Injectable nanofiber-reinforced bone cement with controlled biodegradability for minimally-invasive bone regeneration

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ABSTRACT

Injectable materials show their special merits in regeneration of damaged/degenerated bones in minimally-invasive approach. Injectable calcium phosphate bone cement (CPC) has attracted broad attention for its bioactivity, as compared to non-degradable polymethyl methacrylate cement. However, its brittleness, poor anti-washout property and uncontrollable biodegradability are the main challenges to limit its further clinical application mainly because of its stone-like dense structure and fragile inorganic-salt weakness. Herein, we developed a kind of injectable CPC bone cement with porous structure and improved robustness by incorporating poly(lactide-co-glycolic acid) (PLGA) nanofiber into CPC, with carboxymethyl cellulose (CMC) to offer good injectability as well as anti-wash-out capacity. Furthermore, the introduction of PLGA and CMC also enabled a formation of initial porous structure in the cements, where PLGA nanofiber endowed the cement with a dynamically controllable biodegradability which provided room for cell movement and bone ingrowth. Interestingly, the reinforced biodegradable cement afforded a sustainable provision of \( \text{Ca}^{2+} \) bioactive components, together with its porous structure, to improve synergistically new bone formation and osteo-integration in vivo by using a rat model of femur condyle defect. Further study on regenerative mechanisms indicated that the good minimally-invasive bone regeneration may come from the synergistic enhanced osteogenic effect of calcium ion enrichment and the improved revascularization capacity contributed from the porosity as well as the lactic acid released from PLGA nanofiber. These results indicate the injectable bone cement with high strength, anti-washout property and controllable biodegradability is a promising candidate for bone regeneration in a minimally-invasive approach.

1. Introduction

Treatment of irregular bone defect and comminuted fracture, due to population aging, sports injury and traffic accident, remain as common clinical challenges. Due to the accumulating needs for faster healing after operation, the clinical demand for injectable biomaterials is increasing, which can allow for regeneration of diseased/injured bone tissue with minimally-invasive strategies [1–4]. Currently, poly (methyl methacrylate) (PMMA) is the most commonly used injectable material for bone supporting and pain relief after osteoporosis vertebral fracture [5–7]. Although PMMA has high mechanical strength, its drawbacks such as non-degradability, residuary monomer toxicity, lack of

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bioactivity as well as too high modulus mismatchable strength, leading to osteoporosis complications and bone re-fracturing which greatly impede its translational use in bone defect treatment [5,8]. Therefore, it is urgent and valuable to develop advanced injectable bone substitute biomaterial with good bioactivity for bone regeneration.

Calcium phosphate cement (CPC) developed in the 1980s is regarded as an attractive bone substitute material, due to its excellent biocompatibility and arbitrarily plasticity [9–13]. However, the application of traditional CPC is usually limited by its own brittleness, mismatched balance between the CPC degradation and the new bone regeneration, and poor washout resistance [14–16]. To address the shortcomings of pure CPC, a major strategy is to integrate absorbable polymers into CPC [17]. Poly(lactide-co-glycolic acid) (PLGA), authorized by the US Food and Drug Administration (FDA), is frequently used to regulate the degradation rate of implants to meet clinical needs, due to the property of controllable degradation by regulating the monomer ratio [19–21]. PLGA degradation produces lactic acid (LA) and glycolic acid (GA), which could be metabolized and absorbed by the human body. A high level of LA can make the local microenvironment become weakly acidic, which can facilitate the degradation of CPC. Furthermore, it has been reported that LA promotes vascular growth, which is important for bone regeneration [22–24]. It was indicated that in situ pore formation of implants for bone ingrowth by adding PLGA microspheres, but it still is difficult to form connected pores [25]. It was reported that the strength of CPC could be enhanced by adding PLGA medical sutures to form connected pores after polymer fiber degradation, whereas this type of CPC could not be injected because of the excessively long fibers [26]. Short PLGA fibers were used to improve the brittleness of CPC and concluded that only the fibers with length less than 1 mm could be used to obtain better injectability [27]. The supporting effect of fiber is subjected to its aspect ratio, therefore the reinforced effect of short fiber on CPC strength is extremely limited [27,28]. The low biodegradability of CPC could be greatly improved by incorporating rapidly degradable salts into injectable CPC, which was able to regulate the immune microenvironment of the bone defect [29,30]. Biopolymers were often preferred for improving the injection properties of CPC as well as their anti-washout abilities, but the addition of biopolymers often results in reduced strength of CPC [31,32]. The addition of non-degradable high-strength fibers can significantly improve the toughness and strength of CPC, but their low biodegradability prevents the new bone from growing in [33]. This study will improve the low biodegradability and poor toughness of CPC by adding PLGA nanofibers. Furthermore, the in situ pore formation with the addition of PLGA in CPC promoted the growth of new bone and vascularization property. In addition, the higher the molecular weight of the polymer, the higher the fiber strength [34], which leads to better reinforcement effect on the CPC. However, when the $M_w$ of PLGA was 500 kDa, the viscosity of the polymer solution was too large to lead to airbrush clogging, so the PLGA with $M_w = 400$ kDa was chosen for this study.

Besides mechanical strength and biodegradability, anti-washout property is an important technical determinant for cement used in orthopedic surgery. In order to maintain the cement integrity in the environment of body fluids, the addition of natural polymers to the liquid or solid phase of CPC is a common procedure for enhancing its low cohesive energy [35]. More importantly, previous study has found that it could drastically improve injectability and anti-washout properties by adding a small amount of carboxymethyl cellulose (CMC) [36]. Furthermore, the long-term biocompatibility of CMC has been confirmed in vivo [37]. It is therefore proposed that a combination of PLGA, CPC and CMC can provide a therapeutic tool to significantly facilitate the clinical application, via its improved mechanical strength, timely and controllable degradation, anti-washout property, and injectability. Herein, a new design strategy was proposed to develop a PLGA nanofiber-reinforced injectable bone cement (C/PL/C) in the present study, and the acronyms of different components of the bone cements were used such as calcium phosphate cement/carboxymethyl cellulose (CPC/CMC, C/C), calcium phosphate cement/2.5 wt% poly(lactide-co-glycolic acid) (CPC/2.5 wt% PLGA, C/PL-2.5) and calcium phosphate cement/10 wt% poly(lactide-co-glycolic acid)/carboxymethyl cellulose (CPC/10 wt% PLGA/CMC, C/PL/C-10). PLGA nanofibers increased the strength of CPC and reduced its brittleness, and the degradation of PLGA nanofibers increased the porosity and pore size of CPC. The bone cement showed good anti-washout property and injectability by adding CMC. The physicochemical characteristics of C/PL/C, including pore size variation, degradability, and ion release, were examined. Further, the mechanism involved in the osteogenic differentiation of bone marrow mesenchymal stromal cells (BMMCs) was explored, followed with the osteo-integration and bone regenerative in vivo. Our results suggest that C/PL/C can be the next generation of injectable bone regenerative material (shown in Scheme 1).

## 2. Materials and methods

### 2.1. Fabrication of injectable nanofiber-reinforced CPC

1.1 g poly(lactide-co-glycolic acid) (PLGA, 85:15 lactide: glycolide, $M_w = 400$ kDa) was dissolved in 20 mL dichloromethane (DCM, Taitian, China) by magnetic stirring for 2 h at 20 °C. Then a commercial airbrush (HD-150, Syou Tools, China) was used to airbrush the solution (2 MPa air pressure, 0.5 mm-diameter nozzle). The distance from receiver (wrapped in aluminum foil) to nozzle of airbrush was 30 cm. Then the collected fibers were placed in a vacuum drying oven at 40°C to remove the solvent for 24 h. Next, they were embrittled with liquid nitrogen for 5 min and crushed with mortar immediately. Finally, the short nanofibers (Fig. S1) were sieved out with screens. Calcium phosphate cement powder (Rebone, China) was composed of equimolar tetracalcium phosphate (TTCP, $C_a(P_2O_5)_2$) and dicalcium phosphate anhydrous (DCPA, CaHPO$_4$). CPC, PLGA nanofibers and carboxymethyl cellulose (CMC, Macklin, China) were mixed and stirred in a small beaker, meanwhile, the mixture was ultrason for 5 min. Then pastes were fabricated by mixing the solid-phase composite and 4 wt% NaH$_2$PO$_4$.H$_2$O (Yonghua, China), at a 2:1 powder-liquid ratio. The composition of cements was listed in Table S1.

### 2.2. Evaluation of handling properties of the reinforced cements: setting time, injectability and anti-washout ability

The paste was mixed from 1.2 g of powder for each kind of cements and 0.6 mL 4 wt% NaH$_2$PO$_4$.H$_2$O, which was then injected into Teflon molds (Ø 6 × 12 mm). Cements were placed in a chamber (100% humidity and 37 °C) for curing. According to the ASTM Test Method C 187-98, the setting time of cement samples was tested by Vicat apparatus. The cement was tested every 0.5 min to indicate whether curing or not [37]. Each experiment was repeated three independent times.

According to a previously reported method, the injectability of the CPC was analyzed [38]. Each paste was prepared by mixing the solid phase and liquid for 1 min with a plastic knife and then filled in the 2.5 mL syringe (Kangli, China) for injectability measurement. Injectability was calculated based on the mass percentage of CPC by fully extruding, and the extrusion time remained consistent (the time from solid-liquid mixing to cement to complete extrusion), according to equation (1).

Each experiment was performed three times.

$$I = \frac{M_b - M_{b0}}{M_b}$$

where $I$ refers to injectability, $M_b$ corresponds to the mass of CPC paste which remained in the syringe and cannula after extrusion and $M_{b0}$ corresponds to the mass of CPC paste before extrusion.

The prepared CPC, C/C and C/PL/C-10 past were injected manually into 37 °C phosphate-buffered saline (PBS) solution. Then the samples were placed in a constant temperature shaker and shaken at 120 rpm for...
60 min at 37°C. If the cements did not obviously disintegrate in PBS, the specimens were considered to pass the anti-washout test [39].

2.3. Physicochemical characterisation

2.3.1. Chemical phase composition and surface morphology

The cement specimens were examined using Fourier infrared spectrometer (FTIR, Nicolet is50) and X-ray diffraction (XRD, Rigaku D/Max2550, Cu Karadation, Japan). Surface morphological features of the cement, the energy dispersive spectrometer (EDS) elements analysis and mapping of carbon, calcium and phosphorus on samples surfaces were characterized by scanning electron microscope (SEM, S-4800, Hitachi, Japan).

2.3.2. Evaluation of mechanical properties of cements in vitro

Each sample \((n=5)\) was prepared by mixing vigorously the powders and curing fluids for 60 s until uniformity was achieved. Then these pastes were injected into molds. Cylindrical Teflon molds \((6 \times 12 \text{ mm})\) were selected for the compression experiment. After extrusion of the paste, all specimens were placed to cure for 1 day at room temperature. After setting, each sample was de-molded and undesirable samples (presence of cracks and defects) were removed from the experiment. All cements were polished uniformly on both sides and immersed in PBS solution, which were placed at a constant temperature shaking box (120
Cylindrical samples were tested at a loading speed of 1 mm/min using a universal testing machine (SANS CMT 2503, MTS Industrial Systems, USA). The compressive work-of-fracture \( W_c \) was calculated by the curves of the compression load-displacement, as shown in equation (2). In order to allow for a quantitative comparison of \( W_c \) when the specimen reached a compressive strain of 20%, mechanical tests were stopped. Moreover, the compressive modulus and compressive strength were derived from the slope and the highest point of the stress-strain curves respectively [33].

\[
W_c = \frac{\Omega_s}{A_0^{\frac{1}{l}}}
\]

where \( W_c \) corresponds to the compressive work-of-fracture, \( \Omega_s \) corresponds to the area under the load-displacement curve, \( A_0 \) refers to the original cross-sectional area of specimens and \( l \) indicates the sample’s height.

2.3.3. Ion release of cements

The dynamic release of the Ca\(^{2+}\) from C/PL/C was studied. In brief, the samples of all groups (n = 3) were immersed in 5 mL PBS solution (pH = 7.4). The soaking solution would be collected every certain time. The concentration of Ca\(^{2+}\) of immersion solution was measured with inductively coupled plasma atomic emission spectrometer (ICP-AES, PerkinElmer Optima 2000, USA).

2.3.4. pH values of cement-soaking solution

The pH of the solution after the sample immersing is monitored was 7.4). After 1 day, 3 days, 5 days, 7 days, 14 days, the soaking solution was collected. The concentration of Ca\(^{2+}\) in 5% CO\(_2\) was measured with iCAP-Q inductively coupled plasma atomic emission spectrometer (ICP-AES, PerkinElmer Optima 2000, USA).

2.3.5. Assessment of in vitro degradation of cements

The specimens of all groups (n = 3) were soaked in Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) solution (pH = 7.4) at 37 °C. The solution was refreshed every 4 days. At various time points, the cements were removed from the solution, washed with de-ionized water and dried in a blast dryer at 60 °C for 3 h. The percentage of weight loss was calculated as equation (3).

\[
\text{Weight loss(\%)} = \frac{W_n - W_0}{W_0} \times 100 \%
\]

where \( W_0 \) indicates mass of undegraded sample, \( W_n \) indicates mass of the sample for the corresponding time of degradation.

2.3.6. Assessment of the porosity change of cements

Pore size distribution and porosity of the cement specimens (Ø 6 × 12 mm) were assessed by mercury intrusion porosimetry using Auto Pore 9510 porosimeter (Micrometrics, USA). Each sample was tested three times.

2.3.7. Determination of the LA concentrations

The release of the LA from C/PL/C-10 was studied. The samples (n = 3) were immersed in 5 mL PBS solution (pH = 7.4). After 1 day, 3 days, 5 days, 7 days, 14 days, the soaking solution was collected. The concentration of LA in soaking solution was measured with ion chromatography (IC, ICS-1100, USA).

2.4. In vitro response of bone marrow mesenchymal stromal cells (BMSCs) to C/PL/C-10 cements

2.4.1. Cell culture

BMSCs from Sprague-Dawley (SD) rat were acquired from Shanghai JieSiJie Laboratory Animals Co., LTD. BMSCs were grown in α-MEM with 10% fetal bovine serum (FBS, BI, USA) and 100 mg/mL penicillin and streptomycin (Sigma, USA). Generation 2nd - 3rd of BMSCs was incubated at 37 °C in saturated humidity and 5% CO\(_2\). To test the BMSC response, all cement samples were sterilized by soaking in 75% alcohol for 24 h.

2.4.2. Cell proliferation and adhesion

According to ISO 10993, CPC, C/C, C/PL/C-10 samples (10 mm × 3 mm) were immersed in α-MEM at 37 °C for 24 h to achieve the cement extracts in order to test the cytotoxicity. For live/dead staining assay, BMSCs were treated with the cement extracts for 1/4/7 days, and then replaced with the calcein-AM/PI double stain kit (Beyotime, China) for 30 min. The samples were observed and imaged by using an inverted fluorescence microscope (Olympus, IX71, Japan). To quantify cell proliferation, counting of the live/dead cells and calculation of the cell survival rate were performed according to the following formula:

\[
S_c = \frac{N_l}{N_l + N_d} \times 100 \%
\]

where \( S_c \) indicates Cell survival rate, \( N_l \) indicates the number of live cells, \( N_d \) indicates the number of dead cells.

Cell counting kit-8 (CCK-8, Beyotime, China) test was also utilized to assess the cell growth. BMSCs with the density of 5 × 10\(^4\) cells/well were seeded on 96-well plates (3 mm in height × 10 mm in diameter) of each group in α-MEM medium at 24-well culture plate. After culturing for 1/2/3 days, the medium were replaced with α-MEM medium comprising 10% CCK-8. Subsequently incubated at 37 °C in 5% CO\(_2\), the plate was read by a microplate reader (Epoch, BioTek Instruments, Inc.) to analyze the absorbance at 450 nm was measured. The cell medium free of cement sample were served as control. For SEM observation, BMSCs were seeded and incubated for 3 days in 24-well culture plates at 5 × 10\(^4\) cells per well on plate samples (10 mm × 3 mm) from each cement group. Afterward, the cells were fixed with 2.5% glutaraldehyde, dehydrated through a graded sequence of alcohol solutions and allowed to air-dry overnight. The samples were then analyzed with SEM (S-4800, Hitachi, Japan).

2.4.3. Osteogenic differentiation

For osteogenic differentiation, BMSCs were cultured in the α-MEM medium, and then the medium was replaced with osteogenic inducing medium (OIM). OIM were made from a culture medium with 0.05 mM vitamin C (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA) and 1 × 10\(^8\) M dexamethasone (Sigma, USA). Medium were exchanged every 2 days.

The BMSCs were cultured in 12-well plates with cement-extracts at 1 × 10\(^5\) cells per well in α-MEM and 10% FBS medium in the cell incubator. After the cell density reached 70–80% approximately, the α-MEM medium was switched to OIM and BMSCs were induced to differentiate for 7/14 days. After that, differentiation of BMSCs was tested with alkaline phosphatase (ALP) staining, ALP activity and alizarin red S (ARS) staining.

ALP activity was checked using an ALP assay kit (Beyotime, China) after 7/14 days of culture. The ALP activity results were standardized with total cell protein and the media containing CPC cement extracts were used as a control. After 7/14 days of culture, cell monolayers were washed twice with PBS and were processed with 10% neutrophilic formalin for 15 min. After washing twice with PBS, the samples were stained according to the protocol of the ALP and ARS assay kit and observed with a light microscope (Olympus, IX71, Japan).
2.4.4. Osteogenic-related protein levels

After culturing with cement extracts in OIM for 7 days, BMSCs were lysed on ice in RIPA buffer mixed with phenylmethylsulfonyl fluoride (PMSF, 100 mM). The lysates were centrifuged at 10,000 rpm for 10 min and the total protein concentration was detected by bicinchoninic acid (BCA) method [40]. Further, equivalent amounts of proteins were isolated through SDS-polyacrylamide gel electrophoresis (12.5% or 15%) and transferred onto a polyvinylidene fluoride membrane (PVDF, Millipore, Darmstadt, Germany). Following blocking with 5% skimmed milk for 1 h, the membrane was probed with the following primary antibodies: anti-GAPDH (1:1000), anti-osteopontin (OPN, 1:1000), anti-runt-related transcription factor 2 (Runx-2, 1:1000), anti-osteocalcin (OCN, 1:1000) at 4°C overnight. After being washed with Tris-HCl Buffer Saline and Tween (TBST), the membrane was incubated with secondary antibodies (HRP-labeled, 1:2000) for 1 h. After being washed with TBST, the protein bands were visualized, and quantification was performed using Image J.

2.4.5. Osteogenic-related gene levels

After 7 days of treatment, total RNA was extracted with a 1 mL Trizol RNA Extraction Kit (Invitrogen, Carlsbad, CA) and cell samples were harvested. RNA was reverse transcribed into complementary DNA with the Revert Aid First Strand complementary DNA Synthesis Kit (Thermo Fisher Scientific). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine the gene expression levels of ALP, OCN, Collagen type 1 (Col-1), Osterix, bone morphogenetic proteins 4 (BMP4), Runx-2 and human β-actin which was used as control reference. The sequences information is displayed in Table S2.

2.5. In vitro angiogenesis assay

In this study, human umbilical vein endothelial cells (HUVECs) were introduced as an in vitro model to evaluate angiogenesis. The HUVECs were starved overnight in HG-DMEM with 0.2% FBS. To evaluate angiogenic capacity, growth factor reduced Matrigel (BD Biosciences, San Jose, CA) was mixed with cement extract (1:1, v/v) in 24-well plates and polymerized for 1 h. Cells (1 × 10⁵ cells per well) were then inoculated on the Matrigel surface and incubated in the extract for 3 and 9 h. Microscopic images were taken at random by Image J with the Angiogenesis Analyzer plug-in (NIH, Bethesda, MD) to quantify the tubular network (parameters included number of primary nodes and total segment length).

The migration of HUVECs was identified by the monolayer wound experiment. HUVECs were seeded in 6-well plates with a density of 5 × 10⁴ cells/cm². The monolayer was scraped with a sterile pipette tip to create a cell-free wound zone after 100% cell converged. The cells were then treated with extracts, followed by microscopic evaluation of the wound width of the monolayer after 12 h.

qRT-PCR was performed to examine the gene expression levels of Vascular Endothelial Growth Factor (VGEF), CD 31 and human β-actin which was used as control reference after 3 days of co-culture. The sequences information is displayed in Table S2.

2.6. In vivo evaluation of the reinforced bone cements

2.6.1. Animal model

Experiment protocol was approved by the Animal Committee of Zhongshan Hospital, Fudan University (Shanghai, China). (Ethic approval number: Y2021-228). Thirty-three male rats (2 months old, 300 ± 30 g, Shanghai LASJ Laboratory Animals Co., Ltd., China) were housed separately in stainless steel cages at a temperature of 22°C. All rats were allowed to freely move, eat and drink after surgery. All animals received general anesthesia using pentobarbital sodium (0.1 mL/100 g, Sigma, USA).

The surgical area was shaved and disinfected with 75% ethanol. A 2 cm incision was made to reveal the femoral epiphysis and a surgical drill was then applied to create bone defects (3 mm in depth) in the femoral condyles. Solidified sterilized cement specimens (CPC, C/C, C/PL/C-10) were administered into the defect with a 1 mL syringe (Fig. 7B) and the layers of subcutaneous tissue were closed with absorbable sutures. The rats were subsequently given an intramuscular injection of antibiotics (penicillium, Sigma, USA) for three days at a dose of 40 mg/kg to minimize the risk of pre-operative infection. Further fluorochrome labels were administered at 17 days before sacrificing (30 mg/kg, alizarin, Beyotime, China) and at 3 days before sacrificing (10 mg/kg, calceine, Beyotime, China). Excessive amounts of pentobarbital sodium were given 6 and 12 weeks after implantation. Nine rats were sacrificed at each postoperative time point and the bilateral femoral condyles were fixed in 10% formalin solution (Servotecbio, China) for subsequent experiments. Six rats were sacrificed at 3 days after the surgery and the bilateral femoral condyles were harvested and fixed in 10% formalin solution (Servotecbio, China) for the push-out tests.

To evaluate the angiogenesis effect of cements, a rat model of subcutaneous implantation was developed. Briefly, 12 adolescent male rats (2 months old, 300 ± 30 g) were anesthetized by pentobarbital sodium. After shaving the dorsal spine, a small incision is made on each side of his dorsal spine and extended under the skin with forceps. Two cement cylindrical molds (6 mm in diameter × 12 mm in height, CPC, C/C, C/PL/C-10) were implanted subcutaneously on their dorsal sides and the wounds were then closed with absorbable sutures. Experiments in each cement group were carried out independently on 4 rats. Eight weeks after implantation, the animals were sacrificed with an overdose of sodium pentobarbital for histological analysis.

2.6.2. Push-out test

The cortical bone on the corresponding surface was abraded from the femoral condyles of rats at different times after surgery, then the push-out test was performed using a CMT2503 universal testing machine to assess the strength of the interfacial bond between bone and cement (Fig. 7G). An aluminum rod with a cylindrical tip of 3 mm diameter was used as the push-out tool and tested at a crosshead speed of 2 mm/min. The experiment was stopped when the cement detached or the bone-cement interface fractured, and the highest point of the curve of force-displacement was the push-out force.

2.6.3. Biocompatibility

Harvested viscera including heart, kidney, liver, lung and spleen were kept in 10% formalin for 24 h at 12 weeks after the surgery, and then dehydrated by different grade ethanol. Sections of 50 μm thickness were allotted for tissue staining after embedding of methyl methacrylate (Biotecnology, China) till solidification. Hematoxylin-eosin staining (H&E staining) was performed for histological observation.

2.6.4. Micro-computed tomography (Micro-CT) analysis

The bilateral femoral condyles of the rats were treated for 24 h in 10% formalin of fixing. The reconstruction of femoral condyles were evaluated by Micro CT (Bruker SkyScan1172, Belgium, voltage 100 KV, current 100 μA, pitch 2, filter 0.11 mm Cu). The relevant program (Micro View) was utilized to visualize and analyze the open areas of new osteophytes to the cement. Postoperative bone formation was assessed by measuring bone mineral density (BMD), the ratio of new bone volume to tissue volume (BV/TV) and bone trabecular pattern factor (Tb.pf).

2.6.5. Histological analysis

The harvested bilateral femoral condyles were fixed and then rehydrated in grades of ethanol. Following dehydration, the samples were embedded in paraffin. Following embedding of methyl (Biotecnology, China) till solidification, 300 μm thick sections were allotted for hard tissue staining. Hematoxylin-eosin staining (H&E staining), Verhoff-Van Gieson staining (V&G Staining) and toluidine blue staining were carried out for histological analysis. Immunohistochemistry staining was performed on the harvested subcutaneously samples using standard
protocols on the paraffin sections stained with primary antibodies to CD 31 (1:800, Beyotime, China) followed by counterstaining with hematoxylin and the positive stained cell numbers in the specimen were calculated and compared [36,41].

2.7. Statistical analysis

The data are presented as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was performed in SPSS 20.0 software to analyze statistical differences. The level of significant difference is set as $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Structure and morphology of the composite cements

The SEM micrograph showed that after 72 h of curing, the pore size was significantly increased in samples containing PLGA and CMC. Some individual nanofibers were mixed well in the CPC matrix, but the fiber bundles could be clearly separated from the CPC so the cement would form micron-size holes (Fig. 1A). Furthermore, EDS mapping revealed that homogenous distribution of PLGA nanofibers in the cement (Fig. S2). In order to more accurately reflect the influence of the CMC and PLGA on the pore formation, mercury intrusion porosimetry was used to evaluate the pore size of the sample. The curve shape of the pure CPC was sharp, and the pore size was concentrated at 62.6 nm (Fig. 1B). The pore size distribution of the CPC cement changed from a sharp peak to a broad flat peak after adding CMC, and the pore size distribution was 69.0–349.6 nm. There was an extra shoulder in the C/PL/C-10 group at 551.8 nm compared with the C/C group. In addition, C/PL/C-10 had significantly more micron-sized pores compared to that in the fiber-free group (i.e., CPC and C/C), and these pore structures would accelerate the degradation of the composite bone cement [42].

The infrared absorption spectrum showed that, except for the 1757 cm$^{-1}$ carbonyl group in C/PL/C-10, the other peaks were essentially the same in the three groups, indicating that PLGA was only blended into CPC without chemical reaction. The peak between CPC and C/C was the same, indicating that the CMC was not in the cement system when the soaking liquid dissolved during the curing process (Fig. 1C). The phase composition of each sample after curing was shown in Fig. 1D. A broad peak centered about $2\theta = 17.5^\circ$ which was long LA chain segment in the polymer chain segment of PLGA, but overall PLGA was still an amorphous polymer demonstrated by a broad diffraction peak at $2\theta$ value of 22.0$^\circ$. The XRD peaks of all three groups showed the typical peak of Hydroxyapatite (HA) and unhydrated TTCP [43], but the peak of HA in C/PL/C-10 was significantly weaker than that in the other groups, and the relative intensity between HA and TTCP was significantly reduced, which might be due to inhibitory effect of PLGA nanofiber on the
hydration of CPC and crystallinity of HA [44]. Moreover, because of the low content of PLGA, the diffraction peak of LA is not obvious in C/PL/C-10.

3.2. Physical and chemical of the composite cements

Firstly, we investigated the influence of different PLGA and CMC contents on the anti-washout performance of cement (Supplementary Video 1) CPCs became fragmented after shaking for 60 min, while both CMC-modified cement (C/C) and CMC/nanofiber-modified cement (C/PL/C-10) did not disintegrate during the curing process (Fig. 2A). This is meaning that the anti-collapsibility of the composite cements has been greatly improved after nanofiber introduction. The injectability of all samples were shown in Fig. 2B. When the content of fibers was larger than or equal to 5 wt% without CMC, the paste could not be injected probably because the fiber agglomeration caused clogging of the syringe. However, when a small amount of CMC was added into CPC and CPC/poly(lactide-co-glycolic acid) (C/PL), the injectability was obviously improved (Supplementary Video 2, Fig. S4). The injectability value of CPC was increased from 67.5 ± 1.3% to 93.0 ± 0.63%, the injectability value of C/PL-2.5 was increased from 24.0 ± 4.3% to 92.2 ± 0.6%, and C/PL/C-10 maintained excellent injection performance of 91.7 ± 1.4% (Fig. 2B). However, the setting time of CPC was increased from 30.0 ± 1.2 min to 45.8 ± 1.9 min with the addition of CMC. By contrast, the setting time decreased with the content of the PLGA fibers, therefore the increase of PLGA fiber content could offset the negative effect of CMC on cement curing. Hence, it is possible to control the setting time by adjusting the content of PLGA nanofiber. The setting

Fig. 2. Effect of CMC and PLGA on the operational performance and compressive property of CPC. (A) Anti-washout performance of CPC, C/C and C/PL/C-10 respectively. (B) Injectability (%) of different cement formulations. (C) Compressive strength. (D) Compressive fracture work. (E) Setting time of cements Data are presented as the mean ± SD; n = 3; *significant difference compared with control group, *p < 0.05 and **p < 0.01.
time of C/PL/C-10 reached 21.0 ± 2.2 min, but this cement could not become a paste when the more than 5 wt% PLGA nanofibers were added into the CPCs without CMC or more than 10 wt% PLGA nanofibers with CMC (Fig. 2E). Due to poor performance of the handling, these CPCs were not further analyzed.

The compressive properties of injectable cements with different fiber contents were further analyzed. The compressive strength of CPC was decreased slightly from 5.1 ± 0.3 MPa to 4.7 ± 0.6 MPa after adding CMC, on the contrary, the mechanical strength was enhanced with the increase of fiber content. The strength of the optimal group (C/PL/C-10) reached 6.4 ± 0.2 MPa, which increased by about 30% than the pure CPC (Fig. 2C). It could be clearly observed that the compressive work of fracture for C/PL/C-10 was significantly enhanced by increasing the content of PLGA nanofiber, from 228.7 ± 28.3 kJ/m² (CPC) to 652.9 ± 20.5 kJ/m² (C/PL/C-10) (Fig. 2D).

3.3. Effect of PLGA nanofibers on physicochemical properties of cement

After soaking for 3 months, the SEM analysis showed that the pore size and number continued to increase with the extending of soaking time. After degradation for one month, the PLGA nanofibers retained a relatively complete shape in the pores, but some small cracks appeared.

**Fig. 3.** Effect of PLGA and CMC on the physicochemical properties of CPC. (A) Surface morphology of C/PL/C-10 samples degradation after 1 month, 2 months, and 3 months respectively. (B) Weight loss curves of samples after immersing in Tris-HCl for different time. (C) Pore size distribution of the samples degradation for different time. (D) Porosity of the samples. (E) Average pore diameter of the samples. (F) The pH vibration of PBS after samples soaking. (G) Ca²⁺ released into PBS determined by ICP-AES. Data are presented as the mean ± SD; n = 3; *significant difference compared with control group, *p < 0.05 and **p < 0.01.
After 2 months, obvious cracks began to appear in the fiber structure, the agglomerated fiber bundles had many holes, and the fiber surface became rougher. Compared with the fiber at one month, the fiber structure was basically destroyed after degradation for three months, and the agglomerated fiber was almost degraded, leaving flaky debris and producing large pores in the cement (Fig. 3A). Weight loss analysis was performed by soaking the cement in Tris-HCl solution for different times. The degradation rate of C/PL/C-10 was faster than that of the CPC and C/C groups, while the degradation rate of CPC and C/C is almost the same (Fig. 3B).

The pore size distribution of the C/PL/C-10 was measured by mercury intrusion method after soaking in PBS. The pore size distribution curves could intuitively reflect the impact of PLGA degradation on the pore size of the CPC. After one month of degradation, pore size distribution was not changed obviously after degradation for one month as indicated by SEM, but the number of pores larger than 100 μm was increased in the C/PL/C-10 group. With the extension of degradation time, although the porosity of composite bone cement did not change much, the pore size was increasing. After 3 months, some small peaks appear around 24.1 μm and 298.6 μm (Fig. 3C–E). Macroporosity allowed for cells penetration and fluid flow through the material, stimulating active and passive degradation [45]. Furthermore, pores above 100 μm were conducive to blood vessel growth and bone ingrowth [46]. To analyze the cements interactions in the immersion

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Fig. 4. Proliferation and cell adhesion of bone marrow mesenchymal stromal cells (BMSCs) treated with cement extracts. (A) Live/dead assay of BMSCs. (B) Cells viability according to the live/dead assays. (C) CCK-8 analysis of BMSCs. (D) SEM images of BMSCs on the cement surface after 24 h of incubation (BMSCs are colored for easy viewing). Cell culture medium without extracts was applied as a control. Data are presented as the mean ± SD; n = 5; *significant difference compared with control group, *p < 0.05 and **p < 0.01.
solution, the concentration of Ca$^{2+}$ as well as pH level were determined (Fig. 3F and G). The pH value trend of C/C and C/PL/C-10 was not much different from that of the CPC. The pH level of the C/PL/C-10 decreased a bit more, eventually reaching 6.3. There was no clearly difference in the Ca$^{2+}$ concentration between CPC and C/C during the first 7 days, but afterward the Ca$^{2+}$ concentration of C/C (0.8 ± 0.04 mM) was greater than that of CPC (0.6 ± 0.01 mM). The Ca$^{2+}$ concentration of C/PL/C-10 was higher than that in the other groups from the beginning, and the

Fig. 5. Osteogenic effect on bone marrow mesenchymal stromal cells (BMSCs) treated with cement extracts. ALP staining of BMSCs for (A) 7 days and (B) 14 days. (C) Quantification of ALP activity of BMSCs for 7 days and 14 days. (D) ARS staining of BMSCs for 14 days. (E) Osteogenesis related protein expression of BMSCs cultured in cement extracts for 7 days. (F) Osteogenesis related gene expression of BMSCs cultured in extract solutions for 7 days. Data are presented as the mean ± SD; n = 5; *significant difference compared with CPC group, *p < 0.05 and **p < 0.01.
highest concentration reached 1.1 ± 0.1 mM. Additionally, the amount of released LA in the C/PL/C-10 was increased after degradation, reaching 281.5 ± 7.7 μM finally (Fig. S3).

3.4. Effect of C/PL/C-10 on adhesion and proliferation of BMSCs

The in vitro biocompatibility of the C/PL/C-10 cement was evaluated. The Live/Dead staining revealed that the BMSCs incubated with the cement extracts were alive (Fig. 8A). Cell survival rate of Live/Dead assay and CCK-8 analysis revealed that the BMSCs proliferated well in the C/PL/C-10 group, but the cell proliferation in the CPC and C/C groups was slightly reduced in comparison to the control group (Fig. 4B and C). The SEM images showed that attached BMSCs on the surface of different cements were presented (Fig. 4D), demonstrating that BMSCs had a better adhesive and flat morphology on the C/PL/C-10 surfaces. Taken together, the C/PL/C-10 cement was non-cytotoxic and supplied rough surface for cell adhesion and proliferation.

3.5. Effect of C/PL/C-10 cement on osteogenic differentiation of BMSCs in vitro

ALP staining, ARS staining and relative analysis of osteogenic gene expression were performed to detect the effect of C/PL/C-10 cement on osteogenic differentiation. The ALP expression of BMSCs treated with cement extracts for 7 and 14 days was positive for all the cement groups (Fig. 5A and B). The stained intensity of ALP for C/PL/C-10 group was higher than that of CPC and C/C groups after treatment for 7 and 14 days. As seen in Fig. 5C, ALP activity showed a similar trend to the ALP staining results. In addition, C/PL/C-10 group displayed the strongest ARS staining intensity in the cement groups (Figs. 5D and S8). OPN, Runx-2 and OCN are important markers of osteogenic differentiation at a relatively late stage during bone regeneration. The outcomes in Fig. 5E showed that the extracts of C/PL/C-10 group significantly promoted the expression of OPN, Runx-2 and OCN after 7 days treatment, compared with C/C extracts. The related genes expression (OCN, Runx-2, ALP, Osterix, BMP4 and Col-1) of BMSCs treated with cement extracts is shown in Fig. 5F. After treatment for 7 days, the expression of OCN, Runx-2, ALP, Osterix and BMP4 in the C/PL/C-10 group was much higher than that in the CPC and C/C groups, which was consistent with ALP and ARS expression results, suggesting the promoted effect of C/PL/C-10 cement on osteogenic differentiation.

3.6. Effect of C/PL/C-10 cement on angiogenesis in vitro

Human umbilical vein endothelial cells (HUVECs) were used to evaluate the angiogenesis effect of LA released from the fabricated samples in this study. By using wound healing model in vitro, it was revealed that the migration capacity of HUVECs was enhanced by −19.3%, 6.1% and 49.7% ($p < 0.01$) in the CPC, C/C and C/PL/C-10 groups, when in comparison to the control group (Fig. 6A and B). As can be seen in Fig. 6D by using the tube formation test, we found that the formation of tubular structures was enhanced after incubation with C/PL/C-10 extract. Quantitative results measured with Image J software showed that the number of master junction were 2%, 22% and 49.7% ($p < 0.01$), and 11% ($p < 0.01$) and 75% ($p < 0.01$) in the CPC, C/C and C/PL/C-10 group, respectively in comparison with the control group (Fig. 6D and E). The SEM images showed that attached HUVECs on the surface of different cements were presented in Fig. 6C, which exhibited that C/PL/C-10 surfaces could provide a better adhesive surface for HUVECs. After co-culture for 3 days, the expression of vWF, VEGF and CD 31 in the C/PL/C-10 group was much higher than that in the CPC and C/C groups, which was consistent with wound healing assays and tube formation assays, suggesting the positive effect of C/PL/C-10 cement on angiogenesis (Fig. S9).

3.7. Effect of C/PL/C-10 cement on bone regeneration and osteointegration in vivo

The biocompatibility of C/PL/C-10 cement in vivo was confirmed by the H&E staining for major organs in rats (Fig. S5). The CPC, C/C and C/PL/C-10 cements were injected into the bone defect of rat femoral condyle to explore the bone regeneration and osteointegration (Fig. 7B). Micro-CT examination at 6 and 12 weeks after implantation showed yellow new bone formation surrounding the surface of the cylindrical bone cements at each time of implantation (Fig. 7C). No significant differences in new bone formation were found among the three cement groups 6 weeks after implantation. Twelve weeks after operation, more amount of new bone formation around the implants was identified in the C/PL/C-10 group compared to that in the CPC and C/C groups. Quantitative analysis of the micro-CT revealed that the BV/TV, Tb. pf and BMD values were significantly enhanced by the C/PL/C-10 group compared with the C/C group at the implantation time of both 6 and 12 weeks (Fig. 7D-F), demonstrating the highest bone microarchitecture values in the C/PL/C-10 group. Furthermore, a time-dependent increase in the values of BV/TV, BMD and Tb. pf were observed in all three groups.

The osteo-integration capacity of the implanted cements in the femoral condyle was detected using the push-out tests. With the extension of implantation time, the extrusion force of cement showed an increasing trend, and the force in the C/PL/C-10 group was obviously stronger than that in the C/C and CPC groups (Fig. 7H). Because of the poor anti-wash out and slow curing capacity of CPC, the anti-push-out force of CPC was lowest compared with that in the other groups at any time after implantation. The initial push-out force in the C/C group was unsatisfactory attributed to the prolongation of the curing time and the negative impact of CMC on cement strength, which was only 2.8 ± 1.1 N (3 h) and 10.7 ± 1.7 N (3 d). By contrast, the initial force of C/PL/C-10 group was much stronger due to the improvement of the curing time and the existence of a certain friction effect between the PLGA nanofibers and the bone surface. 1, 6 and 12 weeks after implantation, the extrusion force of the C/PL/C-10 cement was further significantly enhanced because of the osteo-integration of the cement-bone interface, reaching to 148.7 ± 8.0 N at 12 weeks, which was slightly less than the force of rat femoral cortical bone (178.8 ± 4.7 N) (Fig. 7H).

The bone-implant interface of CPC, C/C and C/PL/C-10 cements was further evaluated by histological examination at the femoral defect in rats 6 and 12 weeks after implantation. As indicated by H&E staining, no signs of inflammatory reaction, necrosis and infection after implantation were observed, indicating their satisfied biocompatibility (Fig. 8A, C). Six weeks after implantation, CPC and C/C groups exhibited rare new bone formation, whereas C/PL/C-10 group demonstrated more new bone formed in vivo (Fig. 8A, C). Furthermore, blood vessels were detected in the newly formed bone into the C/PL/C-10 cement, leaving no space between implant and bone. In contrast, after 6 weeks, the CPC group showed a large amount of fibrous tissue at the border of the cement. At 12 weeks, the C/PL/C-10 group showed a significant amount of dense calcification of new bone and vascular formation, accompanied by degradation of the bone cement. Finally, alizarin red and calcein were intraperitoneal injected 4 and 6 weeks after surgery respectively to measure the rate of bone mineralization. The mineralization rate showed that C/PL/C-10 cement significantly promoted mineralization compared with CPC and C/C cement (Fig. 8B, D). The immunohistochemistry assays of the cement implanted subcutaneously for 6 weeks indicated that blood vessel formation marker CD 31 was highly expressed in the C/PL/C-10 groups ($p < 0.01$) compared with the CPC and C/C groups (Figs. S6 and S7), demonstrating the effect of C/PL/C-10 on the acceleration of angiogenesis in vivo.

4. Discussion

Commercial injectable calcium phosphate cement (iCPC) is brittle,
Fig. 6. Angiogenic effect on human umbilical vein endothelial cells (HUVECs) treated with cement extracts. (A) Wound healing assays of HUVECs cells. (B) Quantitative analysis of the wound healing assays. (C) SEM images of HUVECs on the cement surface after 24 h of incubation (HUVECs are in colour for easy observation). (D) Tube formation assays of HUVECs cells. (E) Number of master junction and (F) total segment length calculated with Image J. Data are presented as the mean ± SD, n = 5; *significant difference compared with control group *p < 0.05 and **p < 0.01.
Fig. 7. Bone regeneration and osteo-integration of different cements after implantation into the bone defect of rat femoral condyles. (A) 2D micro-CT images. (B) Establishment of the femoral condyle defect model of C/PL/C-10 group in SD rats. (C) 3D reconstructions of the implants of different groups, grey: cements; yellow: newly formed bone. Quantitative results of (D) BV/TV, (E) T.B.Pf and (F) BMD in the femoral condyle defect determined by micro-CT. (G) Push-out experimental diagram. (H) Push-out force. Data are expressed as the mean ± SD, n = 3, *significant difference in comparison to CPC group, *p < 0.05 and **p < 0.01.
Fig. 8. Histology and fluorescent assay of newly bone formed 6 and 12 weeks after operation. (A) H&E, V&G and toluidine blue staining of undecalcified femoral condyle sections 6 and 12 weeks after implantation. NB: newly formed bone; BV: blood vessels; M: materials. (B) Fluorescent microscope observation of a typical image of calcein and alizarin red staining. Quantification of results of (C) new bone percentage and (D) depositing rate. Data are presented as the mean ± SD, n = 3, *significant difference compared with CPC group, *p < 0.05 and **p < 0.01 analyzed by ANOVA.
slow to degrade, and does not have large pores to allow new bones and blood vessels to grow in [47]. What’s more, it suffers from poor injectability due to solid-liquid separation and tends to disintegrate in the body due to poor washout resistance [48]. Herein, the present bone cement took advantage of the high aspect ratio and controllable degradation properties of PLGA nanofibers to greatly improve the toughness of iCPC and accelerate the degradation of iCPC [27,49]. With the PLGA degradation, the large pores in iCPC could be created, where the blood vessel and new bone would grow into [50]. Furthermore, carboxymethyl cellulose was added into iCPC, which could improve injectability and anti-washout abilities [36].

In the present study, by adding PLGA nanofibers into the solid phase composition, the original brittleness of CPC was significantly improved. The toughness was increased by twice, and the compressive strength were improved to some extent. The mechanical performances of fiber-reinforced CPCs can be explained as follows. The fiber deflects the cracks and bridges the cracks, thereby hindering the propagation of the cracks, and dissipating the energy of the entire matrix due to the formation of new cracks [28]. At the same time, because of the hydrophobicity and the formation of fiber bundles, phase separation between the matrix and the fibers is inevitable, making it is difficult to further improve the compressive strength. Fortunately, the increase in fiber content could reduce the setting time to within the acceptable range for clinical use. This is due to the fact that the liquid phase is absorbed in the gap of the polymer bundle, which is equivalent to reduce the liquid-to-solid ratio [51].

The pore size in the CPC cement was increased by the addition of PLGA and CMC. Although the increase of pore size might restrict the strength improvement of the cement, the large pore could enhance the roughness of the material and enlarge the contact area of the liquid and increase the release of calcium ions. In addition, the acidic products of PLGA degradation reduced the pH of the local environment to achieve autocatalysis and promote degradation [22]. At the same time, acidic substances will also accelerate the release of calcium phosphate [49,52]. On other hand, CPC consumes protons upon CPC dissolution in combination with the flow of body fluids in bone tissue, overcoming potentially negative effects caused by excessive acidity [53]. Taken together, the amount of the Ca$^{2+}$ release in the C/PL/C-10 group was larger than that in the C/C and CPC groups. This leads to an osteogenic environment during material degradation, thereby significantly improving osteo-integration and new bone ingrowth into the defect area.

The in vitro cell culture results showed that the good cytocompatibility of C/PL/C-10 cement for BMSCs, and the cell proliferation and adhesion were even increased when co-cultured with C/PL/C-10 owing to the rougher surface, more large pores and release of Ca$^{2+}$ in the C/PL/C-10 group. Furthermore, the release of Ca$^{2+}$ might further facilitate the differentiation of BMSCs in the C/PL/C-10 group, as demonstrated by ALP staining, ARS staining and qPCR results (analyzing osteogenic markers). Similarly, its surface allowed HUVECs to have the best spreading and adhesion on these samples. Additionally, the cement samples containing PLGA nanofibers promoted vascularization and migration ability of HUVECs due to the promoting effect of Ca$^{2+}$ and LA on angiogenesis. What’s more, the role of LA was even more important because LA could promote angiogenesis by activating several molecular pathways that work together [54-56].

Our in vivo results further showed the biodegradation and cytocompatibility of C/PL/C-10 cement. During injection into the defect within well-shaped and anti-washout property confirmed the shape-maintaining onto the C/PL/C-10 cement, thereby providing a better initial integration and mechanical support, which is helpful for surgical procedure [44]. According to the three-month in vivo degradation results and the pore size change of the cements, it could be concluded that the degradation rate of PLGA in the early stage was relatively slow, whereas, with the formation of LA, the decomposition of PLGA was accelerated under the action of autocatalysis. The macroporous structure (larger than 100 μm) was increased significantly in the C/PL/C-10 cement, providing spaces for the ingrowth of blood vessels and new bone, which was also verified in the Micro-CT image and histology observation. Therefore, dense and mineralized new bones were observed at the surface and in the pores inside the C/PL/C-10 cement. In addition, the histology analysis reflected a poor osteo-integration in the CPC group, as fibrous tissue was observed between the host bone and the implants, which was further demonstrated by the result of push-out test. On the other hand, the addition of CMC and PLGA addressed this problem, which resulted in a better osteo-integration of C/PL/C-10 cement. At 12 weeks after implantation, the C/PL/C-10 cement was very tightly bonded to the host bone due to new bone growth, and the push-out strength of C/PL/C-10 was approximately three time as high as that of the pure CPC group, which was even close to the strength of cortical bone. In summary, under the synergistic effect of Ca$^{2+}$, LA release and macroporous structure formation on osteogenesis and angiogenesis, the osteo-inductive and osteo-integrative capacities of C/PL/C-10 cement was significantly improved, which should have an increasing potential for clinical application.

5. Conclusion

In this study, we fabricated an injectable nanofiber-reinforced calcium phosphate bone cement by introduction of poly(lactide-co-glycolic acid) nanofibers and carboxymethyl cellulose which improved the mechanical properties, biodegradability, injectability and porosity of bone cement. The resulting bone cement showed 3-time reinforcement in mechanical robustness as well as hierarchical porous structure to crease space the ingrowth of blood vessels and new bone. Poly(lactide-co-glycolic acid) nanofiber can be used for adjustment of the cement’s biodegradability, allowing for not only a continuous delivery of calcium ions to enhance the osteogenesis but also of the lactic acid to stimulate vascularization. Taken together, this study introduces a new strategy to design and optimize the relevant resorbable fibers to enhance the mechanical robustness and anti-washout capacity of traditional injectable calcium phosphate cement, promising their high potential for minimally-invasive bone regeneration.

Data availability

The data that support this study are available within the article and its Supplementary data files or available from the corresponding authors upon request.

CRediT authorship contribution statement

Peihao Cai: Methodology, Validation, Formal analysis, Investigation, Resources, &, Data curation. Shunyi Lu: Formal analysis, Investigation, Resources, &, Data curation. Jieqin Yu: Investigation, Data curation. Lan Xiao: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. Jiayi Wang: Methodology, Visualization. Haifeng Liang: Formal analysis, Investigation, Resources, & Data curation. Mengxuan Shi: Formal analysis, Investigation, Resources, & Data curation. Guanjie Han: Investigation, Data curation. Mengxuan Bian: Formal analysis, Investigation, Resources, & Data curation. Shihao Zhang: Methodology, Investigation, & Formal analysis. Tian Zhang: Writing – review & editing, Conceptualization, Methodology. Changsheng Liu: Writing – review & editing, Supervision, Funding acquisition. Libo Jiang: Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing, Supervision, Funding acquisition. Yulin Li: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition.
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