiff-base and photocrosslinking. Together with the swelling, degradation, and rheological data, these findings indicate that the composite system formed a stable yet degradable hydrogel network suitable for early-stage repair implantation.

The SEM-EDS line-scan analysis further strengthens the structural basis of the gradient scaffold. A key concern for layer-by-layer assembled hydrogels is whether the compositional gradient is merely a fabrication concept or can be experimentally verified within the construct. In the present study, the heterogeneous distribution of calcium and phosphorus signals along the longitudinal direction of the multilayer hydrogel supports the successful construction of a spatial HAP gradient. This evidence is important since the biological interpretation of a “gradient” scaffold depends on the verified spatial distribution of the mineral phase. Compared with a homogeneous HAP-containing hydrogel, the gradient design allows the HAP-rich region to face the bony footprint and the HAP-free or HAP-reduced region to face the tendon stump. This spatial arrangement directly follows the principle of region-dependent material instruction proposed in previous gradient enthesis scaffold studies [37,38].

The expanded mechanical characterization helps clarify the scaffold’s mechanical role. HAP incorporation increased compressive stiffness and strength, while keratin-containing formulations showed additional reinforcement of the hydrogel matrix. Quantitatively, the compressive modulus increased from 52.9 ± 10.4 kPa in the GC group to 148.4 ± 9.9 kPa in the K+HAP@GC group, while the compressive strength increased from 31.3 ± 7.0 kPa to 96.7 ± 7.0 kPa. Rheological testing demonstrated stable gel-like behavior, with the storage modulus remaining higher than the loss modulus during the testing period. In addition, cyclic compression showed that HAP-containing and gradient hydrogels maintained repeated loading–unloading profiles more effectively than GC, indicating improved mechanical robustness under repetitive deformation.

These findings, however, do not imply that the hydrogel reproduced the absolute mechanical properties of the native tendon-to-bone insertion. Native tendon and bone possess much higher mechanical moduli than the kPa-level hydrogels reported here [40]. Instead, the scaffold is engineered to provide temporary local support, viscoelastic stability, and a relative variation in stiffness within a hydrated repair microenvironment. This distinction is essential, as the current scaffold should be considered a mechanically supportive and biologically instructive material rather than a direct mechanical substitute for the native enthesis. Indeed, the gradient HAP architecture provides localized physical cues rather than complete biomechanical equivalence to native tendon, fibrocartilage, and bone.

The *in vitro* osteogenic results indicated that the HAP-containing formulations provided osteogenesis-supportive cues for BMSCs. Compared with GC, K+HAP@GC induced stronger ALP staining, higher *Alp* and *Runx2* expression, and increased OPN deposition, suggesting that the combination of HAP and keratin created a more favorable microenvironment for osteogenesis-associated responses. This finding is consistent with previous reports showing that HAP, as a bone-like calcium phosphate phase, can en-

hance osteogenic commitment of mesenchymal stem cells by providing calcium/phosphate-related biochemical cues and an osteoconductive matrix environment [41]. In the present scaffold design, this osteogenesis-supportive formulation was incorporated into the HAP-rich layer facing the bony footprint. Consistently, the improved micro-CT outcomes *in vivo* showed more favorable bone-side reconstruction in the K+gHAP@GC group, supporting the functional relevance of the HAP-rich bone-facing region during tendon-to-bone healing. However, osteogenesis alone does not constitute successful tendon-to-bone integration. The key challenge is to reconstruct a transitional interface that mediates load transfer between the soft tendon and the hard bone. In this regard, the increased *Col2a1*-related matrix expression *in vitro* and the larger SO-FG-positive region *in vivo* suggest that K+gHAP@GC supported fibrocartilage-like matrix formation rather than isolated bone regeneration.

The fibrocartilage-related findings should be interpreted cautiously. In the present study, *Sox9* was not significantly increased, whereas *Col2a1* expression and SO-FG-positive matrix deposition were enhanced. This pattern should not be overinterpreted as definitive evidence of complete chondrogenic differentiation or native fibrocartilage regeneration. A more appropriate interpretation is that, at the selected observation time point, the scaffold exerted a stronger influence on cartilaginous extracellular matrix production than on early chondrogenic lineage commitment. The SO-FG-positive fibrocartilage-like area increased from 13.2 ± 1.8 % in the Blank group and 17.8 ± 1.1 % in the GC group to 38.3 ± 2.2 % in the K+gHAP@GC group. This quantitative improvement supports enhanced fibrocartilage-like matrix formation at the repaired interface. Nevertheless, fibrocartilage is a complex transitional tissue with both fibrous and cartilaginous components, and its complete identification requires a broader marker panel and spatial tissue characterization. Therefore, the current data support fibrocartilage-like matrix formation and interface remodeling, but do not fully establish complete regeneration of the native fibrocartilage transition zone.

Moreover, the YAP1 immunofluorescence results provided preliminary evidence that BMSCs responded to the HAP-containing hydrogel microenvironment through altered mechanosensitive localization. Compared with GC and K@GC, the HAP-containing hydrogels, particularly K+HAP@GC, showed more evident nuclear localization of YAP1. This observation is consistent with previous hydrogel-based biomaterials studies showing that matrix stiffness, degradability, cell spreading, and dimensionality can regulate YAP1 subcellular localization in MSCs [42]. More broadly, YAP1 is recognized as an important mechanotransducer through which stem cells sense substrate stiffness and cell-material interactions [43]. In the present scaffold, the HAP-rich region may provide a relatively stiffer and more mineralized microenvironment, thereby con-

tributing to YAP1-associated cellular responses. However, YAP1 inhibition, downstream TEAD target-gene analysis, and integrin–FAK pathway validation were not performed. Therefore, these data should be interpreted as supportive evidence for a YAP/TAZ-associated mechanosensitive response, rather than as proof that YAP/TAZ signaling causally mediates the scaffold's osteogenic or repair-enhancing effects.

Keratin-associated angiogenic support represents another important feature of the scaffold. *In vitro*, keratin-containing hydrogels enhanced HUVEC migration, scratch closure, capillary-like tube formation, and *VEGFA/NOS3* expression. In recent years, keratin-based biomaterials have been explored in regenerative medicine because of their biocompatibility, degradability, and capacity to provide biologically active proteinaceous matrices; experimental studies have also suggested that keratin-related components can support endothelial migration or tissue repair-associated biological responses [28,44]. Enhanced VEGF immunoreactivity at the repaired TBI in the K+gHAP@GC group provides further *in vivo* evidence supporting this rationale. Taken together, these findings suggest that keratin contributed an angiogenesis-supportive biological function beyond its possible participation in hydrogel network formation.

This angiogenesis-supportive effect is relevant to tendon-to-bone healing. VEGF is a central angiogenic regulator, and previous studies have shown that properly timed VEGF-mediated angiogenesis can facilitate tendon repair, whereas persistent or dysregulated VEGF activity may be detrimental [45]. In the context of rotator cuff repair, VEGF has also been reported to enhance tendon-bone healing by promoting angiogenesis-related responses [46]. In addition, angiogenesis and osteogenesis are closely coupled during skeletal repair, and VEGF signaling is an important mediator of this coupling [47]. Therefore, the increased *VEGFA/NOS3* expression *in vitro* and VEGF-positive staining *in vivo* provide a reasonable biological explanation for the improved repair microenvironment observed in the K+gHAP@GC group. Nevertheless, the upstream molecular mechanism remains incompletely defined since hypoxia-related signaling, VEGF/HIF-1 α pathway activation, endothelial recruitment markers, and vessel maturation markers were not systematically examined. Thus, keratin should be described as providing angiogenesis-supportive activity rather than a fully defined angiogenic signaling mechanism.

The *in vivo* results further demonstrated that K+gHAP@GC promoted a more integrated repair response rather than simply increasing tissue filling. Micro-CT analysis showed improved bone-side reconstruction; histological staining revealed a larger fibrocartilage-like region and more organized collagen deposition; VEGF immunofluorescence indicated enhanced expression of angiogenesis-related markers; and biomechanical testing

confirmed improved stiffness and failure load. This is particularly relevant since functional tendon-to-bone repair requires not only histological tissue formation but also recovery of load-bearing capacity. The consistency among these structural, histological, angiogenesis-related, and mechanical outcomes is important. A scaffold may increase the amount of repair tissue without improving interface quality if the regenerated tissue remains disorganized, scar-like, or mechanically weak. In contrast, K+gHAP@GC appeared to support coordinated bone remodeling, fibrocartilage-like matrix deposition, collagen organization, local VEGF expression, and mechanical restoration. These findings suggest that the scaffold improved the structural-to-functional maturation of the repaired TBI, although the current data do not allow direct prediction of re-tear reduction in a clinical setting.

In addition to these repair outcomes, the early *in vivo* mass-retention assay provided preliminary information on the temporal stability of K+gHAP@GC during the initial healing phase. The scaffold exhibited progressive mass loss during the first 21 days after implantation, with approximately 62% of its initial dry mass remaining at day 21. This result suggests that while K+gHAP@GC did not undergo rapid structural collapse immediately after implantation, it retained part of its material framework during the early period when cell infiltration, matrix deposition, and interface remodeling are expected to occur. At the 8-week repair endpoint, no gross residual hydrogel was observed during specimen harvest. However, given that quantitative mass recovery was only performed up to 21 days, the degradation process between day 21 and week 8 should be interpreted qualitatively rather than as a fully characterized long-term degradation profile.

Taken together, the favorable repair outcome of K+gHAP@GC is likely related to the combined contribution of its major design elements. The GelMA/OCMC matrix provided a hydrated and crosslinked framework for cell attachment and tissue ingrowth. Schiff-base-associated interactions and UV photocrosslinking contributed to network formation and gel stability. The HAP gradient supplied spatially organized mineral and relative stiffness cues for bone-side reconstruction and fibrocartilage-like interface remodeling. The YAP1 localization results suggest that cells responded to the HAP-containing microenvironment through mechanosensitive behavior. Keratin further enhanced the system by participating in network formation and supporting pro-angiogenic endothelial responses. Although the present study does not fully separate all possible interaction effects among these components, the comparisons among GC, HAP@GC, K@GC, gHAP@GC, and K+gHAP@GC support the integrated design rationale of the final scaffold. This combined design represents the major novelty of the present work compared with homogeneous or purely mineral-gradient hydrogel systems. Accordingly, K+gHAP@GC should be regarded as a single

integrated gradient hydrogel scaffold with spatially varied HAP distribution, rather than a four-phase multiphasic construct separately reproducing the native tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone zones.

Several limitations of the present study should be acknowledged. First, although the rat rotator cuff repair model is widely used for preliminary evaluation, it cannot fully reproduce the size, chronic degeneration, fatty infiltration, poor tendon quality, and complex loading environment of human massive RCTs. Validation in a large-animal chronic tear model will therefore be necessary before clinical translation can be considered. Second, the present study mainly evaluated repair at an 8-week time point. Longer-term observation is required to determine whether the fibrocartilage-like interface, bone reconstruction, vascular remodeling, YAP/TAZ-associated localization pattern, and biomechanical improvements are maintained during later remodeling. Third, although YAP1 nuclear localization and VEGF-positive staining were observed by immunofluorescence, the causal roles of mechanotransduction and angiogenic pathways were not directly verified by pathway inhibition, gene silencing, downstream target gene analysis or protein-level pathway validation. Future studies should examine YAP/TAZ target genes, integrin-FAK signaling, cytoskeletal organization, VEGF/HIF-1 α -related pathways, and pharmacological or genetic inhibition. Fourth, long-term scaffold degradation and its temporal coupling with tissue ingrowth after day 21 require further quantitative validation. Finally, the optimal HAP concentration, gradient profile, layer thickness, interlayer bonding strength, keratin dosage, scaffold scale-up, and implantation strategy require further optimization for clinically relevant tendon-to-bone repair conditions.

Conclusions

In summary, this study developed a keratin-incorporated, gradient HAP-reinforced GelMA/OCMC hydrogel scaffold for rotator cuff tendon-to-bone repair. K+gHAP@GC provided spatial mineral cues, keratin-associated bioactivity, and local mechanical support, thereby improving bone-side reconstruction, fibrocartilage-like matrix formation, collagen organization, VEGF-positive staining, and biomechanical performance *in vivo*. These findings suggest that a mineral-protein gradient hydrogel strategy may improve the structural and functional quality of tendon-to-bone healing, although its causal mechanisms, long-term stability, and translational feasibility require further validation.

List of Abbreviations

CCK-8, cell counting kit-8; FBS, fetal bovine serum; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction; TBI, tendon-bone interface; OCMC, oxidized carboxymethyl cellulose;

GelMA, gelatin methacryloyl; gHAP, gradient hydroxyapatite; HAP, nano-hydroxyapatite; SEM, scanning electron microscope; FTIR, Fourier transform infrared spectrometer; MA, methacrylic anhydride; DN, double network; DI, deionized; RCTs, rotator cuff tears; BM-SCs, Bone Marrow Mesenchymal Stem Cells; HUVECs, Human umbilical vein endothelial cells; SD, Sprague-Dawley; cDNA, complementary deoxyribonucleic acid; ITS, Insulin-Transferrin-Selenium; TGF- β_1 , transforming growth factor- β_1 ; PFA, paraformaldehyde; H&E, hematoxylin and eosin; SO-FG, Safranin O-Fast Green; ROI, region of interest; BV/TV, bone volume fraction; SD, standard deviation; Micro-CT, Micro-computed tomography; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *Sox9*, SRY-box transcription factor 9; *NOS3*, nitric oxide synthase 3; *Col2a1*, collagen type II alpha 1 chain; *VEGFA*, vascular endothelial growth factor A; *Alp*, alkaline phosphatase; *Runx2*, runt-related transcription factor 2.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Author Contributions

GLY and YGZ contributed to the design of this work. YGZ, SXC, GLY and WNS performed the experiments and data acquisition. YM, MCW and JFX contributed to the interpretation of data. WNS, GLY, XL, JFX, MCW, YM and ZHY analyzed the data. YGZ, GLY and SXC drafted the work. GLY, DMZ, ZHY and CLP revised critically for important intellectual content. All authors read and approved the final manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Ethics Approval and Consent to Participate

All animal procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals. The study was approved by the Institutional Animal Care and Use Committee of the Southern Medical University (Approval No. SMUL202411016). All animal experimental procedures were performed in accordance with ARRIVE guidelines 2.0.

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Conflict of Interest

The authors declare no conflict of interest.

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