

## Original Article

# Core decompression combined with implantation of $\beta$ -tricalcium phosphate modified by a BMSC affinity cyclic peptide for the treatment of early osteonecrosis of the femoral head

Guozong Wang<sup>1</sup>, Hua Xin<sup>2</sup>, Guanghao Tian<sup>3</sup>, Kuisheng Sheng<sup>4</sup>, Nianping Zhang<sup>5</sup>, Shui Sun<sup>6</sup>

<sup>1</sup>Department of Joint Surgery, People's Hospital of Rizhao, Rizhao 276800, Shandong, China; <sup>2</sup>Department of Neurology, People's Hospital of Rizhao, Rizhao 276800, Shandong, China; <sup>3</sup>Department of Internal Medicine, Lixia People's Hospital, Jinan 250014, Shandong, China; <sup>4</sup>Department of Orthopedics, Rizhao City Traditional Chinese Medicine Hospital, Rizhao 276800, Shandong, China; <sup>5</sup>The Teaching and Research Section of Surgery, The First Clinical College of Shandong University of Traditional Chinese Medicine, Jinan 250355, Shandong, China; <sup>6</sup>Department of Joint Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong, China

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**Abstract:** Early intervention of osteonecrosis of the femoral head (ONFH) is very important. At present, the therapeutic effect on early ONFH is not completely satisfactory. D7 peptide has special affinity towards bone marrow mesenchymal stem cell (BMSC). Taking advantage of the adsorption/freeze-drying strategy, we constructed D7 cyclic peptide-modified  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) scaffolds. The functional  $\beta$ -TCP scaffolds can enhance adhesion, spreading and proliferation of BMSCs compared with unmodified  $\beta$ -TCP scaffolds, which was confirmed in cytological experiments. In rabbit model of early ONFH, functional  $\beta$ -TCP scaffolds were stuffed into the cavities after core decompression (CD). Radiographic and histological examination confirmed that CD followed by filling of functional  $\beta$ -TCP scaffolds can obviously improve the therapeutic effect of early ONFH. Our study provides a new option for curing early ONFH.

**Keywords:** Osteonecrosis of the femoral head,  $\beta$ -tricalcium phosphate, affinity cyclic peptide, bone marrow mesenchymal stem cell, core decompression

## Introduction

It is estimated that, in the United States, 10,000 to 20,000 new cases of osteonecrosis of the femoral head (ONFH) occur annually [1]. It is thought by many that early treatment of ONFH can alter natural progression of the disease significantly [2]. Early appropriate treatment before collapse is very important for the prognosis of ONFH. Core decompression (CD) is commonly used to treat early ONFH [3]. But there are complications that can occur, such as cartilage injury and subtrochanteric fracture [4]. And it is believed that the residual cavity results in weakening of the support force to the femoral head which makes the necrotic femoral head easy to collapse [5]. CD has been combined with some bone graft materials which

were stuffed into the cavities after CD to elevate the efficacy of CD on ONFH and avoid the collapse of the necrotic femoral head [6].

Biomaterial is an important part of tissue engineering (TE) [7].  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) has been paid much attention in the field of TE due to its good biocompatibility and osteoconductivity. It is often used as bone graft in orthopaedics. ONFH has been treated with CD followed by stuffing  $\beta$ -TCP into the bone tunnels [8]. But in the cure of early ONFH,  $\beta$ -TCP is not a satisfactory biomaterial because of its faster degradation rate and absence of intrinsic biological stimulatory activity [9, 10].

In order to give biomaterials some special functions, surface modification is a widely adopted

strategy. Studies on biomaterials modified by peptide with specific affinity towards specific cells have gradually increased [11-14]. In our previous study, through phage display technology, cyclopeptide CDNVAQSVC (D7) was found and identified to have special affinity towards C57BL/6 mouse bone marrow mesenchymal stem cells (BMSCs) [15]. The role of BMSCs is crucial in repair of necrotic femoral heads due to secretion of cytokines and osteogenesis [16]. Surface modification of  $\beta$ -TCP with D7 peptide can construct functional  $\beta$ -TCP which can recruit BMSCs. Because of lack of viable mesenchymal stem cells (MSCs), the ability of regeneration and repair of necrotic area of femoral head is poor [17]. So our study provides a new idea to treat ONFH using endogenous BMSCs sufficiently.

In this study, we fabricated functional  $\beta$ -TCP scaffolds which were modified by cyclopeptide D7 ( $\beta$ -TCP-D7). Then we studied the influence of  $\beta$ -TCP-D7 on adhesion, spreading and proliferation of C57BL/6 mouse BMSCs through experiments *in vitro*. We further studied whether binding of D7 peptide to BMSCs has species specificity. Finally, we studied the curative effect of CD followed by filling of  $\beta$ -TCP-D7 scaffolds on early ONFH. Our study is dedicated to providing a novel option to treat early ONFH.

## Materials and methods

### Cell culture

Human BMSCs (cat. no. HUXMA-01001), rabbit BMSCs (cat. no. RBXMX-01001) and C57BL/6 mouse BMSCs (cat. no. MUBMX-01001) were purchased from Cyagen Biosciences, Inc. (Santa Clara, CA, USA). The cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM; cat. no. 01-051-1A; Biological Industries, Ltd., Kibbutz Beit-Haemek, Israel) which containing L-glutamine, antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin; cat. no. 15140122; Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (cat. no. 10099141; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. Replace the culture medium every two to three days. When growing to 80-90% cell density, cells were passaged. Cells at passage 4-5 were selected to complete cytological experiments.

### Synthesis of peptides

A cyclic peptide with high affinity towards C57BL/6 mouse BMSCs, CDNVAQSVC, was identified using phage display in our previous study and designated as D7 [15]. The peptide V7 (CVAVQNDSC), which has the same amine acid residues as D7, but in different order, was selected as the negative control. The peptide RGD consists of arginine, glycine and aspartic acid. We selected RGD as the positive control. Through solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl chemistry (Scilight-Peptide Inc., Beijing, China), experimental peptides were synthesized. In order to label peptides with fluorescein-5-isothiocyanate (FITC), the amino terminal of peptide D7, V7 and RGD were attached by aminohexanoic acid. Peptides were preserved in a refrigerator of -20°C. The peptides were dissolved in PBS (cat. no. 02-024-1A; Biological Industries, Ltd.) to make peptide solution of 1 mg/ml to complete the subsequent experiments.

### Synthesis of functional $\beta$ -TCP scaffolds modified by cyclopeptide D7

A previously described method was used to synthesize functional  $\beta$ -TCP scaffolds modified by cyclopeptide D7 [18]. Cylindrical  $\beta$ -TCP (height, 2 mm; diameter, 6 mm) was obtained from Shanghai Bio-lu Biomaterials Co., Ltd. (Shanghai, China). Through the adsorption/freezing-drying strategy, cyclopeptide D7 was adsorbed to  $\beta$ -TCP. In short, a solution of 100  $\mu$ g/ml was produced by dissolving D7 peptide in PBS. Then  $\beta$ -TCP was placed in this solution and agitated gently for 24 h to ensure that D7 peptide molecules were fully adsorbed to the surface of  $\beta$ -TCP scaffolds. Wash the scaffolds after incubation with PBS five times to eliminate unadsorbed peptide molecules. The composites were freeze-dried for 1 h *in vacuo* and preserved in moisture-proof containers at -20°C. The synthesized material was observed by scanning electron microscope (SEM). At the same time, functional  $\beta$ -TCP scaffolds modified by FITC-D7 peptide were fabricated and examined with ImageXpress Micro Confocal (Molecular Devices, LLC, Sunnyvale, CA, USA).

### The effect of functional $\beta$ -TCP-D7 scaffolds on cytological behavior of BMSCs *in vitro*

The effect of  $\beta$ -TCP and  $\beta$ -TCP-D7 on adhesion, spreading and proliferation of C57BL/6 mouse

BMSCs was compared. Materials for testing were sterilized by ultraviolet light on the super clean table before use.

### *Cell adhesion assay*

Cell adhesion test was completed using Cell Counting Kit-8 (CCK-8; cat. no. 96992; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) reagent with some modifications [19]. Briefly, C57BL/6 mouse BMSCs were made into cell suspension of  $6 \times 10^4$  cells/ml using DMEM without serum. Then 200  $\mu$ l of the cell suspension prepared above was added to 96-well plates which contained  $\beta$ -TCP or  $\beta$ -TCP-D7 fabricated in 100  $\mu$ g/ml or 500  $\mu$ g/ml D7 peptide solution and incubated at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. After 3 hours, rinse the scaffolds sufficiently with PBS for three times and then put them into CCK-8 solution. The absorbance of the solution at 450 nm was measured after incubation at 37°C for 24 h. The experiment was repeated at least three times.

### *Cell spreading assay*

In cell spreading assay,  $\beta$ -TCP-D7 scaffolds were fabricated in 500  $\mu$ g/ml D7 peptide solution. The spreading morphology of C57BL/6 mouse BMSCs growing on  $\beta$ -TCP or  $\beta$ -TCP-D7 was observed with SEM. Briefly, cells were made into cell suspension of  $4 \times 10^4$  cells/ml. Then 200  $\mu$ l of the cell suspension prepared above was added to 96-well plates which contained pure  $\beta$ -TCP scaffolds or functional  $\beta$ -TCP-D7 scaffolds and incubated at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere for 24 h. The scaffolds loading BMSCs were fixed with 2.5% glutaraldehyde and preserved at 4°C. Then the BMSC-loaded scaffolds were fixed with 1% osmic acid, dehydrated with gradient ethanol, sputter-coated with gold and observed with SEM.

### *Cell proliferation assay*

The cell proliferation of C57BL/6 mouse BMSCs growing on  $\beta$ -TCP and  $\beta$ -TCP-D7 scaffolds fabricated in 500  $\mu$ g/ml D7 peptide solution was evaluated using CCK-8 reagent following a previously described procedure with some modifications [19]. Briefly,  $1 \times 10^3$  cells were seeded on  $\beta$ -TCP or  $\beta$ -TCP-D7. On day 3 and 7, the absorbance of the solution at 450 nm was

measured after incubation in CCK-8 solution at 37°C for 3 h. The experiment was repeated at least three times.

### *Species specificity of D7 peptide affinity towards BMSCs*

The cyclopeptide D7 has extremely high affinity for C57BL/6 mouse BMSCs [15]. In our present study, human and rabbit BMSCs were selected to further study the species specificity of D7 peptide affinity towards BMSCs. The cells grew to 70-90% confluence in 24-well plates. Then the cells were incubated with peptide D7, V7 or RGD (10  $\mu$ M) which were pre-labeled with FITC at 37°C for 1 h. In order to show the cytoskeleton, the cells were incubated with rhodamine phalloidin (cat. no. CA1610; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) of 80 nM for 30 min at RT. The nuclei were stained with DAPI (cat. no. C0065; Beijing Solarbio Science & Technology Co., Ltd.) of 10  $\mu$ g/ml at RT for 10 min. The stained cells were examined by fluorescence microscope.

### *In vivo evaluation of therapeutic effect on early ONFH using core decompression combined with implantation of $\beta$ -TCP-D7 scaffolds*

**Laboratory animals:** 24 healthy male New Zealand rabbits, weighting  $2.64 \pm 0.46$  kg and aged 3 months, were prepared for subsequent experiment. Our current research complied with Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our animal experiments were authorized by the Experimental Animal Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University (Shandong, China).

### *Preparation of early ONFH in rabbits*

A previously described method was used to prepare the rabbit model of early ONFH [20]. In short, the rabbits were anesthetized using 3% pentobarbital sodium (1 ml/kg) which were injected into the ear vein. Then the animals were fixed to the lateral position. Through the lateral approach, the soft tissue of the left hip joint was cut layer by layer to expose the femoral head. The femoral head was frozen 20 times with liquid nitrogen for 10 s each time and thawed with warm saline. The process was repeated three times. Suture incision layer by layer. Four weeks after surgery, rabbits were

selected randomly and the femoral heads were separated, fixed in 4% paraformaldehyde and decalcified in 0.5 M EDTA. Then the femoral head specimens were made into 4- $\mu$ m-thick paraffin sections along the frontal plane. Hematoxylin and eosin staining was completed to evaluate the effect of animal modeling of early ONFH.

### *Surgical procedure*

Eighteen New Zealand rabbits of early ONFH were divided equally into three groups. Rabbits in the first group were treated with CD. Rabbits in the second group were treated with CD followed by filling of  $\beta$ -TCP (4 $\times$ 14 mm). Rabbits in the third group were treated with CD followed by filling of  $\beta$ -TCP-D7 (4 $\times$ 14 mm) fabricated in 500  $\mu$ g/ml D7 peptide solution. All experimental materials were sterilized by  $\gamma$ -irradiation before operation. Anesthesia and position were the same as mentioned above. The rabbits were operated according to a previously described technique [21]. In short, under aseptic conditions, the left greater trochanter was exposed through a standard lateral approach. A bone tunnel of 4 $\times$ 14 mm from the greater trochanter to the femoral head was fabricated with a 4 mm surgical drill to complete CD. Then, the bone tunnels were filled with  $\beta$ -TCP or  $\beta$ -TCP-D7. Suture incision layer by layer.

### *Radiographic evaluation*

12 weeks after surgical treatment, rabbits were sacrificed. Left femurs were separated for micro-computed tomography (Micro-CT) examination. Three-dimensional (3D) reconstruction was produced after scanning with a 23.4  $\mu$ m resolution. From 3D images, the shape of the femoral head was observed. The bone mineral density (BMD, mg/cm<sup>3</sup>) was measured.

### *Histological evaluation*

After radiographic evaluation, the femoral heads were decalcified with 0.5 M EDTA. Then the femoral head specimens were made into 4- $\mu$ m-thick sections along the frontal plane. Hematoxylin and eosin staining was completed and the stained sections were observed using an optical microscope. Vacant bone lacunae percentage was counted. Immunohistochemistry (IHC) staining was completed to evaluate the expression of COL1 in bone matrix using mouse

anti-collagen I (COL1) antibody (1:2000, cat. no. GTX26308; GeneTex, Inc., Irvine, CA, USA). IHC staining was also performed using mouse anti-osteocalcin (OCN) antibody (1:200, cat. no. ab13420; Abcam plc, Cambridge, UK) to assess the expression of OCN in bone matrix.

### *Statistical analysis*

Data was expressed as mean  $\pm$  standard deviation. In cell proliferation, two groups were compared using student's *t*-test. Multiple groups were compared using one-way analysis of variance and LSD-*t* test in cell adhesion assay or Dunnett-*t* test in radiographic and histological evaluation. The significant level was set as *P*<0.05. Data analysis was carried out with SPSS v24.0 (IBM Corp., Armonk, NY, USA) software.

## **Results**

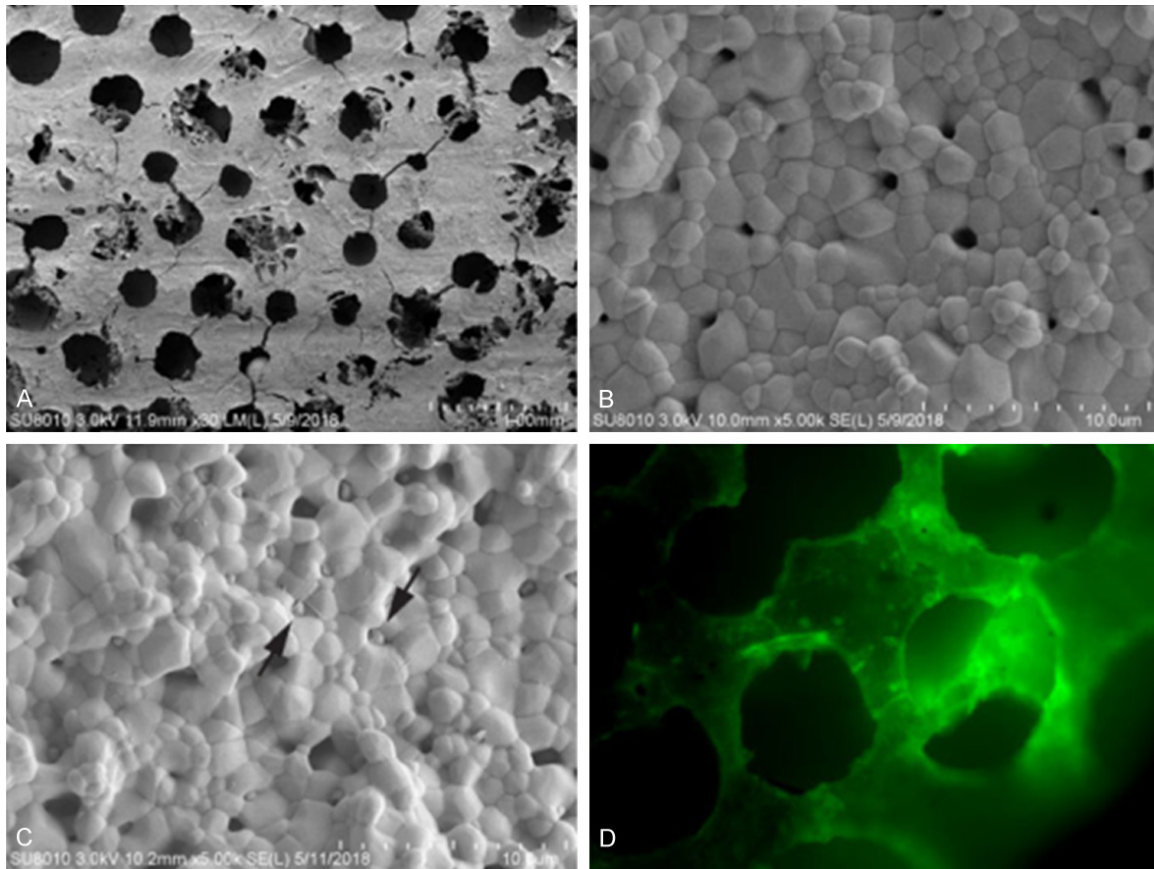
### *Synthesis of functional $\beta$ -TCP-D7*

The SEM images showed that  $\beta$ -TCP presented irregular porous structure (**Figure 1A**). At the same time, we observed that the surface of  $\beta$ -TCP was also full of microporous structure (**Figure 1B**).  $\beta$ -TCP was incubated in 100  $\mu$ g/ml D7 peptide solution. The SEM images showed that peptide molecules were adsorbed onto the microporous structure of  $\beta$ -TCP (**Figure 1C**). Cyclopeptide D7 was pre-labeled with FITC and the functional  $\beta$ -TCP scaffolds were constructed. After construction,  $\beta$ -TCP showed green fluorescence (**Figure 1D**). These phenomena indicated that the cyclic peptide D7 could be adsorbed on the surface of  $\beta$ -TCP successfully. These results also indicated that the functional  $\beta$ -TCP-D7 scaffolds were fabricated successfully.

### *Effect of $\beta$ -TCP-D7 on in vitro cytological behavior of C57BL/6 mouse BMSCs*

In cell adhesion experiment, the number of cells adhering to  $\beta$ -TCP-D7 was significantly more than that of unmodified  $\beta$ -TCP according to the optical density (OD) of both (*n*=3; \*\**P*<0.01; **Figure 2B**). This phenomenon reflected that the  $\beta$ -TCP-D7 scaffolds have better property and potential for BMSC adhesion. The average OD value increased with increased scaffold construction concentration of D7 peptide. The subsequent experiments were per-





**Figure 1.** Morphology of functional  $\beta$ -TCP-D7. A. The SEM images showed that  $\beta$ -TCP presented irregular porous structure. B. The surface of  $\beta$ -TCP was also full of microporous structure. C. Peptide molecules were adsorbed onto the microporous structure on the surface of  $\beta$ -TCP (denoted by the arrowheads). D. The D7 peptide was pre-labeled with FITC and the functional  $\beta$ -TCP scaffolds were constructed. After construction,  $\beta$ -TCP showed green fluorescence.

formed using the  $\beta$ -TCP-D7 scaffolds constructed in 500  $\mu$ g/ml D7 peptide solution.

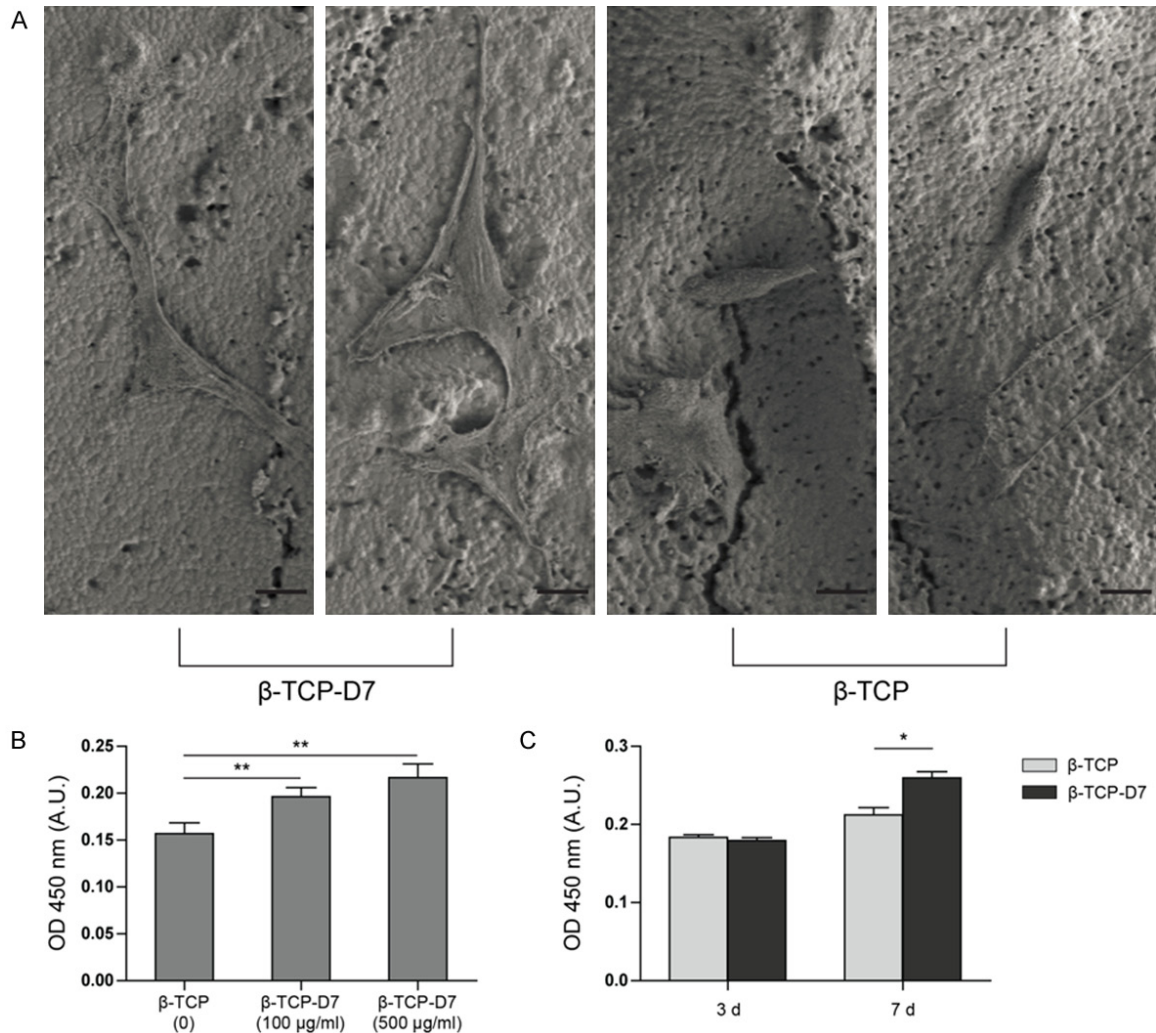
In cell spreading experiment, C57BL/6 mouse BMSCs were seeded and incubated on  $\beta$ -TCP-D7 or unmodified  $\beta$ -TCP scaffolds for 24 h and observed with SEM. The cells growing on  $\beta$ -TCP-D7 scaffolds exhibited good spreading morphology and expanded much better than those on pure  $\beta$ -TCP scaffolds (**Figure 2A**).

The CCK-8 reagent was selected to assess the effect of  $\beta$ -TCP-D7 or pure  $\beta$ -TCP scaffolds on cell proliferation. As shown in **Figure 2C**, the number of cells growing on  $\beta$ -TCP-D7 increased with increased incubation time, which showed that the functional  $\beta$ -TCP-D7 scaffolds didn't affect viability and proliferation of BMSCs ( $n=3$ ;  $*P<0.05$ ).  $\beta$ -TCP-D7 exhibited higher OD value compared with unmodified  $\beta$ -TCP after 7 days in proliferation test ( $n=3$ ;  $*P<0.05$ ; **Figure 2C**), proving that the number of cells growing on the

former was more than the latter. The result reflected that  $\beta$ -TCP-D7 scaffolds can enhance BMSC proliferation compared with pure  $\beta$ -TCP scaffolds.

#### *D7 peptide can bind to BMSCs without species specificity*

The cyclopeptide D7 has extremely high affinity towards C57BL/6 mouse BMSCs. In our present study, we selected human and rabbit BMSCs to further study the species specificity of D7 peptide affinity for BMSCs. The cells were incubated with peptide D7, V7 or RGD which were pre-labeled with FITC. Then the stained cells were examined by fluorescence microscope. Cells incubated with FITC-D7 or FITC-RGD emitted strong fluorescence signal, whereas cells incubated with FITC-V7 emitted weak or no fluorescence signal (**Figure 3**). These results showed that cyclopeptide D7 can bind to BMSCs without species specificity.



**Figure 2.** Effect of  $\beta$ -TCP-D7 on *in vitro* cytological behavior of C57BL/6 mouse BMSCs. A. The SEM images showed that cells growing on  $\beta$ -TCP-D7 scaffolds exhibited good spreading morphology and expanded much better. Scale bar, 10  $\mu$ m. B.  $\beta$ -TCP-D7 scaffolds have better property and potential for BMSC adhesion. C.  $\beta$ -TCP-D7 scaffolds didn't affect viability and proliferation of BMSCs.  $\beta$ -TCP-D7 scaffolds can enhance proliferation of BMSCs compared with unmodified  $\beta$ -TCP scaffolds; n=3; \*P<0.05; \*\*P<0.01.

#### Animal modeling assessment and evaluation of surgical treatment

The model of early ONFH in rabbits was prepared using liquid nitrogen freezing method (Figure 4A-C). Four weeks after modeling operation, the rabbits were randomly selected and the femoral heads were obtained, made into paraffin sections and analyzed by H&E staining. The typical osteonecrotic area of femoral head could be observed under microscope. The vacant bone lacunae number of the femoral heads after modeling was much higher than that of normal femoral heads (Figure 4D-I).

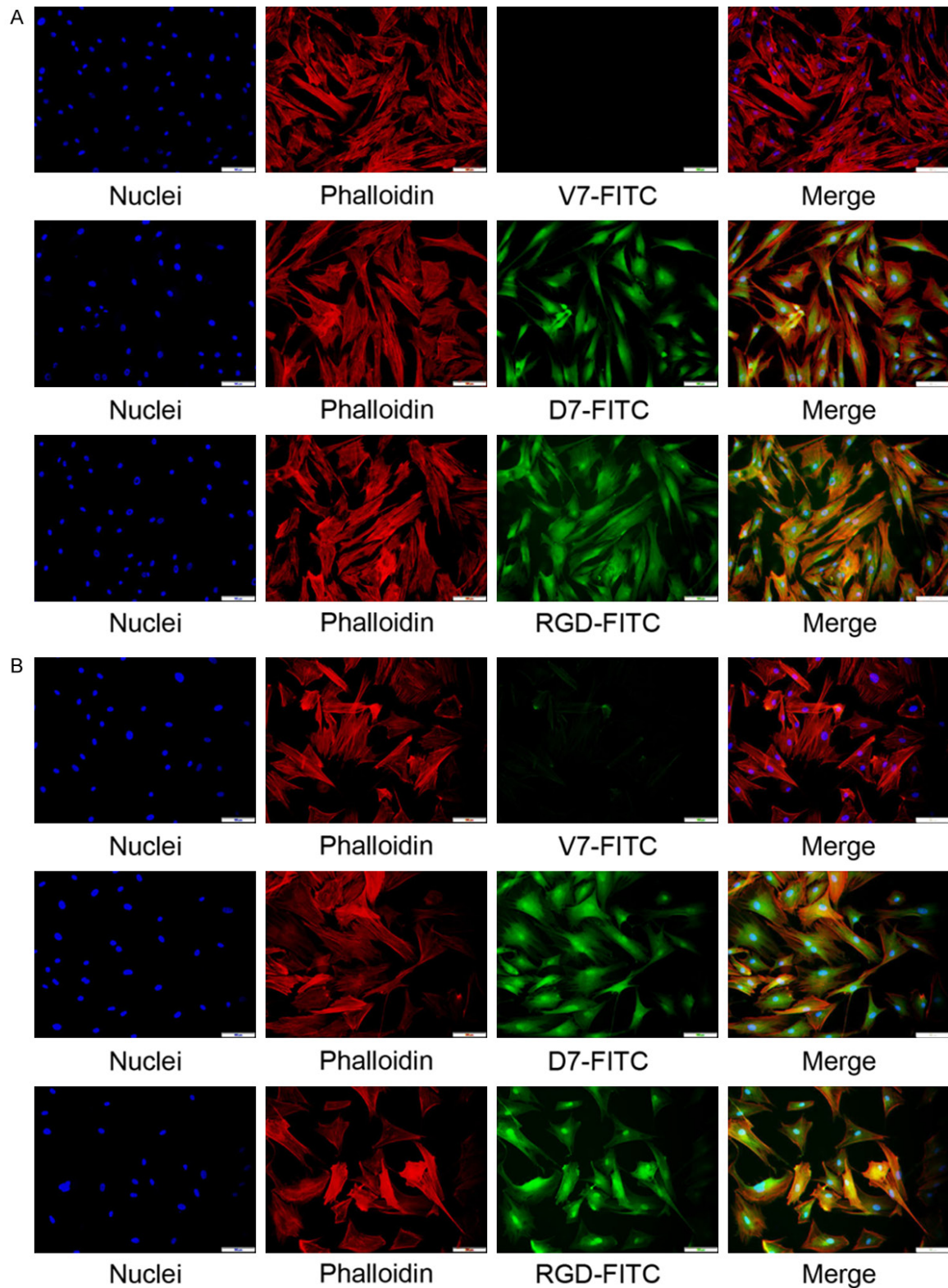
Figure 5 demonstrated the procedure of CD followed by filling of scaffolds. Except for pure CD,

$\beta$ -TCP or  $\beta$ -TCP-D7 were stuffed into the cavities after CD. There was no infection in rabbits after operation.

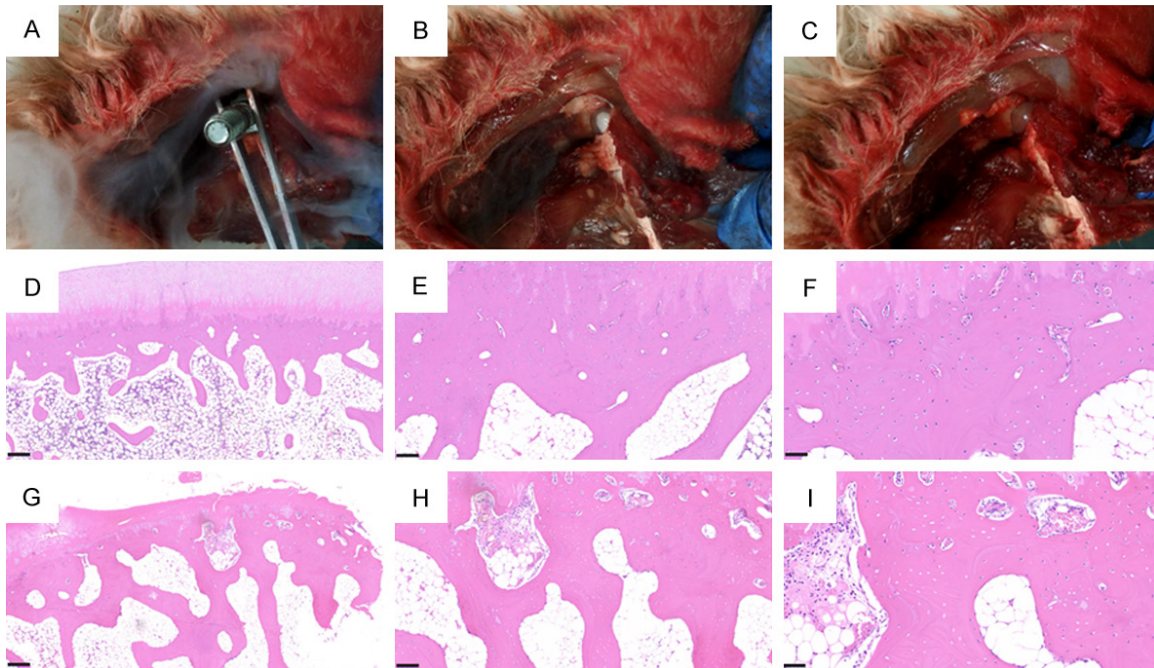
#### Radiographic evaluation

Twelve weeks after surgery, Micro-CT analyses were performed. The 3D reconstructions showed that the femoral heads became flattened and the load area collapsed in groups of CD and CD+ $\beta$ -TCP scaffolds. The contour of the femoral heads in CD+ $\beta$ -TCP-D7 group was complete (Figure 6A). The BMD of femoral heads in group of CD+ $\beta$ -TCP-D7 scaffolds was higher compared to the other two groups (n=3; \*\*P<0.01; \*P<0.05; Figure 6B). These results showed that the therapeutic effect of ONFH in

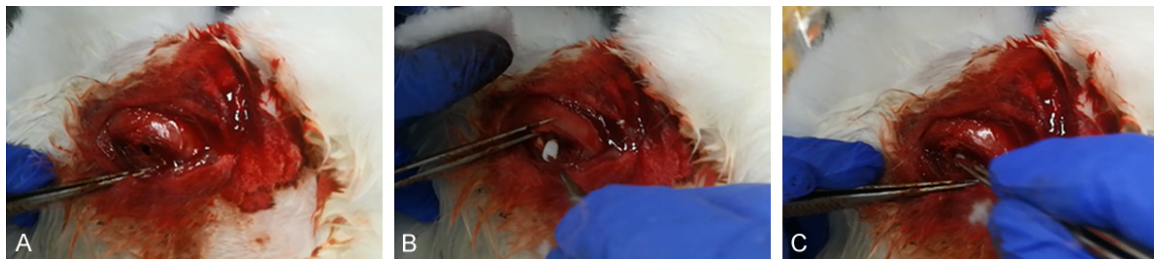




**Figure 3.** D7 peptide can bind to BMSCs without species specificity. Human (A) and rabbit (B) BMSCs were incubated with peptide D7, V7 or RGD which were pre-labeled with FITC. Then cells were observed under fluorescence microscope. Cells incubated with FITC-D7 or FITC-RGD emitted strong fluorescence signal, whereas cells incubated with FITC-V7 emitted weak or no fluorescence signal. The cytoskeleton was stained with rhodamine phalloidin. The nuclei were stained with DAPI; scale bar, 100  $\mu$ m.



**Figure 4.** The model of early ONFH in rabbits was prepared using liquid nitrogen freezing method. (A) Femoral head was frozen by liquid nitrogen. (B) The state of femoral head after frozen by liquid nitrogen. (C) The state of femoral head after thawed with warm saline. Four weeks after modeling operation, the vacant bone lacunae number of the femoral heads after modeling (G-I) was much higher than that of normal femoral heads (D-F). The result confirmed the feasibility of modeling by liquid nitrogen freezing method; magnification,  $\times 50$ ; scale bar, 200  $\mu\text{m}$  (D, G);  $\times 100$ , 100  $\mu\text{m}$  (E, H);  $\times 200$ , 50  $\mu\text{m}$  (F, I).



**Figure 5.** Core decompression followed by filling of scaffolds was performed. A. A bone tunnel of 4 $\times$ 14 mm was made from the greater trochanter to the femoral head. B. A scaffold was stuffed into the cavity after CD. C. The filled scaffold was compacted.

group of CD+ $\beta$ -TCP-D7 scaffolds was superior to the other two groups.

#### *Histological evaluation*

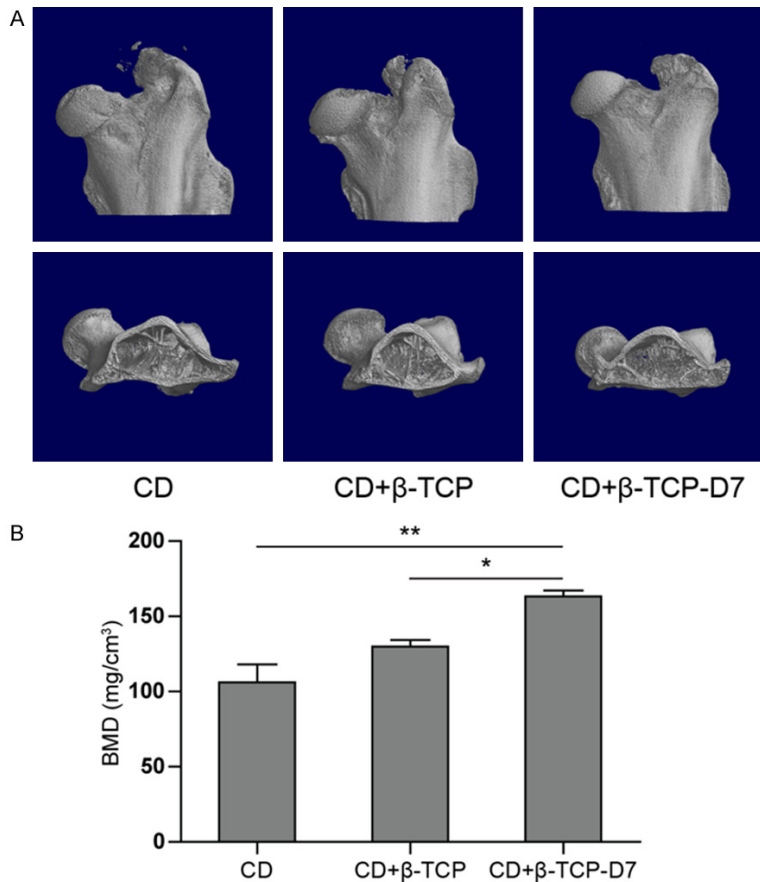
Twelve weeks after surgical treatment, the femoral heads of the rabbits were obtained to evaluate the repair of necrotic femoral heads by histological analyses. Tissue sections were stained by hematoxylin and eosin. Samples in CD+ $\beta$ -TCP-D7 group showed the lowest vacant bone lacunae percentage ( $n=3$ ;  $**P<0.01$ ; **Figure 7**). In order to evaluate the therapeutic effect of three different treatments, IHC staining was completed and analyzed with IPP 6.0

software (Media Cybernetics, Inc., Rockville, MD, USA) to evaluate the expression of COL1 and OCN. The result showed that the expression of COL1 and OCN in CD+ $\beta$ -TCP-D7 group was highest according to the value of integrated optical density (IOD) ( $n=3$ ;  $**P<0.01$ ; **Figures 8 and 9**). These findings indicated that, of three treatments, CD+ $\beta$ -TCP-D7 scaffolds was the best.

#### **Discussion**

ONFH is a common disease and can lead to disability. Currently, most patients with ONFH require total hip arthroplasty (THA) eventually. If





**Figure 6.** Radiographic evaluation of therapeutic effect. Twelve weeks after surgery, Micro-CT analyses were performed. A. The 3D reconstructions showed that the femoral heads became flattened and the load area collapsed in CD and CD+β-TCP groups. The shape of the femoral heads treated with CD+β-TCP-D7 was complete. B. The BMD of femoral heads treated with CD+β-TCP-D7 was highest. These results showed that the therapeutic efficacy of ONFH treated with CD+β-TCP-D7 was superior to the other two groups;  $n=3$ ; \* $P<0.05$  and \*\* $P<0.01$ .

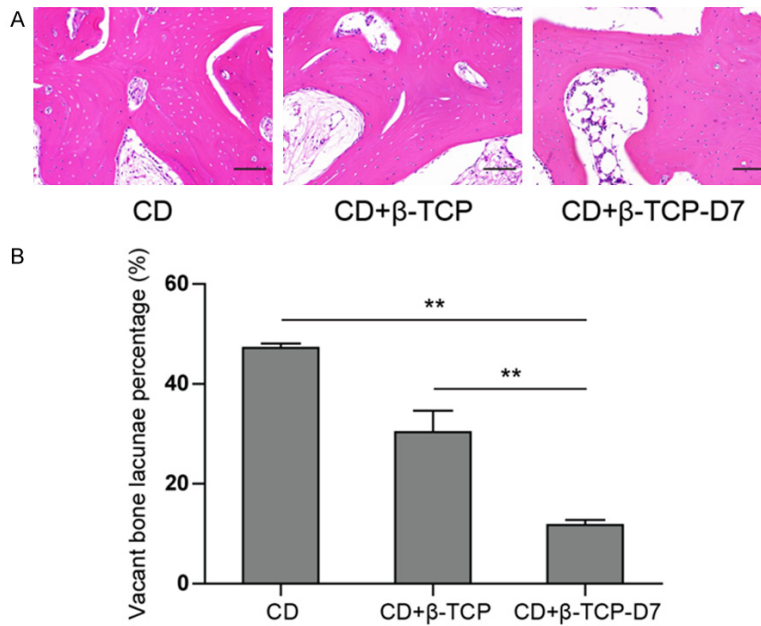
ONFH is diagnosed early before the femoral head collapses, joint-preserving procedures may be selected. CD is a common surgical option for early ONFH. Studies have showed that ONFH is due to elevated intraosseous pressure and decreased blood supply [22]. The rising intraosseous pressure can be reduced and vascularization can be promoted by CD [23]. But it was reported that CD alone has not shown any ability to halt progression of the disease and prevent femoral head from collapsing [24].

The left cavity after CD has been filled with some materials such as tantalum rod or calcium graft materials et al to improve the therapeutic effect [6, 25]. β-TCP is a totally absorbable bioceramic. It has excellent biocompatibility

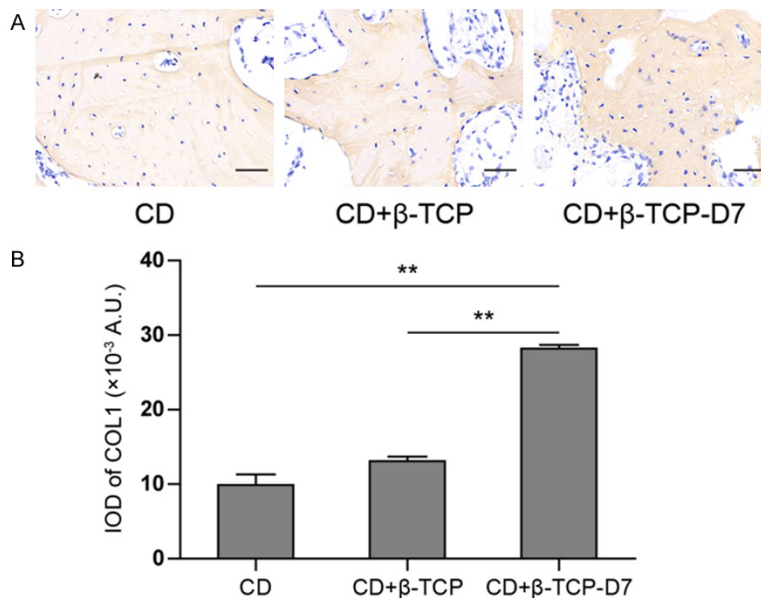
and osteo-conductivity. For these characteristics, β-TCP has become one of widely used bone graft materials. CD followed by filling of β-TCP has been used to cure early ONFH [8]. But the osteogenesis is slower than the absorption of β-TCP [9]. Meanwhile, β-TCP can not be osteogenic [9]. Because the ability of repair and regeneration of necrotic femoral head is weak, the femoral head may collapse due to the imbalance between the weaker ability of osteogenesis and the faster degradation of β-TCP [6].

The role of BMSCs in the repair of necrotic femoral head is very important due to the potential of angiogenesis and osteogenesis [16]. A study showed that CD followed by filling of BMSC-loaded bone matrix scaffold could enhance the repair effect of ONFH [26]. It is feasible to use scaffolds as carriers to recruit BMSCs and promote tissue repair. Surface modification of materials using cell affinity peptides is a common strategy. It has been reported that pig peritoneum-derived acellular matrix modified by E7 peptide, a BMSC affinity peptide, could recruit BMSCs sufficiently and exhibit superior effect on the repair of rabbit cartilage tissue defect [27]. D7 peptide is a BMSC affinity cyclic peptide. Therefore, the functional scaffold, β-TCP-D7, was fabricated and stuffed into the cavity after CD to recruit BMSCs sufficiently to enhance the repair ability of necrotic femoral head, match the degradation rate of β-TCP and elevate the therapeutic efficacy of early ONFH.

Cyclopeptide D7 was found to have high affinity towards C57BL/6 mouse BMSCs through phage display [15]. In our current experiment, we found that D7 peptide can bind to BMSCs without species specificity (Figure 3). Functional β-TCP scaffolds were fabricated by adsorption of D7 peptide on the surface of β-TCP th-



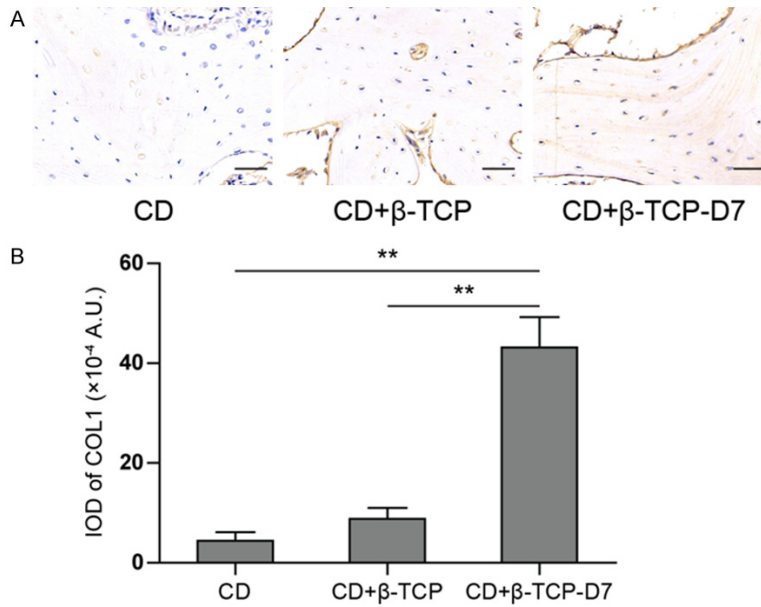
**Figure 7.** Histological evaluation of therapeutic effect by H&E staining. Twelve weeks after surgical treatment, tissue sections of rabbit femoral heads were made and hematoxylin and eosin staining was completed. A. The most vacant bone lacunae number of femoral heads appeared in CD group and the least in CD+β-TCP-D7 group; magnification,  $\times 100$ ; scale bar, 100  $\mu\text{m}$ . B. Compared with the other two groups, samples treated with CD+β-TCP-D7 showed the lowest vacant bone lacunae percentage. The result showed that the therapeutic efficacy of CD followed by filling of β-TCP-D7 scaffolds was superior to the other two methods;  $n=3$ ;  $**P<0.01$ .



**Figure 8.** Histological evaluation of therapeutic effect by IHC staining of COL1. A. The expression of COL1 in CD+β-TCP-D7 group was highest according to the IOD value of three groups; magnification,  $\times 200$ ; scale bar, 50  $\mu\text{m}$ . B. The result indicated that, of three groups, the therapeutic effect of CD+β-TCP-D7 was better than CD and CD+β-TCP;  $n=3$ ;  $**P<0.01$ .

rough adsorption/freeze-drying strategy (Figure 1C, 1D). Subsequently, the adhesion test showed that BMSCs were much easier to adhere to D7 peptide-modified β-TCP scaffolds (Figure 2B). The characteristics of the successfully constructed β-TCP-D7 scaffolds to enhance cell adhesion *in vitro* became the basis of subsequent experiments on the therapeutic effect of early ONFH using CD followed by filling of β-TCP-D7.

The therapeutic effect of CD+β-TCP-D7, CD+β-TCP and pure CD on early ONFH in rabbits was studied. Twelve weeks after surgery, the femoral heads became flattened and the load area collapsed in CD and CD+β-TCP groups. The shape of the femoral heads treated with CD+β-TCP-D7 was complete (Figure 6A). Although the intraosseous pressure of the femoral head is reduced, the blood supply is improved and the pain of the hip is relieved after CD, the mechanical support to the femoral head is weakened. Collapse of the femoral head and subtrochanteric fracture may occur [4, 5, 28]. The support to the femoral head treated with CD+β-TCP was enhanced because β-TCP was filled into the left cavity after CD. The osteogenic repair was slower than the degradation of the material due to the weak repair ability of necrotic femoral head [29]. Meanwhile, it was impossible for rabbits to avoid bearing weight after surgery. So the femoral head also collapsed. In CD+β-TCP-D7 group, better therapeutic effect was obtained due to enhanced repair ability of necrotic femoral head because



**Figure 9.** Histological evaluation of therapeutic effect by IHC staining of OCN. A. The expression of OCN in CD+β-TCP-D7 group was highest according to the IOD value of three groups; magnification, ×200; scale bar, 50 μm. B. The result indicated that, of three treatments, the therapeutic effect of CD+β-TCP-D7 was better than CD and CD+β-TCP; n=3; \*\*P<0.01.

angiogenesis and osteogenesis of the necrotic femoral head was elevated due to the recruitment of BMSCs by β-TCP-D7.

The BMD of the femoral heads treated with CD+β-TCP-D7 scaffolds was highest (**Figure 6B**). In order to further evaluate the therapeutic effect of three different methods, we analyzed vacant bone lacunae rate and expression content of COL1 and OCN in bone matrix of the femoral heads. The vacant bone lacunae percentage in CD+β-TCP-D7 is the lowest (**Figure 7**). The content of COL1 and OCN in bone matrix of femoral heads in CD+β-TCP-D7 group was highest (**Figures 8 and 9**). These results are also consistent with our previous analysis.

In our current study, through the adsorption/freezing-drying strategy, we fabricated functional β-TCP scaffolds which were modified by cyclopeptide D7 (β-TCP-D7). Through *in vitro* cytological experiments, we found that the adhesion, spreading and proliferation of BMSCs can be enhanced by β-TCP-D7 scaffolds. The functional β-TCP scaffolds were stuffed into the bone tunnels of rabbit early necrotic femoral heads after CD. The result of radiographic and histological analyses showed that the therapeutic efficacy of CD followed by filling of β-TCP-D7 was better compared with CD followed by filling of β-TCP and pure CD on early

ONFH. Our study provides a novel option to treat early ONFH.

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#### Disclosure of conflict of interest

None.

**Address correspondence to:** Shui Sun, Department of Joint Surgery, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwuwei Road, Jinan 250021, Shandong, China. E-mail: m15-169106255@163.com

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