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Original Article

BMP-2/CPC scaffold with dexamethasone-loaded blood clot embedment accelerates clinical bone regeneration

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Abstract: Delayed repair caused by prolonged inflammatory response might lead to clinical nonunion and failed bone regeneration. The design of desirable biomaterials requires precise regulation of the initial osteoimmune responses and efficient osteoinductive capacity to facilitate later bone regeneration. Herein, a Dex-loaded blood clot-embedded BMP-2/CPC scaffold (Dex/blood/BMP-2/CPC) was fabricated for clinical bone regeneration with the sequential release of dexamethasone (Dex), a clinical immunosuppression drug, and BMP-2, a potent osteogenic growth factor. The introduction of Dex at a BMP-2/Dex ratio of 1/6 effectively facilitated M2 polarization of macrophages and exerted a synergetic effect on BMP-2-mediated osteogenic differentiation. The highest *in vivo* bone regenerative efficacy was achieved by Dex/blood/BMP-2/CPC scaffold with a 1/6 BMP-2/Dex dose, exhibiting significantly enhanced endochondral ossification and attenuated bone resorption in an ectopic model, as well as reduced fibrosis at the orthotopic defect site. Blood clot embedment further provides nutrition and cytokines for the endochondral ossification process. On these bases, a pilot clinical trial was carried out and the Dex/blood/BMP-2/CPC scaffold was demonstrated to accelerate fracture healing, improve therapeutic quality, and eliminate local inflammation compared to current bone regenerative treatment. Taken together, this work designed an immunoregulatory and osteoinductive Dex/blood/BMP-2/CPC scaffold, which might provide new insights for future biomaterial development (Trial registration: ChiCTR2100047693).

Keywords: BMP-2, calcium phosphate cement, dexamethasone, blood clot, clinical bone regeneration

Introduction

Large bone defects that cannot be healed by the body itself require surgical intervention with the assistance of bone substitutes, which sparks interests of researchers in developing bone regenerative materials. A thorough understanding of the physiological process of bone regeneration is the critical prerequisite for designing desirable biomaterials. Specifically, a typical bone healing process involves three sequential stages, including inflammatory phase, endochondral ossification and bone remodeling [1]. Many reports uncovered the important role of inflammatory phase in the early stage in ensuing endochondral ossification and bone remodeling in recent years [2-4]. Prolonged inflammatory response can delay bone regeneration and lead to bone nonunion in clinic. Therefore, immunoregulatory capacity and osteoinductive activity are equally important in designing applicable bone substitutes for improved clinical treatment.

Numerous bone substitutes have been developed in recent decades to avoid the risks of autografts and allografts including infection, rejection, donor site morbidity and limited supply. Among these substitutes, calcium phosphate cement (CPC) has been widely applied in clinic for its biocompatibility, osteoconductivity, as well as similar chemical composition and crystal structure to natural bone [5-8]. Bone morphogenetic protein-2 (BMP-2), as a potent

osteogenic growth factor, can effectively promote osteogenic differentiation, accelerate fracture healing, and endow biomaterials with enhanced osteoinductivity [9]. In 2002, recombinant human BMP-2 was approved by FDA for the treatment of spinal fusion in clinic, and its therapeutic performance has been widely acknowledged [10]. A BMP-2-incorporated CPC scaffold has been developed and tested clinically in our previous study [11]. However, after implantation of BMP-2/CPC, wound exudation and delayed fracture healing were observed in some individual cases, indicating that individual differences in immune microenvironment could lead to exceptional failure of even the most potent therapy. These phenomena inspired us that early-stage inflammatory regulation might facilitate the clinical therapeutic effect of the BMP-2/CPC scaffold. Therefore, dexamethasone (Dex), a common clinical drug for immunosuppression, was incorporated for immunoregulation. Our previous study demonstrated that adequate dosage of Dex inhibited excessive macrophages infiltration, expedited a balanced M2/M1 polarized ratio of macrophages in the early stage, and promoted BMP-2-mediated endochondral ossification process [12]. Moreover, Dex has been reported to induce osteogenic differentiation of BMSC [13-20] and synergize osteogenesis with BMP-2 [21-24]. Based on the above, we hypothesized that a sequential release of Dex and BMP-2 could modulate the early-stage inflammatory phase, accelerate the ensuing endochondral bone formation process, and promote high-quality bone regeneration. To our best knowledge, there is no clinical report on the synergistic effect of BMP-2 and Dex so far.

Preoperative blood drainage and collection are common and mature techniques in clinical orthopedic surgeries, which provide a rich source of platelets and autologous growth factors to promote bone regeneration [25, 26]. Herein, a BMP/CPC scaffold with Dex-loaded blood clot embedment was fabricated for clinical bone regeneration. As shown in **Scheme 1A**, Dex was mixed into autologous blood and backfilled to form a blood clot embedding the BMP-2-loaded CPC scaffold (Dex/blood/BMP-2/CPC scaffold). The immunoregulatory and synergistic osteogenic effect of Dex and BMP-2 was systematically studied. The physiochemical property of CPC scaffold and drug release were determined. The *in vivo* osteogenic efficacy was studied with an ectopic bone formation model in mice and an orthotopic critical-sized calvarial defect regeneration model in rats. Accordingly, a practical surgical method of the Dex/blood/BMP-2/CPC scaffold for bone defect treatment was established, and preliminary clinical studies were carried out for therapeutic effect verification.

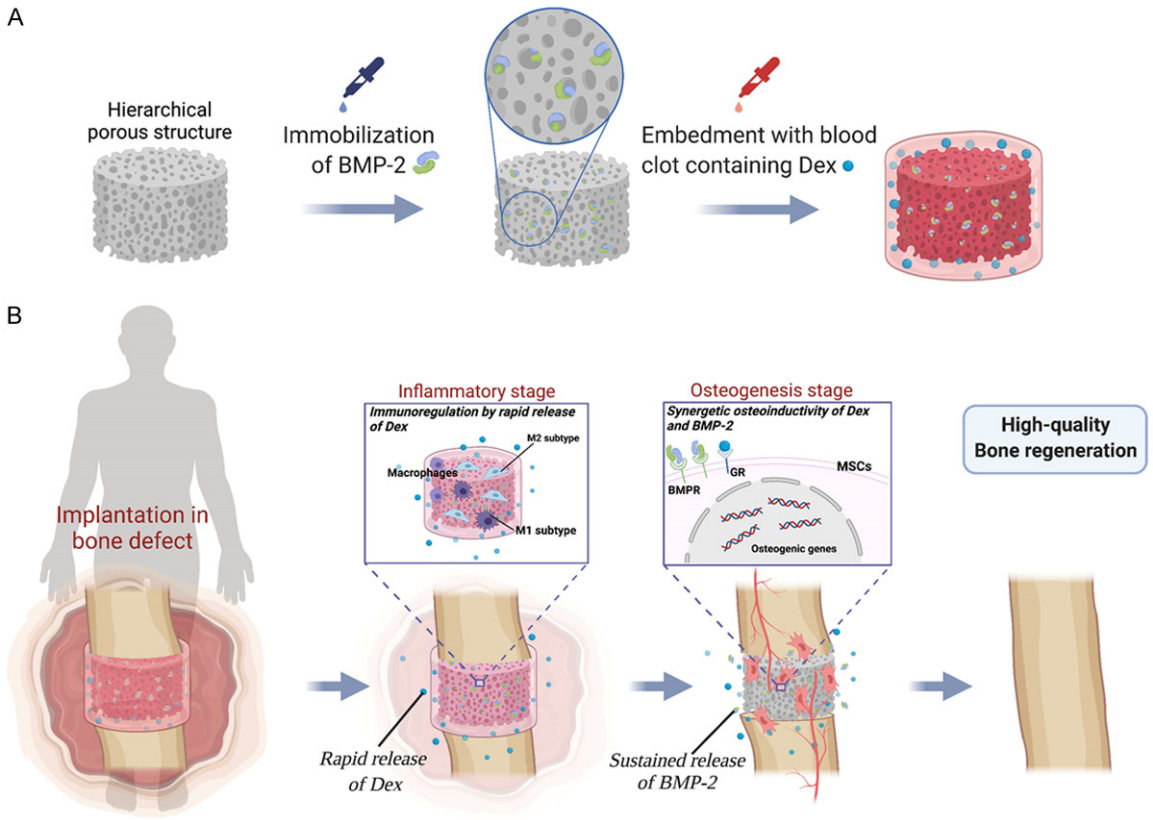
Material and methods

Materials

Recombinant human BMP-2, CPC scaffolds and BMP-2-incorporated CPC scaffolds (CFDA License No. 2013:3460199) were provided by Shanghai Rebone Biomaterials Co., Ltd. (Shanghai, China). Dexamethasone sodium phosphate injection (CFDA License No. H37-021969) was purchased from Cisen Pharmaceutical Co., Ltd. (Shandong, China). Fetal bovine serum (FBS) was from Gibco® (Thermo Fisher Scientific Inc., MA, USA). α-MEM culture medium was from HyClone™ (GE Healthcare, UK). Human BMP-2 ELISA kit, tetracycline hydrochloride, alizarin red and calcein were from Sigma-Aldrich (St. Louis, MO, USA). Nonidet P-40 (NP-40) and p-Nitrophenyl phosphate (disodium, hexahydrate) (PNPP-Na) were from Sangon (Shanghai, China). Trizol reagent, PrimeScript RT reagent kit and SYBR Premix Ex TaqTM were from Takara (Tokyo, Japan). All of the reagents were purchased and used without further purification.

Cell experiments

rBMSCs extraction and culture: This study was performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23 Rev. 1985). Rat bone marrow stromal cells (rBMSCs) were extracted from femur bone marrow. After flushing out with 15 mL α-MEM culture medium supplemented with 10% FBS and 1% antibiotics (100 U mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin sulphate), the bone marrow suspension was transferred into a 75 cm^2 polystyrene tissue culture flask and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was refreshed every 2 days until about 90% confluence was achieved. Passages 3-8 of the rBMSCs were utilized for the *in vitro* experiments in this study.



Scheme 1. Schematic illustration of the spatiotemporal delivery of Dex and BMP-2 from blood clot-embedded CPC scaffold for clinical bone regeneration. A. The fabrication of Dex/blood/BMP-2/CPC scaffold. Dex was mixed into autologous blood and backfilled to form a blood clot embedding the BMP-2-loaded CPC scaffold. B. Blood clot served as Dex carrier and a supply of nutrition and angiogenic cytokines, and Dex first acted as immune-modulator and later synergistically enhanced BMP-2-induced osteoinductivity. Together, Dex/blood/BMP-2/CPC scaffold exerted superior regenerative performances with attenuated inflammation and shortened repairing time.

Evaluation of in vitro osteoinductivity: ALP activity and mineralization staining were assessed to evaluate the osteoinductive capacity of different dose ratios of BMP-2 and Dex. Eight groups were set: blank control, Dex, BMP-2, BMP-2/Dex (1:1), BMP-2/Dex (1:3), BMP-2/Dex (1:6), BMP-2/Dex (1:9) and BMP-2/Dex (1:12). In all groups, 1 μg mL^{-1} of BMP-2 was applied, dose ratio of BMP-2 and Dex was weight ratio, and the concentration of Dex in Dex group was 6 μg mL^{-1}.

rBMSCs were seeded in the 24-well plate at a density of 3×10^4 cells per well, and at 24 hours post-seeding the medium was changed to experimental medium. After 4, 7 and 14 days, the medium was removed and 500 μL of 1% NP-40 solution was added to obtain cell lysates. Then 50 μL of the cell lysates from each sample was pipetted to 96-well plates. 200 μL of 1 mg mL^{-1} pNPP-Na solution containing 0.1 mol L^{-1} glycine and 1 mmol L^{-1} $MgCl_2{\cdot}6H_2O$ was added and incubated for 30 min at 37°C. The reaction was quenched by adding 100 μL NaOH (0.1 mol L^{-1}). ALP activity was quantified by the absorbance at 405 nm using a microplate reader (SPECTRAmax 384, Molecular Devices, USA). Total protein content of each cell lysate was determined using a BCA protein assay kit (Beyotime, Jiangsu, China). ALP levels were normalized with total protein content, and these experiments were performed in quintuplicates.

Mineralization of rBMSCs was analyzed on day 14 and 21 using Alizarin Red staining. Cells were stained with 1% Alizarin Red (pH=4.2) after fixed with 2.5% glutaraldehyde. Inverted light microscope (TE2000U, Nikon, Japan) was applied to observe the rBMSCs mineralization.

Expression of osteogenic genes: The expression of osteogenic genes in rBMSCs were mea-

sured on day 4, 7 and 14 by a real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) system (Bio-Rad, Hercules, CA, USA). Four groups were set: blank control, Dex, BMP-2 and BMP-2/Dex (1:6).

After 4, 7 and 14 days of culture, RNA was extracted from cells and reverse transcribed into complementary DNA (cDNA) using Trizol reagent and PrimeScript RT reagent kit (Takara, Tokyo, Japan) according to manufacturer's instructions. Then osteogenic differentiation markers including runt-related transcription factor 2 (Runx2), osteocalcin (OCN), osteopontin (OPN) and Col I were evaluated, with β-actin used as a housekeeping gene. The relative expression level (fold change) was calculated with the Livak method using $2^{\Delta\Delta}$Ct. All experiments were performed in triplicate. Primer sequences used here are listed in Table S1.

Polarization of macrophages: RAW264.7 cell line was used to determine the immunoregulatory effect of Dex and purchased from Cell Bank of the Chinese Academy of Science. Four groups were set: Control, Dex, BMP-2, and BMP-2/Dex (1:6).

RAW264.7 cells were seeded in a 24-well plate at a density of 1×10^5 cells per well, and at 24 hours post-seeding the medium was changed to experimental medium. After 7 days, RAW264.7 cells were detached and washed with PBS. Then, cells were resuspended with cell staining buffer and blocked by rat anti-mouse CD16/32 (Biolegend, USA) at 4°C for 5 min. Then, cells were stained with rat anti-mouse CD197 Alexa Fluor 647 antibody (BD, USA) for determining M1 phenotype and rat anti-mouse CD206 Alexa Fluor 647 antibody (BD, USA) for determining M2 phenotype. The samples were analyzed on an Accuri C6 flow cytometer (BD, USA) and data were analyzed using the FlowJo workstation (Tree Star, USA).

To determine the expression of inflammatory genes by RT-qPCR, RAW264.7 cells were seeded in a 24-well plate at a density of 1×10^5 cells per well, and cultured with experimental medium for 7 days. Markers for proinflammation, IL-1β and IL-6, and for antiinflammation, Arg1 and IL-10, were determined and normalized with GAPDH. The relative expression level (fold change) was calculated with the Livak method using $2^{\Delta\Delta}$Ct. All experiments were performed in triplicate. Primer sequences used here are listed in Table S1.

Scaffold preparation and characterization

Scaffold preparation: Schematic illustration of the preparation of BMP-2/Dex-loaded, blood clot-embedded CPC scaffold (BMP/Dex/blood/CPC) was shown in **Figure 2D**.

For *in vitro* drug release analyses, porous CPC scaffolds (Ø 3×4 mm) were sterilized by gamma radiation at 10 kGy before use. BMP-2 solution was dropped onto CPC scaffolds and lyophilized to obtain a BMP-2-loaded CPC scaffold (5 μg of BMP-2 per scaffold). Eyeball blood of rat was extracted under sterile condition, and mixed with dexamethasone sodium phosphate injection (30 μg of Dex in 100 μL of blood). The BMP-2-loaded CPC scaffold was placed in a 96-well plate, and 100 μL of Dex/blood mixture along with 1 unit of thrombin was added to form a blood clot embedding the scaffold. For comparison, Dex solution was directly dropped onto BMP-2-loaded CPC scaffold without blood clot embedment.

For animal experiments, BMP-2-loaded CPC scaffolds were prepared as above-mentioned, and autologous blood was extracted, mixed with dexamethasone sodium phosphate injection, and filled into the implanted site to embed the scaffold in situ. Scaffold sizes, drug doses and blood volumes for different experiments are listed in **Table 1**.

Characterization of CPC scaffold: The macroporous morphology and the microstructure of CPC scaffolds were observed by scanning electron microscope (SEM, JSM-6360LV, JEOL). The chemical composition of CPC was characterized by X-ray diffraction (XRD, Rigaku Co., Japan) and energy dispersive spectroscopy (EDS, Bruker QUANTAX 400-30, German).

Releasing profiles of BMP-2 and Dex: In vitro releasing profiles of BMP-2 and Dex from CPC scaffolds and blood clot embedded CPC scaffolds were evaluated. Briefly, scaffolds were placed in test tubes and immersed in 2 mL PBS at 37°C under the vibration of 30 rpm. At each time point, the supernatant was collected and replaced with fresh PBS. The amounts of released BMP-2 and Dex were quantitatively analyzed by human BMP-2 ELISA kit and high-

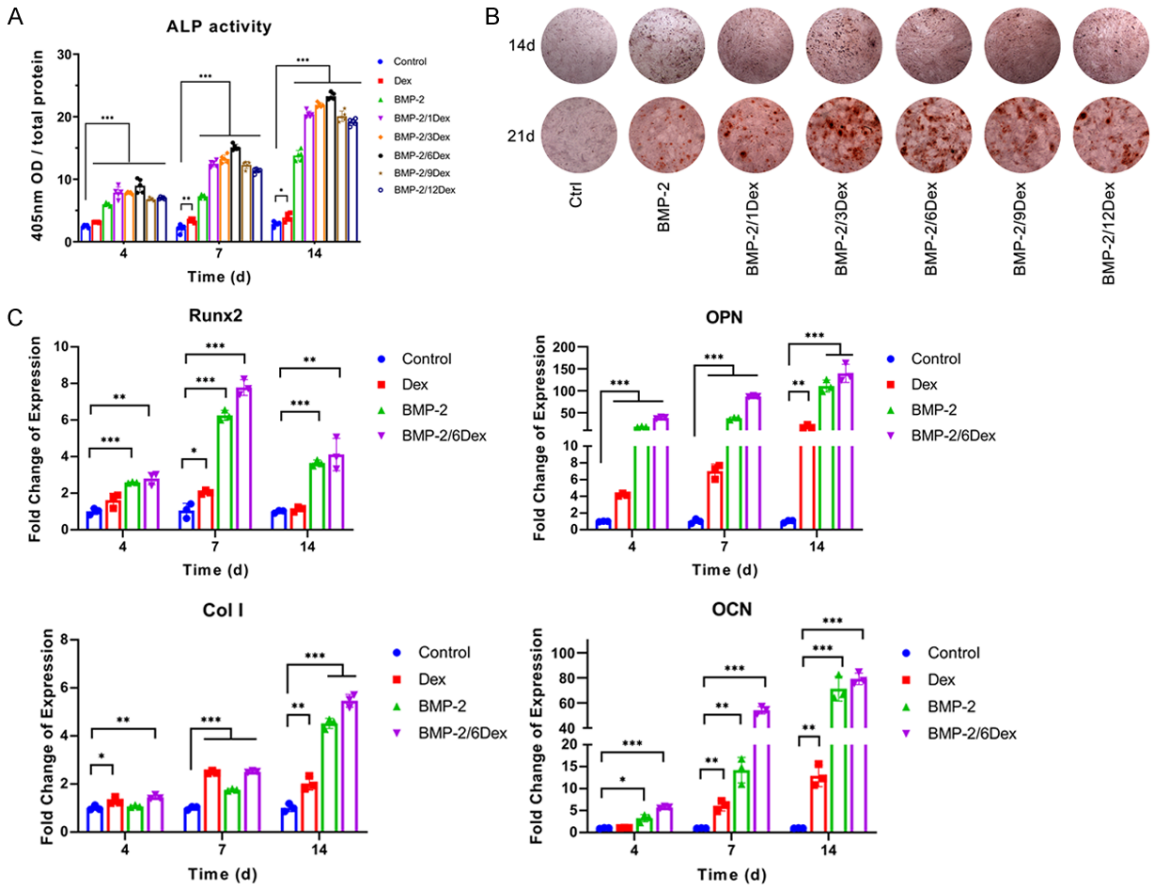


Figure 1. Effect of Dex on BMP-2-induced *in vitro* osteoinductivity at different ratios: (A) ALP activity and (B) Alizarin red staining of BMSC cultured with different dose ratios of BMP-2 and Dex. The combined application of BMP-2 and Dex induced significantly higher ALP activity and mineral deposition of BMSC than singular application of BMP-2 or Dex, with an optimal BMP-2/Dex dose ratio at 1/6. (C) Expression of osteogenic genes (Runx2, Col I, OPN and OCN) at the optimal dose ratio (BMP-2/6Dex). Dex induced a limited and insignificant upregulation of Runx2 (a key transcription factor of osteoblast differentiation), while it synergistically enhanced BMP-2-induced Runx2 expression. Dex alone stimulated Col I and OPN mRNA expression, and Dex-stimulated Col I mRNA level at 4 days and 7 days was even higher than BMP-2 alone.

performance liquid chromatography (HPLC, Waters 2795, USA), respectively.

Animal experiments

The animal experiments were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Animal Research Committee of Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

Evaluation of ectopic bone formation: An animal model of ectopic bone formation in thigh muscle pouches of mice was applied to evaluate the osteoinductivity of BMP/Dex/blood/CPC scaffold. Five experimental groups were set: 1/0+, 1/3+, 1/6-, 1/6+ and 1/9+. Annotations of the experimental groups are listed in **Table 1**.

20 male C57BL/6 mice (Silaike Inc. Shanghai, China) with an average weight of 25 g were used and randomly divided into 5 groups (n=4). 50 μL of blood was drawn from the eyeballs, and specific dose of Dex was added to the blood according to group settings (**Table 1**). BMP-2-loaded CPC scaffold (Ø 1×5 mm, 5 μg of BMP-2 per scaffold) was implanted into the created muscle pouch along the hind limb, and 50 μL of the Dex/blood was dropped onto the scaffold. At each time point, animals were sacrificed with an overdose of pentobarbital and

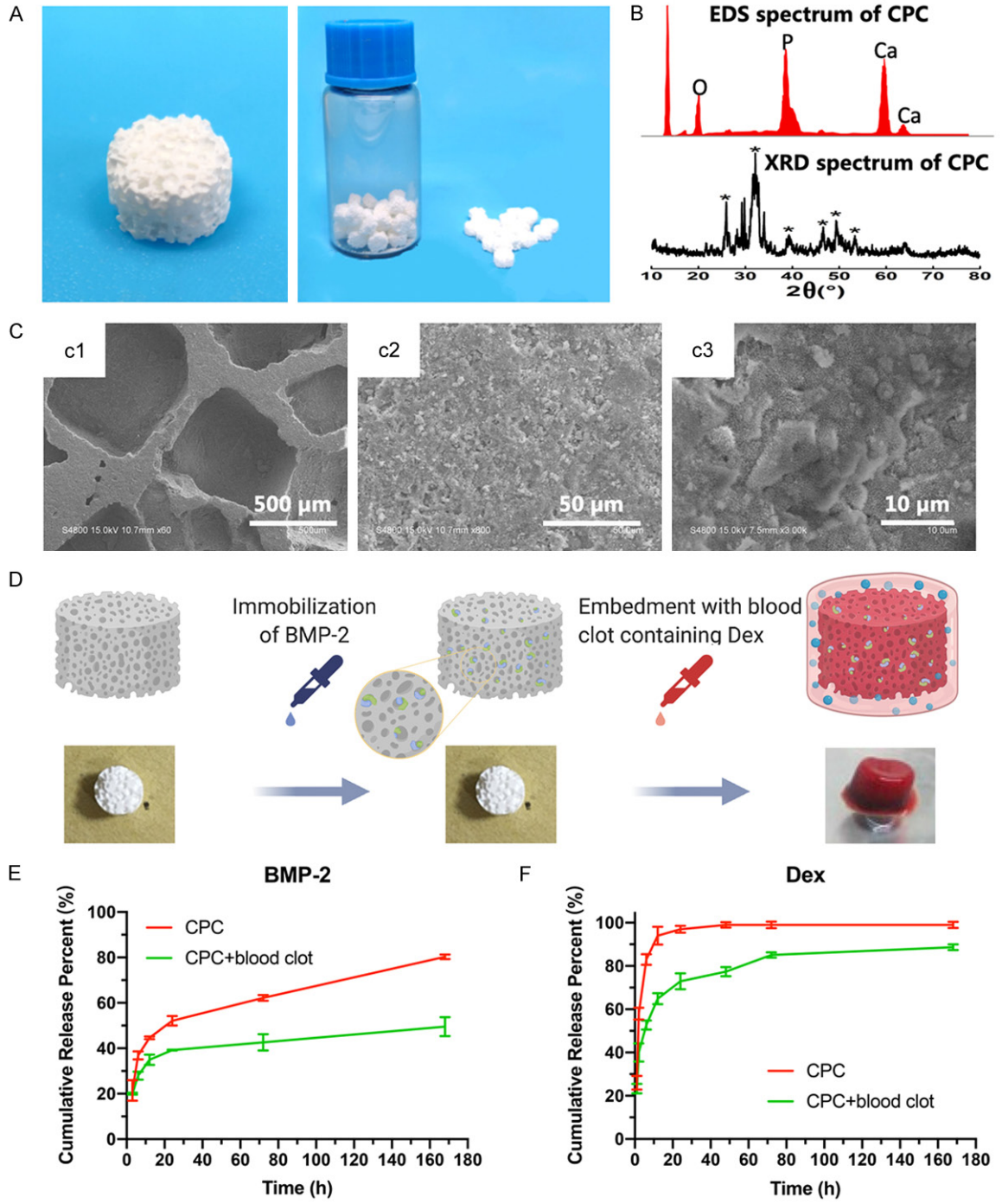


Figure 2. Physicochemical characterization of CPC scaffold and the release profiles of Dex and BMP-2. (A) Digital photograph of porous CPC scaffolds (Ø 3×4 mm). (B) EDS element distribution and wide angle XRD spectra of CPC (Asterisks indicate specific peaks of hydroxyapatite: 28.2°, 32.8°, 39.7°, 46.6°, 49.5° and 52.1°). (C) SEM images of the macroporous structures (c1) and surface hydroxyapatite crystal morphology of CPC scaffold (c2 and c3). (D) Construction of a sequential release of Dex and BMP-2 based on CPC scaffolds and blood clot embedment: the BMP/Dex/blood/CPC scaffold. Cumulative release curves of BMP-2 (E) and dexamethasone (F) from blood clot-embedded CPC scaffold and CPC scaffold. Without blood clot embedment, the release profile of BMP-2 exhibited an initial release of ~50% during the first 24 hours and a slow sustained release during the rest of the time course, with a release percentage of 70% at 7 days, while Dex exhibited a burst release of 95% in 12 hours. After embedded in blood clot, the burst release of Dex was alleviated, and Dex was gradually released in 7 days, which avoided the side effects of local high concentration of Dex by the burst release. The initial release percentage of BMP-2 decreased from ~55% to ~40%, and the subsequent sustained release rate was slowed, with a release percentage of 50% at 7 days.

Table 1. Scaffold sizes, drug doses and blood volumes for different experiments

Experiment	Scaffold size	Dose (per scaffold)		
		BMP-2	Dex	blood
In vitro drug release analyses	Ø 3×4 mm	5 μg	30 μg	100 μL
Ectopic bone formation in thigh muscle pouches of mice	Ø 1×5 mm	5 μg	0-45 μg according to experimental group	50 μL
Orthotopic bone regeneration of rat critical-sized calvarial defect	Ø 5×2 mm	10 μg		100 μL

the implants were retrieved. At week 2 and 4, two specimens from each group were weighed for both wet bone and ash content (calcinated at 800°C for 4 hours), and two samples from each group were used for histological analyses.

Evaluation of orthotopic bone regeneration: An animal model of rat critical-sized calvarial defect was applied to evaluate the bone regenerative capacity of Dex/blood/BMP-2/CPC scaffold. Five experimental groups were set (n=4): 1/0+, 1/3+, 1/6-, 1/6+ and 1/9+. After anesthesia by intraperitoneal injection of pentobarbital (Nembutal, 3.5 mg/100 g), 100 μL of blood was drawn from the eyeballs, and specific dose of Dex was added to the blood according to group settings (**Table 1**). A 1.0-1.5 cm longitudinal incision was made on the scalp to expose the calvarium, and two critical-sized defects were created using a 5 mm diameter trephine bur (Fine Science Tools, USA). BMP-2-loaded CPC scaffolds (Ø 5×2 mm, 10 μg of BMP-2 per scaffold) were implanted into the defects, 100 μL of the Dex/blood was filled to embed the scaffolds, and then the overlying muscle and skin were sutured. At week 12 post-implantation, animals were sacrificed with an overdose of pentobarbital, and the calvarial samples were obtained for micro-CT, sequential fluorescent labeling and histological analyses.

Synchrotron radiation-based micro-CT (SRμ-CT): SRμCT measurements were performed at beamline BL13W of Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, China) using a monochromatic beam with energy of 30 keV. A VHR detector (Photonic-Science Company, Britain) with pixel size of 9 μm was used to record images. Then the 2D slices were reconstructed to 3D structure and quantitatively analyzed by software VG Studio MAX (Volume Graphics, Germany). A cylinder area with the defect site of radius in the center was selected as region of interests (ROI) for quantifications of bone volume/tissue volume (BV/TV) and trabecular thickness (Tb.Th).

Sequential fluorescent labeling and VG staining: Briefly, 25 mg/kg tetracycline (TE), 30 mg/kg alizarin red (AR) and 20 mg/kg calcein (CA) were injected at week 2, 5 and 8 respectively, and 3 days after the last injection the animals were euthanized to obtain the radius specimens. The obtained specimens were embedded in PMMA and cut into ~40 μm thick slices. These undecalcified sections were observed for fluorescent labelled new bone formation under CLSM. The excitation/emission wavelengths of the fluorophores were 405/580 nm (TE), 543/617 nm (AR) and 488/517 nm (CA). The graphic quantifications were analyzed by Image Pro Plus.

After fluorescence observation, the sections were counter-stained with Van Gieson's picro fuchsin (VG) and Stevenel's Blue to observe the mineralized bone tissue microscopically.

Histological analyses: After fixation with 4% paraformaldehyde, the extracted bones were decalcified with 12.5% EDTA, dehydrated in a graded series of alcohol, and embedded in paraffin. 4 μm thick sections were stained with Masson's trichrome and hematoxylin/eosin (HE), respectively, and observed using an inverted light microscope (TE2000U, Nikon Corp., Japan).

Pilot clinical trial

Patient selection: Both BMP-2-loaded CPC scaffolds (1 mg BMP-2/g CPC scaffold) and dexamethasone sodium phosphate injection (5 mg/mL) were commercialized clinical medical apparatuses or medicines, and blood clot was formed using autologous blood of the patients. Clinical protocols were approved by Ethic Committees of Guizhou Provincial People's Hospital ((2019) 28), and all patients gave informed consent before joining the study. 10 bone defect patients were enrolled in Guizhou Provincial People's Hospital in China. Details of surgical inclusion and exclusion criteria are described below.

Inclusion criteria: 1. Age 20-50 years. 2. Tibial plateau fractures or proximal humeral fractures that involve collapse and require implantation of bone-grafting materials. 3. An understanding of the rehabilitation protocol and willing to follow it. 4. An agreement to postoperative visits and tests. 5. Signed an informed subject consent form.

Exclusion criteria: 1. Patients with systemic illness including diabetes, bleeding disorder, hypertension, etc. 2. Patients with orthopedic medical history including arthritis.

Surgical procedure: All operations were conducted by doctors at the level of associate chief physician or above. Procedures were performed under general anesthesia in a tertiary care medical center.

The patients were first treated by reduction of fracture, correction of displacement and the angle of the deformity, and the use of metal implants were fixed. The collapse of the cancellous bone was filled with 2 g BMP-2-loaded CPC scaffolds (1 mg BMP-2/g CPC scaffold), which was compacted and prevented from filling material into the joint cavity. Autologous blood of the patient was drained and collected during the surgery, mixed with dexamethasone sodium phosphate injection (5 mg/mL) according to a BMP-2/Dex dose ratio of 1:6, and backfilled into the implantation site to form blood clot before suture.

Rehabilitation, X-ray imaging and function scoring: Post-operative follow-ups were conducted, and the assessment was based on clinical examination and X-ray radiographic imaging. After the surgery, all patients were observed according to the following indexes: allergic or toxic reactions, rash or high fever. Rehabilitation exercises were required 6 weeks post-operation. X-ray radiography at different time intervals after implantation was employed to observe the osseointegration of the implanted scaffolds to host bone and the degradation of the material.

The time of clinical healing and fracture reunion were determined by observation of fracture lines together with other physical criteria as described below. Criteria of clinical healing: 1) no local tenderness, vertical percussion pain or abnormal movements; 2) the ability of (upper limbs) stretching forward and bearing 1 kg for up to 1 minute or (lower limbs) continuously walking on the ground without crutches for 3 minutes and no less than 30 steps; 3) X-ray film showing massive bone trabeculae crossing barely discernible fracture line. Criteria of fracture reunion: 1) meet the criteria of clinical healing; 2) X-ray film showing homogeneous bone structure and obliterated fracture lines [27].

The functional recovery was recorded at each follow-up time point using IOWA knee and ankle score [28] in cases of tibial plateau and calcaneal fractures, and Neer shoulder score [29] in cases of proximal humeral fractures. The IOWA knee and ankle score is a questionnaire and clinical-examination-based evaluation of the function of the knee and ankle. The knee score is a five-category measurement, which includes activities of daily living, freedom from pain, gait, aid dependence, deformity and range of movement. The ankle score is a four-category measurement of function, freedom from pain, gait and range of movement. Neer shoulder score has three parts: scoring of pain during the previous week by patients (verbal rating scale); clinical testing of function (muscle strength, reaching ability, and stability) and active range of motion; and an anatomical or radiological evaluation. Both IOWA and Neer scores are grouped into excellent (90 to 100), good (80 to 89), fair (70 to 79) and poor (under 70) categories.

Statistical analyses

Results were expressed as mean ± SD (standard deviation). All data were generated from at least three independent experiments. Statistical analysis was conducted using one-way ANOVA with post hoc tests. A value of $P<0.05$ was considered statistically significant.

Results and discussion

Synergistic effects of BMP-2 and dexamethasone on osteogenic differentiation

Compared with the well-acknowledged osteoinductivity of BMP-2, the effect of Dex on cell osteogenic differentiation was two-sided. On the one hand, Dex has been commonly applied as one of the formulas of osteogenic conditional culture media, and has been reported to

induce osteogenic differentiation of BMSC [13-20]. On the other hand, pharmacological concentration or chronic administration of Dex inhibits mineralization of osteoblasts and lead to bone loss [30]. Despite the negative effects of Dex on bone mineralization, combined application of Dex and BMP-2 could lead to an enhanced mineralization than BMP-2 alone, and many literatures have demonstrated the synergistic osteoinductivity of Dex and BMP-2 [19-22]. On these bases, the optimal synergistic dose ratio of Dex and BMP-2 was investigated in this study.

The osteoinductivity of BMP-2/Dex at gradient concentrations was evaluated via ALP activity analyses. As shown in Figure S1, Dex at the concentrations of 30-6000 ng mL^{-1} exhibited a certain but limited osteoinductivity, without significant difference among different concentrations, and the highest ALP activity at 14 days was only ~2 times of the blank control group, while Dex at a high concentration (12 μg mL^{-1}) decreased its ALP activity. BMP-2 at concentrations above 1 μg mL^{-1} efficiently induced osteogenic differentiation for 4 days, and the ALP activity was upregulated with increasing BMP-2 concentration. Though higher dose of BMP-2 resulted in significantly enhanced osteogenic differentiation, the high dose was also accompanied by high cost and high risk of side effects.

Herein, BMP-2 at 1 μg mL^{-1} was selected in this study, added with different dose ratios of Dex, to study its synergistic effect on BMP-2 induced BMSC osteogenic differentiation. The combined application of BMP-2 and Dex induced significantly higher ALP activity and mineral deposition of BMSC than singular application of BMP-2 or Dex, indicating synergy of Dex on BMP-2 osteoinductivity. With a BMP-2/Dex dose ratio of 1/6 (BMP-2 1 μg mL^{-1}, Dex 6 μg mL^{-1}), a highest ALP activity was achieved (**Figure 1A**), and more mineral nodules formed by BMSC at 21 days was observed (**Figure 1B**), indicating an optimal synergistic osteoinductivity. With a higher Dex concentration above 6 μg mL^{-1}, though an enhancement of BMP-2 osteoinductivity was still observed, the ALP activity decreased probably due to the negative effects of Dex at pharmacological concentration on osteogenic differentiation [30].

Osteogenic genes expression at the optimal dose ratio (BMP-2/Dex=1/6) was further investigated (**Figure 1C**). The results indicated that Dex induced a limited and insignificant upregulation of Runx2 (a key transcription factor of osteoblast differentiation), while it synergistically enhanced BMP-2-induced Runx2 expression. Dex alone stimulated Col I and OPN mRNA expression, and Dex-stimulated Col I mRNA level at 4 days and 7 days was even higher than BMP-2 alone. Synergistically enhanced osteogenic-related genes expression by the optimal dose ratio of BMP-2 and Dex (1:6) was demonstrated.

Design and characterization of a controlled release system of Dex and BMP-2 (Dex/blood/BMP-2/CPC)

Suitable carrier is vital to controlled release of drug, activity preservation and performance. In this study, clinically applicable CPC scaffold (Shanghai Rebone Biomaterials Co., Ltd., Shanghai, China) was chosen as the carrier to design a dual controlled release system of BMP-2 and Dex. Cylindrical CPC scaffolds (Ø 4 mm×3 mm) were massively prepared by a salt leaching and molding method (**Figure 2A**, **2B**), with ~500 μm macropores formed by NaCl granules (**Figure 2C**, **2Cc1**) and extensive needle-like hydroxyapatite crystals in the surface (**Figure 2C**, **2Cc2**). The scaffolds were designed to fill defect sites of different sizes and shapes with easy operability and sufficient macroporosity for tissue ingrowth. As shown in **Figure 2B**, EDS and wide-angle XRD analyses indicated a composition of hydroxyapatite with low crystallinity, which is similar to the inorganic phase of natural bone tissue.

Autologous blood could spontaneously coagulate into blood clot and function as a reservoir of natural growth factors essential for tissue healing and regeneration. It was reported that following blood clot formation, degranulation would lead to the secretion of growth factors from platelets [31]. In addition, it was reported that platelets release anti-microbial proteins and play an important role in preventing bacterial infection [32]. Therefore, autologous blood clot could be applied as a natural biomaterial of clinical feasibility, without risk of immune rejection.

As indicated by the release curves (**Figure 2E**, **2F**), a sustained release of BMP-2 and a burst release of Dex were detected without blood clot

embedment. The sustained release behavior of BMP-2 was attributed to the inherent binding affinity of CPC to BMP-2 due to the calcium-binding domains of the protein [33]. Our previous study demonstrated that the immobilization of protein on CPC scaffold, which was mainly based on physical adsorption, would not influence the structure and bioactivity of BMP-2 [11]. In contrast, as a small molecule drug, low affinity of Dex and CPC scaffold led to a burst release and high local concentration of Dex, which might be detrimental to bone regeneration. To avoid a high local concentration of Dex, autologous blood was mixed with Dex and coagulated to embed the BMP-2-loaded CPC. With Dex-loaded blood clot embedment, the burst release of Dex was alleviated, and Dex was gradually released in 7 days. The initial release percentage of BMP-2 decreased and the subsequent sustained release rate was slowed. The results indicated that Dex/blood/BMP-2/CPC scaffold realized a controlled sequential release of BMP-2 and Dex with the embedment of blood clot.

Ectopic bone formation of Dex/blood/BMP-2/CPC scaffold

The *in vivo* osteoinductivity of Dex/blood/BMP-2/CPC was evaluated with an animal model of ectopic bone formation in thigh muscle pouches of mice. Five experimental groups were set (1/0+, 1/3+, 1/6+, 1/9+, and 1/6-), and the groups were named by BMP-2/Dex dose ratios and the existence of blood clot embedment (+: with blood clot; -: without blood clot). **Figure 3A** presented the induced ectopic bones at 2 weeks and 4 weeks post-implantation. At week 4, all groups underwent bone resorption due to the lack of bony microenvironment, resulting in decreased wet weights, but the ash weights increased, indicating further mineralization of the bone.

By comparing the weights data (**Figure 3A**), the effects of different component of Dex/blood/BMP-2/CPC scaffold were analyzed. The incorporation of Dex promoted BMP-2-induced ectopic bone, as both wet weight and ash weight were up-regulated in 1/3+, 1/6+ and 1/9+ groups, and blood clot significantly enhanced ectopic bone formation by comparing the weights data of 1/6- group and 1/6+ group. Among all groups, 1/6+ exhibited the highest wet weight and ash weight of ectopic bone. The significantly enhanced ectopic bone formation at later stage by Dex, especially the inorganic composition (ash weight), indicated that Dex promoted the maturation and mineralization of the newly formed bone.

As shown in histological images (**Figures 3B**, S2, S3), four groups with blood clot embedment (1/0+, 1/3+, 1/6+ and 1/9+) exhibited more blood cells in the ectopic bone than group without embedment (1/6-). There were two possible sources of blood cells: one was the residue of the embedding blood clot, the other was from neovascularization, which indicated the potential of promoting angiogenesis by blood clot embedment. Therefore, the embedding blood clot was believed to provide nutrition and angiogenic cytokines at the ectopic site, and hence increase the amount of bone formation. Besides, the histomorphometry of formed ectopic bone tissue varied in different groups. At week 2, the most intensive bone tissue and calcified cartilage tissue were found in 1/6+ group, the maximum uncalcified cartilage tissue was found in 1/6- group, and discontinuous stripped ectopic bone tissue and many fat vacuoles were found in 1/0+ group. These results might be ascribed to attenuated bone resorption in 1/6+ group as the incorporation of Dex could restrain excessive macrophages infiltration [34], and increased blood vessels by the embedment of blood clot which could facilitate endochondral ossification [35].

Above ectopic bone formation results demonstrated that the Dex/blood/BMP-2/CPC with blood clot embedment and BMP-2/Dex ratio of 1/6 elicited excellent osteoimmunomodulatory property and boosted osteoinductivity.

Repair of rat critical-sized calvarial defect

The orthotopic bone defect repairing capacity of Dex/blood/BMP-2/CPC scaffold was evaluated via a rat critical-sized calvarial defect model. The procedure of the surgery was shown in **Figure 4A**. As intuitively presented by micro-CT images and its quantitative analyses (**Figure 4B**), 1/6+ group exhibited significantly increased BV/TV and Tb.Th. To be noted, 1/9+ exhibited a decreased bone volume and trabecular thickness at week 12 compared to 1/0+, indicating that an excessive local concentration of Dex exerted a negative effect on

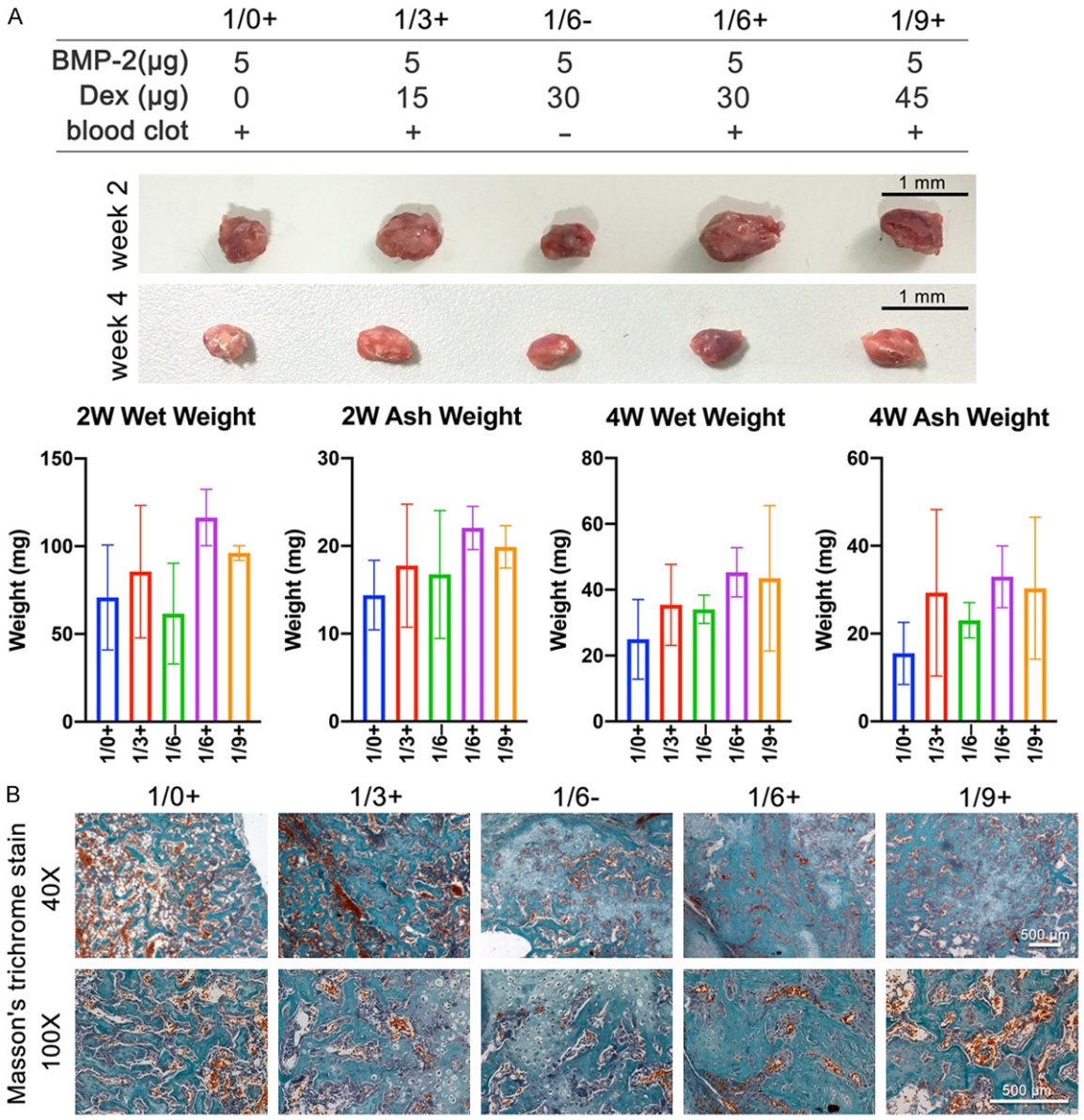


Figure 3. Ectopic bone formation in thigh muscle pouches of mice induced by CPC scaffolds with different ratios of BMP-2 and Dex, with and without blood clot embedment. Five experimental groups (1/0+, 1/3+, 1/6+, 1/9+, and 1/6-) were named by BMP-2/Dex dose ratios and the existence of blood clot embedment (+: with blood clot; -: without blood clot). A. The ectopic bone tissue samples, wet weights and ash weights. The maximum ectopic bone was induced with 1/6+ group. At week 2, 1/6+ was 2-fold to 1/6- in wet weight, and 1.3-fold in ash weight; at week 4, 1/6+ was 1.36- and 1.44-fold to 1/6- in wet and ash weight, respectively. Larger ectopic bones were induced by groups 1/3+, 1/6+ and 1/9+ than 1/0+, indicating the synergistic effect of Dex. Among all groups, 1/6+ exhibited the highest wet weight and ash weight of ectopic bone, with 1.65-fold wet weight and 1.6-fold ash weight to 1/0+ group at week 2, and 1.8-fold wet weight and over 2-fold ash weight at week 4. B. Masson's trichrome staining of ectopic bone sections 2 weeks post-implantation. Four groups with blood clot embedment (1/0+, 1/3+, 1/6+ and 1/9+) exhibited more blood cells in the ectopic bone than the group without embedment (1/6-). And at week 2, the most intensive bone tissue and calcified cartilage tissue were found in 1/6+ group, the maximum uncalcified cartilage tissue was found in 1/6- group, and discontinuous stripped ectopic bone tissue and many fat vacuoles were found in 1/0+ group.

bone regeneration. The time course of new bone mineralization during bone regeneration was intuitively observed by sequential fluorescent labeling (**Figure 5**). At early stage (week 2,

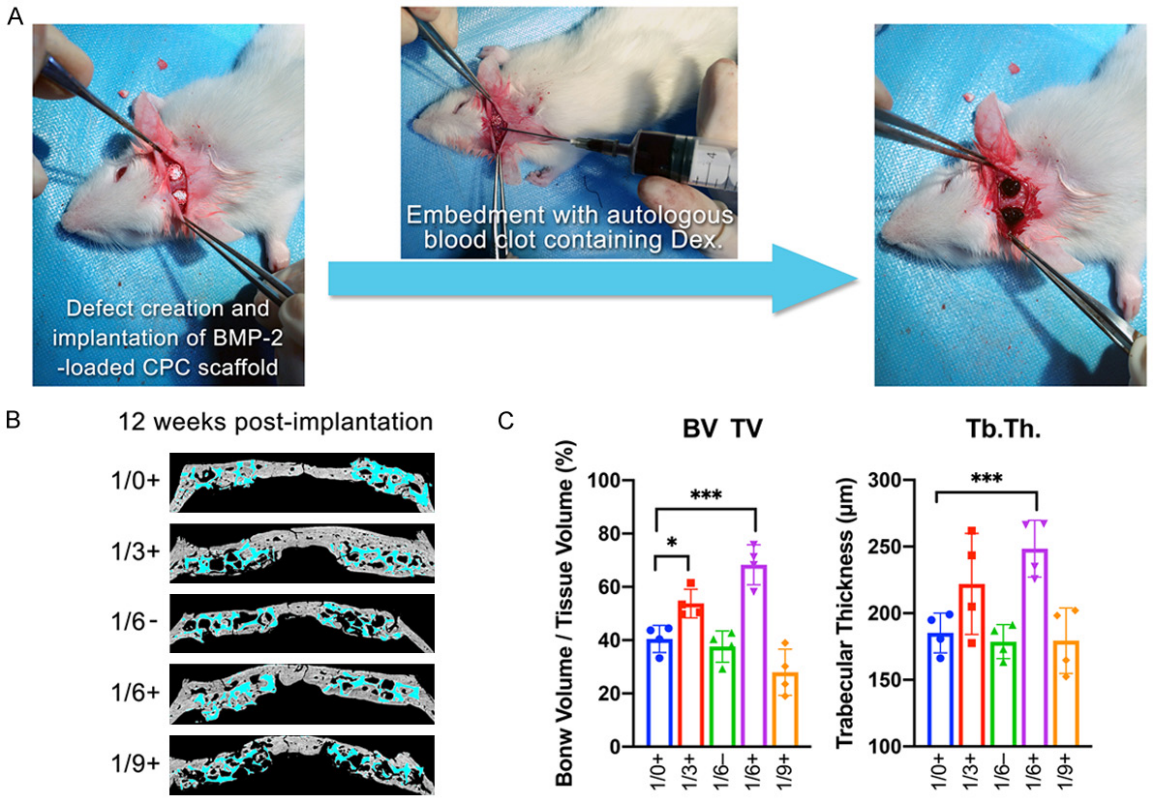


Figure 4. Bone regeneration of rat cranial defect by CPC scaffolds with different ratios of BMP-2 and Dex, with/without blood clot embedment. (A) Surgical procedure of cranial defect creation and autologous blood clot embedment. (B) Micro-CT section images and (C) the corresponding quantification of BV/TV and Tb.Th. 1/6+ group exhibited significantly increased BV/TV and Tb.Th. To be noted, 1/9+ group exhibited a decreased bone volume and trabecular thickness compared to 1/0+ group.

yellow fluorescence, TE), the bone mineralization amount of 1/0+ was significantly lower than that of the other groups, and 1/6+ induced significantly higher bone mineralization than the other groups. At later stage (week 8, green fluorescence, CA), the mineralization amount of 1/6+ decreased due to the primary completion of regeneration with a significantly more rapid mineralization rate. Moreover, the mineralization rate of 1/6 was slower than the blood clot embedded groups, which verified the function of blood clot on ossification.

As shown in **Figure 6**, 12 weeks post-implantation, all groups achieved bridging of the defect with new bone ingrowth into the macropores of CPC scaffold, with the most intensive newly formed bone tissue which completely filled the macropores of the scaffold and a highest degree of bone maturity observed in 1/6+. In contrast, extensive fibrosis was observed in the boneless areas of 1/0+ due to undesired inflammatory response and insufficient osteoinductivity. Besides, bone marrow was observed in the cavities of 1/3+, 1/6+ and 1/9+ groups, indicating successful bone remodeling. In consistent with the ectopic bone formation results, weakened maturity of mineralization and less bone marrow was observed in 1/6-. Intriguingly, the largest cavities were observed in 1/9+, as a consequence of increased bone resorption by excessive dosage of Dex, emphasizing the significance of adequate dosage of Dex. These results demonstrated the excellent therapeutic efficiency and application prospect of Dex/blood/BMP-2/CPC scaffold with BMP-2/Dex ratio of 1/6 in bone defects repair.

Effect of Dex on immunomodulation and macrophages polarization

It is well accepted that M2 macrophages reduce inflammatory response and promote tissue

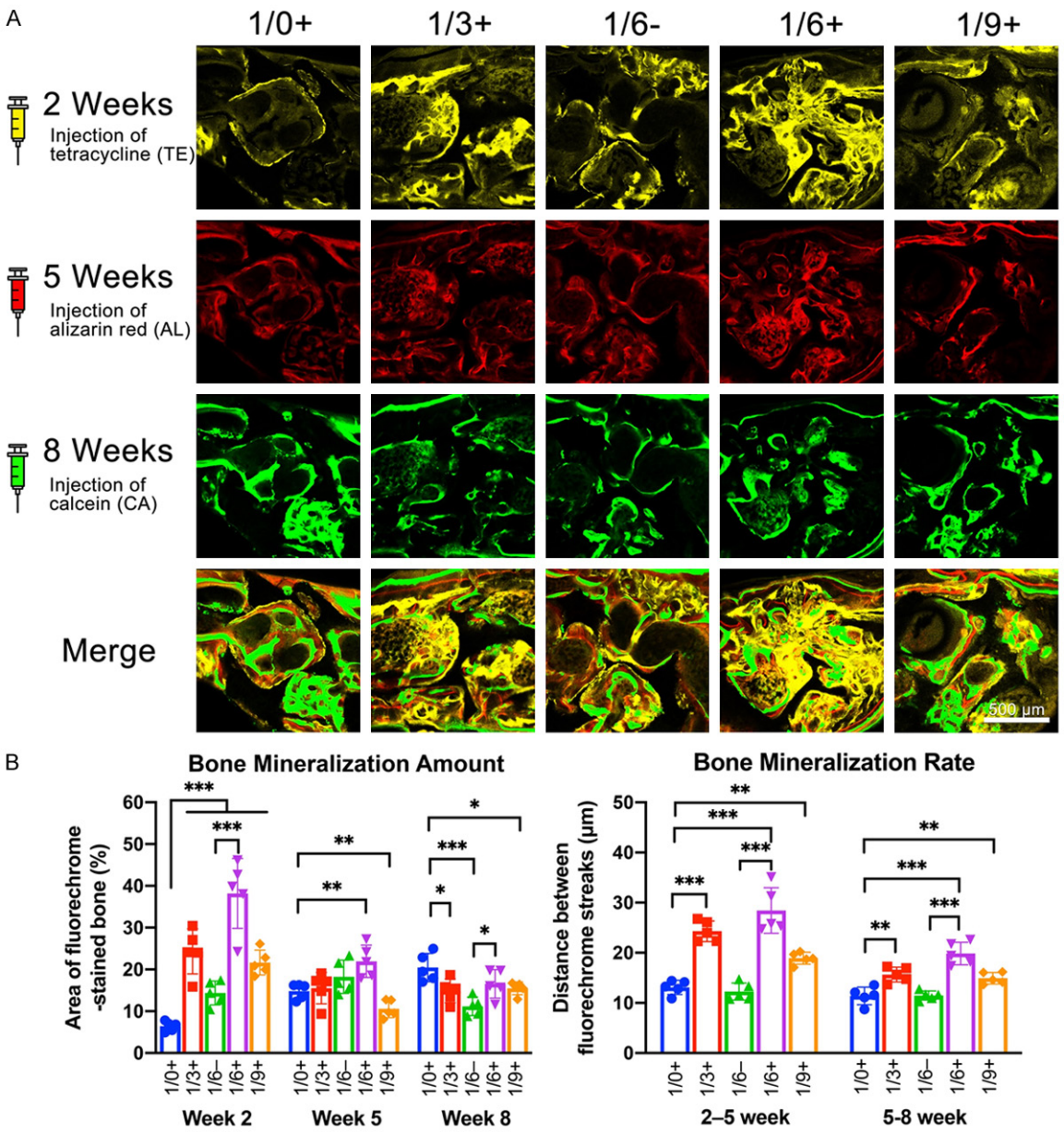


Figure 5. Time course of new bone mineralization during orthotopic bone regeneration by sequential fluorescence labeling. A. Fluorescence observation by CLSM. B. Quantification of the amount and rate of bone mineralization. At early stage (week 2, yellow, TE), 1/0+ exhibited the lowest bone mineralization amount, whereas 1/6+ induced the highest amount. At week 5, all groups exhibited similar bone mineralization amount except 1/9+, which was significantly lower than other groups due to the negative effect on bone regeneration by excessive local concentration of Dex. At later stage (week 8, green, CA), the overall mineralization amount decreased due to the primary completion of regeneration.

regeneration [36, 37]. Dex/blood/BMP-2/CPC scaffold with BMP-2/Dex ratio of 1/6 boosted ectopic bone formation and orthotopic bone regeneration. However, rapid bone resorption in ectopic bone formation and numerous fibers infiltration in calvarial bone repair were observed in 1/0+ group according to the histological analysis, which suggested the immunoregulatory effect of Dex. Therefore, the effect of Dex on the polarization of macrophages was studied with flow cytometry. As shown in **Figure 7A**, the introduction of Dex decreased the expression of CD197 (M1 marker) from 37.6% to 12.1%, and increased the expression of

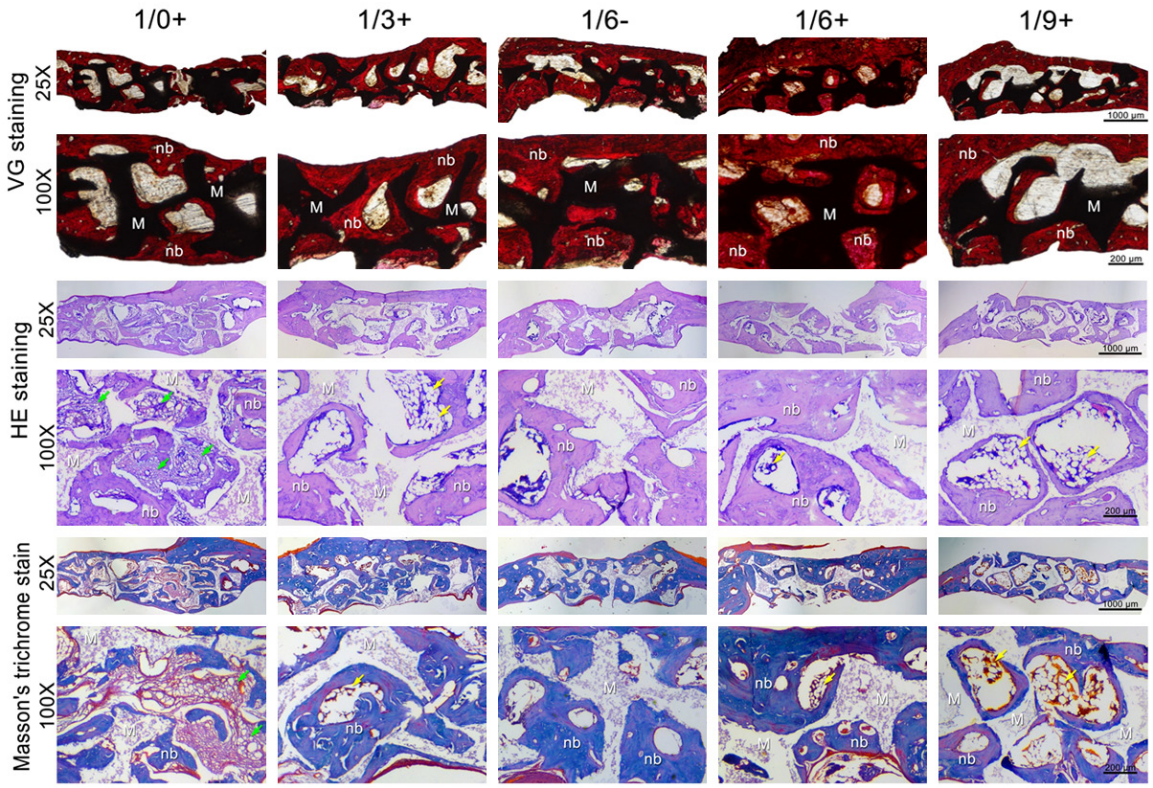


Figure 6. Undecalcified orthotopic bone sections stained with VG, and decalcified sections by HE and Masson's trichrome staining (M: material; nb: new bone; green arrow: fibrous structure; yellow arrow: medulla ossium flava). VG staining presented that at 12 weeks post-implantation, all groups achieved bridging of the defect with new bone ingrowth into the macropores of CPC scaffold. Specifically, the most intensive newly formed bone tissue which completely filled the macropores of the scaffold was observed in 1/6+ group. Boneless cavities were significantly larger in 1/0+ and 1/9+ groups, and the tissue composition of the cavities were further identified via HE and Masson's trichrome staining, which provided more detailed information of the regenerative tissue. The regenerated bone tissue of 1/0+ was significantly less than 1/3+ and 1/6+, and extensive fibrosis was observed in the boneless areas. 1/6+ group exhibited a highest degree of endochondral ossification and bone maturity at week 12 (redness in Masson's trichrome staining indicated high maturity of mineralization). In contrast, bone marrow was observed in the cavities of 1/3+, 1/6+ and 1/9+ groups, whereas weakened maturity of mineralization and less bone marrow were observed in 1/6-. However, the largest cavities were observed in 1/9+.

CD206 (M2 marker) from 32.6% to 37.0%, which verified the anti-inflammation function of Dex. Moreover, the expression of CD197 in Dex group was much less than that in BMP-2 and BMP/6Dex groups. Additionally, the pro-inflammation related genes (IL-1β and IL-6) and anti-inflammation related genes (Arg1 and IL-10) were detected (**Figure 7B-E**). BMP-2 significantly enhanced the gene expression of IL-1β and IL-6, and BMP/6Dex group significantly enhanced the gene expression of Arg1 and IL-10 compared to other groups. Specifically, the introduction of Dex (BMP-2/6Dex) sharply decreased the expression of IL-1β and IL-6. To conclude, the incorporation of Dex in BMP/6Dex group suppressed M1 phenotype polarization of macrophages and the expression of pro-inflammation related genes compared to BMP-2 group, and facilitated the expression of anti-inflammation related genes. Moreover, adequate immunomodulation by Dex could facilitate cartilage formation and ensure endochondral ossification, as previously reported [12].

Pilot clinical trial

Both ectopic and orthotopic animal models demonstrated the enhanced osteogenic capacity of the BMP-2-loaded CPC scaffolds with adequate dosage of Dex and blood clot embedment. To evaluate the clinical therapeutic performance of Dex/blood/BMP-2/CPC scaffold, a clinical trial was conducted in Guizhou

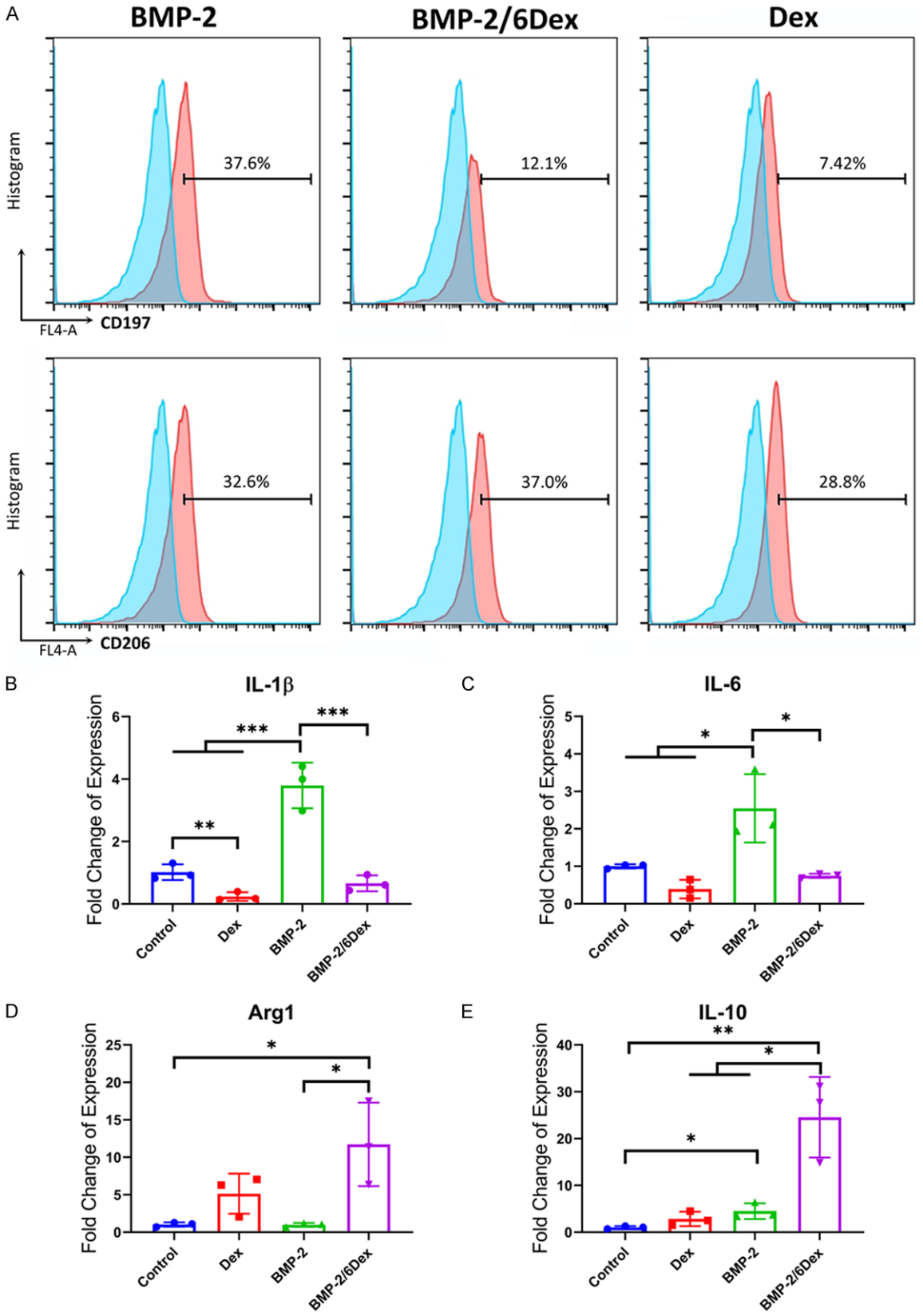


Figure 7. The effect of Dex on macrophage polarization. A. Macrophage subpopulations characterized by flow cytometry. The introduction of Dex decreased M1 marker CD197 expression from 37.6% to 12.1%, and increased M2 marker CD206 expression from 32.6% to 37.0%, verifying the anti-inflammatory function of Dex. Moreover, CD197

expression in Dex group was significantly suppressed compared to BMP-2 and BMP/6Dex groups. B-E. Expressions of typical proinflammatory (IL-1β, IL-6) and anti-inflammatory genes (Arg1, IL-10). BMP-2 significantly enhanced the expression of IL-1β and IL-6, whereas BMP/6Dex significantly upregulated the expression of Arg1 and IL-10 and sharply weakened the expression of IL-1β and IL-6.

Provincial People's Hospital. All components of BMP/Dex/blood/CPC scaffold were commercially and clinically applicable products. 5 tibial plateau fractures and 5 proximal humeral fractures were enrolled. Demographics and clinicopathologies of the patients were listed in **Table 2**. The statistical results were collected and compared to the clinical performance of BMP-2-loaded CPC scaffold in our previous study [11].

7-10 days post-operation, skin wound healing at the surgical incisions was acquired in all cases, with no wound exudation, toxic effect, skin rash or high fever occurred, and liver and kidney functions, routine blood and urine tests and C-reactive protein of all patients were normal. In comparison, in our previous study, in a few cases applying BMP-2-loaded CPC scaffolds without dexamethasone/autologous blood clot, wound exudation occurred and it delayed wound healing. In this study, the Dex/blood/BMP-2/CPC scaffold could attenuate immune inflammation and avoid wound exudation at the early stage by local release of Dex, without potential negative effects of systemic administration.

All cases were followed up for 12-24 months. During follow-up, no osteomyelitis, fractures, or obvious collapses after the bone defect repair was observed, and no plate and screw loosening or other complications occurred. All patients achieved clinical healing by 3 months post-surgery, which was accelerated compared to our previous study applying BMP-2-loaded CPC scaffolds. The average function scores and the excellent rates of function scores at each time point were also higher than BMP/CPC scaffold in previous study, indicating shortened repairing time and higher repairing qualities by the Dex/blood/BMP-2/CPC scaffold treatment proposed in this study. 3 months post-surgery, the excellent and good rates of the tibial plateau and proximal humeral fractures were 60% and 80% respectively, while they were 25% and 57% at the same time point in our previous study [11]. By 6 months post-surgery, all patients achieved bony reunion and the excellent and good rates were 100%.

Two typical cases of tibial plateau fracture and proximal humeral fracture were selected and shown in **Figure 8**. In the typical case of the tibial plateau fracture (**Figure 8A**), at 1-month post-operation, the anatomic shapes of the bone defects were recovered after surgery, and the bone filler materials were closely integrated with the host bone with flurry fracture lines at the interface, and the fracture lines were invisible after 6 months. In our previous study [11], tibial plateau fracture defects repaired by BMP-2/CPC scaffolds, fracture lines were clearly visible 1 month post-operation, became fuzzy at month 2, progressively faded and disappeared at month 4. In the typical case of the proximal humeral fracture (**Figure 8B**), fuzzy fracture lines were observed at 1-month post-operation, and disappearance of the fracture lines was observed in 3 months. In our previous study [11], proximal humeral fracture repaired by BMP-2/CPC scaffolds did not exhibit fuzzy fracture lines until month 2. The accelerated healing of bone fracture was in accordance with the preclinical results that the introduction of Dex and blood clot facilitated rapid bone regeneration in the early stage. The above results demonstrated the superior therapeutic efficiency of the Dex/blood/BMP-2/CPC scaffold treatment than previously reported BMP-2/CPC scaffold.

Delayed healing and nonunion still remained a great challenge that hampered the development of clinical bone regenerative treatment. BMP-2/CPC scaffold was previously developed to address this issue and had successfully applied for many bone defect patients. However, due to individual differences in immune microenvironment, undesired inflammatory responses including wound exudation and delayed wound healing were observed in some individual cases applying BMP-2/CPC scaffolds. Dex was found to regulate inflammatory response and synergize BMP-2-mediated endochondral bone formation. Hence, a Dex-loaded blood clot-embedded BMP-2/CPC scaffold was

Table 2. Patients' information, AO fraction classifications, and functional scores (applying Iowa knee and ankle score in cases of tibial plateau fractures, and Neer shoulder score in cases of proximal humeral fractures)

Defect site/score system	Gender	Age	Average age	AO Fracture Classification	Wound exudation, toxic effect, skin rash or high fever	1 month	Average score/ Excellent and good rate	3 months	Average score/ Excellent and good rate	6 months	Average score/ Excellent and good rate
Tibial plateau (IOWA score)	M	45	42	41-B3	/	40	37±4.58 (0%)	81	80±2.24 (60%)	93	91±1.58 (100%)
	M	38		41-B3	/	32		77		90	
	M	48		41-B2	/	41		83		92	
	F	41		41-B2	/	32		80		89	
	F	36		41-B3	/	40		79		91	
Proximal humerus (Neer score)	M	33	39	11-C3	/	32	37±3.87 (0%)	81	80.6±3.51 (80%)	94	94.6±1.14 (100%)
	F	37		11-B3	/	34		75		95	
	F	42		11-C2	/	41		80		93	
	F	43		11-B3	/	40		84		95	
	M	42		11-B3	/	38		83		96	

The 5 tibial plateau fracture patients included 3 males and 2 females, with an average age of 42, and the AO fracture classification of B2 or B3. The 5 proximal humeral fracture patients included 2 males and 3 females, with an average age of 39, and the AO fracture classification of B3, C2 or C3. After the fracture was reduced and the displacement corrected, the collapse of the cancellous bone was filled with BMP-2-loaded CPC scaffolds and dexamethasone/autologous blood mixture. 3 months post-surgery, the excellent and good rates of the tibial plateau and proximal humeral fractures were 60% and 80% respectively, while they were 25% and 57% at the same time point in our previous study. By 6 months post-surgery, all patients achieved bony reunion and the excellent and good rates were 100%.

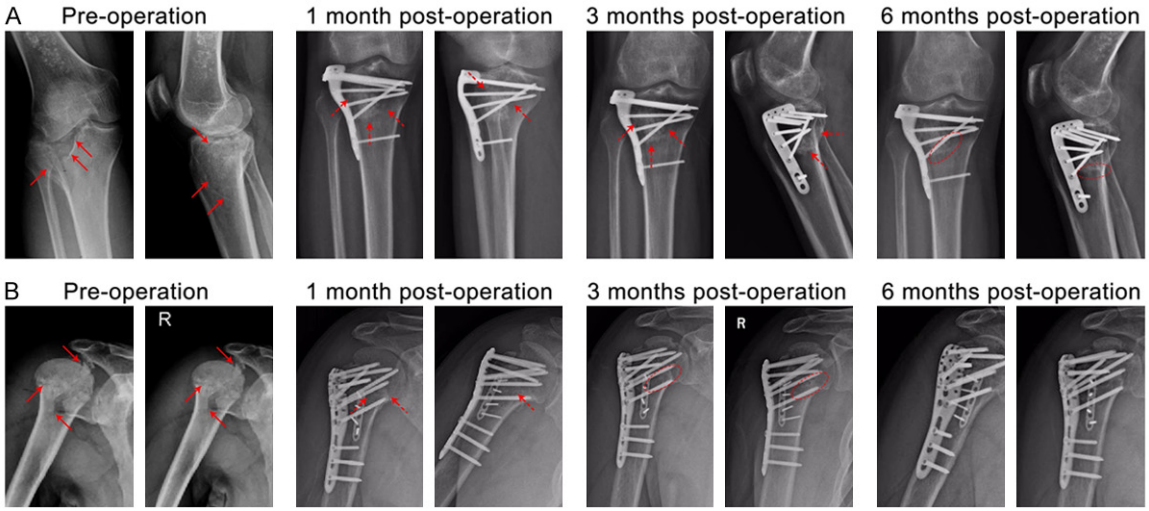


Figure 8. Preliminary clinical study of the Dex/blood/BMP-2/CPC scaffold: typical cases of (A) tibial plateau fracture and (B) proximal humeral fracture. Red solid arrows indicated the fracture lines; red dashed arrows indicated fuzzy fracture lines, which were considered as clinical healing; red dashed circles indicated the disappearance of the fracture lines, which was considered as bony reunion. In the typical case of the tibial plateau fracture, at 1-month post-operation, the anatomic shapes of the bone defects were recovered after surgery, and the bone filler materials were closely integrated with the host bone with flurry fracture lines at the interface, and the fracture lines were invisible after 6 months. In the typical case of the proximal humeral fracture, fuzzy fracture lines were observed at 1-month post-operation, and disappearance of the fracture lines was observed in 3 months.

designed to attenuate inflammation and accelerate bone regeneration in clinical treatment. As anticipated, blood clot embedment realized sequential release of Dex and BMP-2 from the Dex/blood/BMP-2/CPC scaffold. The rapid release of Dex within the first 48 h promoted M2 polarization of macrophages, alleviated local inflammation, and reduced ectopic bone resorption and fibrosis of orthotopic defect site. The residual Dex released after 48 h from the blood clot embedment synergized the BMP-2-mediated osteogenic efficacy, as proven both *in vitro* and *in vivo*. The immunoregulatory efficacy and enhanced osteoinductive activity of the Dex/blood/BMP-2/CPC scaffold were further verified in a preliminary clinical study. Compared to our previously reported cases applying BMP-2/CPC scaffold, Dex/blood/BMP-2/CPC scaffold accelerated the healing of fracture, exhibited higher excellent and good rates in all time points, and exhibited no local inflammation in all cases. Taken together, this work had designed an immunoregulatory and osteoinductive Dex/blood/BMP-2/CPC scaffold with significant improvement in therapeutic efficiency compared to current bone regenerative treatment, which might pave the way for future bone repairing material development and arouse broad interests among researchers in the fields of bone regenerative therapy.

Conclusion

In this study, an immunoregulatory and osteoinductive Dex/blood/BMP-2/CPC (BMP-2/Dex dose ratio of 1/6) scaffold was fabricated with sequential release of Dex and BMP-2. With the blood clot embedment, a large proportion of Dex released rapidly and promoted M2 polarization of macrophages. The residual Dex released over a longer period exerted synergetic effect on BMP-2-mediated osteogenesis. And the BMP/Dex/blood/CPC scaffold facilitated ectopic bone formation and orthotopic bone regeneration. Moreover, the preliminary clinical study verified the improved therapeutic performance of the Dex/blood/BMP-2/CPC scaffold in bone defect regeneration. To conclude, the Dex/blood/BMP-2/CPC scaffold combined immunoregulatory efficacy and enhanced osteoinductive activity, providing new insights for advancing current bone defect treatment and a clinically applicable strategy of sequential drug delivery.

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Disclosure of conflict of interest

None.

Abbreviations

BMP-2, Bone morphogenetic protein-2; CPC, Calcium phosphate cement; Dex, Dexamethasone; BMSC, Bone marrow stomal cells; FDA, Food and Drug Administration; NP-40, Nonidet P-40; PNPP-Na, P-Nitrophenyl phosphate; FBS, Fetal bovine serum; rBMSCs, Rat bone marrow stromal cells; NIH, National Institutes of Health; ALP, Alkaline phosphatase; RT-qPCR, Reverse transcription-polymerase chain reaction; cDNA, Complementary DNA; Runx2, Runt-related transcription factor 2; OCN, Osteocalcin; OPN, Osteopontin; Col I, Type 1 collagen; PBS, Phosphate puffer solution; IL-1β, Interleukin-1β; IL-6, Interleukin-6; IL-10, Interleukin-10; Arg1, Arginase 1; SEM, Scanning electron microscope; XRD, X-ray diffraction; EDS, Energy dispersive spectroscopy; HPLC, High-performance liquid chromatography; SRμCT, Synchrotron radiation-based micro-CT; SSRF, Shanghai Synchrotron Radiation Facility; ROI, Region of interests; BV/TV, Bone volume/tissue volume; Tb.Th, Trabecular thickness; TE, Tetracycline; AR, Alizarin red; CA, Calcein; CLSM, Confocal laser-scanning microscopy; PMMA, Polymethyl methacrylate; VG, Van Gieson's picro fuchsin; EDTA, Ethylene diamine tetraacetic acid; HE, Hematoxylin/eosin; ANOVA, Analysis of variance; AO, Arbeitsgemeinschaftfür osteosynthesefragen.

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Table S1. Primer sequences used in real-time PCR analysis

Primer	Sequences
Runx2-for	GCTTCTCCAACCCACGAATG
Runx2-rev	GAACTGATAGGACGCTGACGA
Col I-for	TGGATGGCTGCACGAGT
Col I-rev	TTGGGATGGAGGGAGTTTA
OCN-for	GCCCTGACTGCATTCTGCCTCT
OCN-rev	TCACCACCTTACTGCCCTCCTG
OPN-for	CCAAGCGTGGAAACACACAGCC
OPN-rev	GGCTTTGGAACTCGCCTGACTG
IL6-for	ATAGTCCTTCCTACCCCAATTTCC
IL6-rev	GATGAATTGGATGGTCTTGGTCC
IL-1β-for	GTATGGGCTGGACTGTTTC
IL-1β-rev	GCTGTCTGCTCATTCACG
Arg1-for	CAGAAGAATGGAAGAGTCAG
Arg1-rev	CAGATATGCAGGGAGTCAC
IL10-for	GAGAAGCATGGCCCAGAAATC
IL10-rev	GAGAAATCGATGACAGCGCC
GAPDH-for	TGACCACAGTCCATGCCATC
GAPDH-rev	GACGGACACATTGGGGGTAG
β-actin-for	CACCCGCGAGTACAACCTTC
β-actin-rev	CCCATACCCACCATCACACC

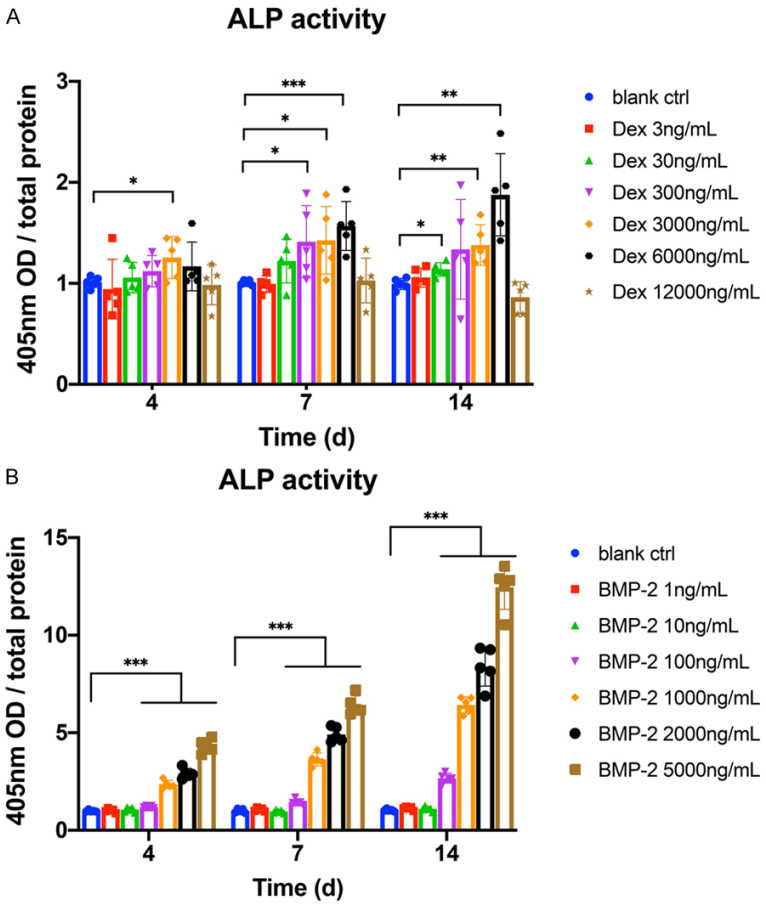

Figure S1. ALP activity of BMSCs induced by BMP-2 and Dex at different concentrations. Dex at the concentrations of 30-6000 ng mL^{-1} exhibited a certain but limited osteoinductivity, with a highest ALP activity at 14 days which was only ~2 times of the blank control group, while Dex at a high concentration (12 μg mL^{-1}) decreased its ALP activity. BMP-2 at concentration above 1 μg mL^{-1} efficiently induced osteogenic differentiation for 4 days, and the ALP activity was dose-dependent with BMP-2 concentration. Though higher dose of BMP-2 resulted in significantly enhanced osteogenic differentiation, the increasing dose was also accompanied by high cost and high risk of side effects.

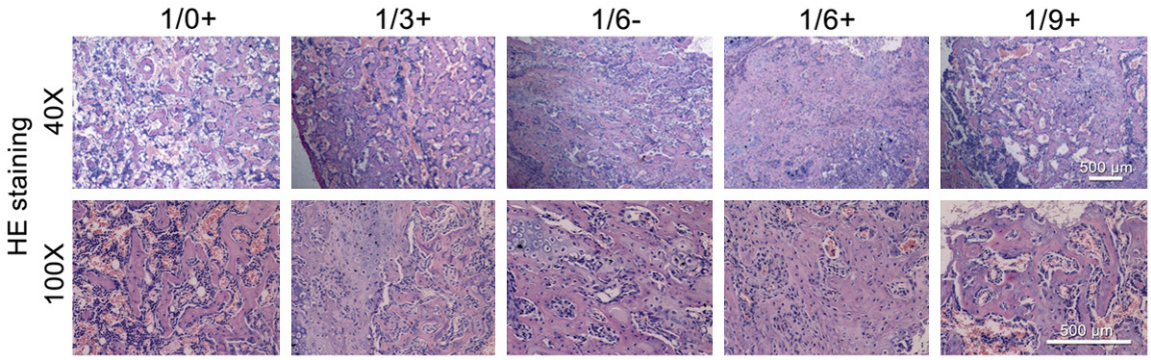

Figure S2. HE staining of ectopic bone sections at 2 weeks post-implantation.

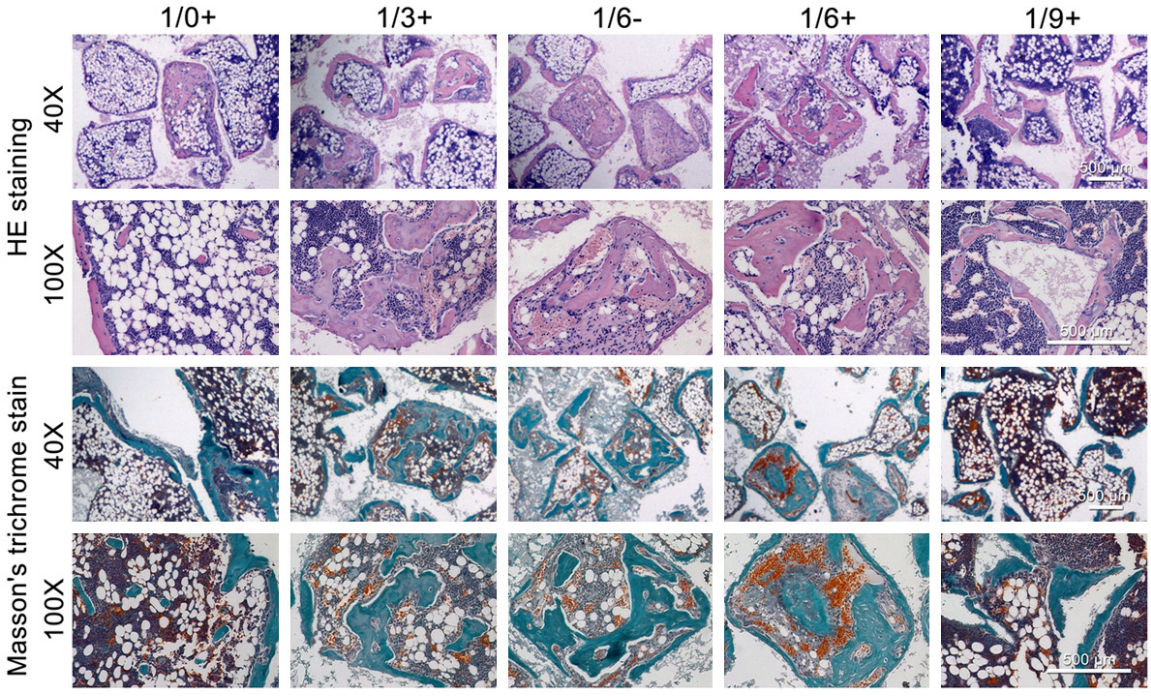

Figure S3. HE and Masson's trichrome staining of ectopic bone sections at 4 weeks post-implantation.