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A new bone repair scaffold combined with chitosan/ hydroxyapatite and sustained releasing icariin

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Icariin, a plant-derived flavonol glycoside, has been proved as an osteoinductive agent for bone tissue engineering. A new bone repair scaffold was generated by thorough mixing of icariin and chitosan/ hydroxyapatite (icariin-CS/HA) using freeze-drying technigue. Characteristics of morphology, mechanical properties, biocompatibility, drug release behavior and bone repair abilities *in vivo* were evaluated. The results show that drug loading process of icariin did not affect physical structure of CS/HA composite significantly but decreased mechanical properies of CS/HA composite, which happened with a high dosage; icariin-CS/HA had favorable cell compatibility and promoted osteogenic differentiation of hBMSCs; the controlled release of icariin was satisfactory and the release retained after 90 d *in vitro*. In addition, icariin-CS/HA scaffolds had favorable osteoconduction and osteoinduction *in vivo*, and could fill bone defect sites and stimulate newborn bone tissues formation at early stage. On the basis of these data, icariin-CS/HA is believed to be an optical bone repair scaffold for tissue engineering.

icariin, chitosan, hydroxyapatite, sustained release, biomimetic, bone tissue engineering, scaffold

Epimedium sagittatum is a traditional Chinese medical herb and widely used in the therapies of fractures, bone and joint diseases, impotence and senility in China for hundreds of years. Icariin ($C_{33}H_{40}O_{15}$, molecular weight: 676.67), a typical flavonol glycoside, is considered to be a major pharmacological component of *Epimedium sagittatum*. In previous studies[1] we found that icariin had osteoinductive activity. It enhanced proliferation of bone marrow-derived mesenchymal stem cells (BMSCs) via accelerating cell cycles, and promoted osteogenic differentiation of BMSCs by increasing the expressions of alkaline phosphatase (ALP), osteocalcin and type I collagen. More evidence has indicated icariin can improve the osteogenesis from mesenchymal stem cells and suppress the activities of osteoclasts *in vitro*[2,3], thereby it exerts its bone-protective functions by increasing bone formation and inhibiting bone resorption[4,5]. Additional studies have demonstrated that icariin has the ability to enhance the expression of osteogenic-related mRNA level in osteoblasts[6], and has a direct stimulatory effect on the proliferation and differentiation of pre-osteoblastic MC3T3-E1 cells in a BMP- and Runx2-dependent manner[7]. Taken together, such results indicate that icariin is a potential osteogenic inductive agent and can be used in bone repair. What is more, icariin is chemically stable, and has high melting point, thus benefiting its extraction from raw herb and combination to form artificial bone material usually used for bone defect

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repair and/or drug-loading scaffolds.

In this study, we combined icariin with chitosan/hydroxyapatite (icariin-CS/HA) scaffolds, tested the feasibility of icariin-CS/HA scaffolds for bone tissue engineering by investigating cell biocompatibility, drug release behavior and repair bone defects *in vivo*.

1 Materials and methods

1.1 Materials

Icariin (98.3% purity) was purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Chitosan (degree of deacetylation ≥90.0%, viscosity <100 cps, biomedical grade) was purchased from Shanghai Bo'ao Biological Technology Co. (Shanghai, China). Analytical grade acetic acid, calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Guangzhou Xinmei Chemical Industry Co. (Guangzhou, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from the HyClone Co. (South Logan, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromile (MTT) were purchased from the Gibco BRL (Grand Island, USA).

1.2 Preparation of icariin-CS/HA scaffolds by *in situ* hybridization

A certain amount of icariin was dissolved in ethanol, and then added to chitosan solution which was prepared by dissolving chitosan in 2% (v/v) acetic acid aqueous solvent. Then the icariin-CS solution was stirred for 2 h, followed by the slow addition of $Ca(NO_3)_2$ and KH_2PO_4 (ratio of Ca/P=1.67) to prepare icariin-CS/HA solution precursor (concentration of CS was 2.5%, the final mass ratio of CS/HA was 1/2). Whereupon, the precursor was homogenized by 4 h vigorous stirring and centrifuged to remove the air bubbles. The resulting solution was cast by a glass mold (4.0mm in diameter, 15mm in length) and placed at 4℃ for 6 h, −10℃ for 3 h, then freeze-dried. After they were fully dried, icariin-CS/HA scaffolds were soaked in 4% (wt/v) NaOH and icariin-saturated deionized water for 10 h, and then rinsed with icariin-saturated deionized water for several times to reach neutral pH. Finally, scaffolds were frozen and freeze-dried. We prepared 10^{-7}, 10^{-6}, 10^{-5} mol icariin-CS/HA scaffolds with the same procedures as the preparation of CS/HA scaffolds but without touching icariin as the controls. All scaffolds were sterilized with 20 kGy ^{60}Co and stored in vacuum packages at room temperature before subsequent uses.

1.3 Morphology and mechanical properties

The micro-structure of icariin-CS/HA scaffolds was examined by scanning electron microscopy (SEM, Hitachi Co., Tokyo, Japan) and HE staining. The density, porosity and pore diameter of scaffolds were evaluated with the methods described by Pei et al.[9]. And the mechanical properties of scaffolds in wet state were detected with universal testing machine (5567 type, Instron Co., Canton, USA).

1.4 Cell biocompatibility

(i) Cell preparation. Human bone marrow-derived mesenchymal stem cells (hBMSCs) were isolated and cultured using the method described by Sekiya et al.[8]. Cells in the third generation were used in investigations.

(ii) Cell proliferation. 1.5 g sterilized scaffolds minces were soaked in 15 mL DMEM supplemented with 10% FBS and penicillin-streptomycin and kept shaking at 37℃ for 72 h. Then solid material was removed by millipore filtration (pore size = 20 μm), thus the supernatant was collected for further use as the conditional cell media. The hBMSCs were seeded at a density of 2×10^3 cells/well in a 96-well plate and incubated for 24 h prior to the addition of 150 μL icariin-CS/HA (icariin dosages were 10^{-7}, 10^{-6} and 10^{-5} mol separately) conditional cell media, while control cells were with fresh DMEM. After 48 h, MTT (20 μL, 5 mg/mL) was added to the media and the cells were kept culturing for another 4 h. Finally, the culture media was removed before DMSO (150 μL) was added to dissolve purple formazan, and absorbance (OD) at 570 nm (ref at 630 nm) was measured by universal microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, USA). The cell proliferation rate (100%) = $OD_{treatment}/OD_{control}\times100\%$.

(iii) Alkaline phosphatase (ALP) activity. hBMSCs were cultured in 6-well plates at a density of 1×10^6 per well, and were treated with icariin-CS/HA conditioned and the control media as above for 10 d. Cells were then lysed in 100 μL deionized water and homogenized by ultrasound at 4℃. ALP activity and total protein content in cell lysates were measured using an alkaline phosphatase activity kit and a micro-BCA Assay kit (Beyotime Co., Haimen, China), respectively, and ALP ac-

tivity was normalized for the corresponding total protein concentration (U/g).

(iv) Scanning electron microscopy (SEM) observation. hBMSCs were seeded on scaffolds at a density of $1\times10^5/cm^2$. 10 d later, cells of the scalffolds were rinsed with PBS and fixed with glutaraldehyde. SEM was performed to observe the cells attached on to the scaffolds surfaces.

1.5 Icariin release behavior *in vitro*

The release behavior of icariin from icariin-CS/HA scaffolds was measured by ultra performance liquid chromatography (UPLC, Waters Co., Milford, USA). Briefly, icariin-CS/HA scaffolds were soaked in 5 mL phosphate-buffered solution (PBS, pH 7.4) and maintained at 37℃ and kept shaking gently at 10 rpm. At 1, 2, 3, 5, 10, 15, 20, 30, 60 and 90 d, the 5 mL PBS was pipetted out (stored at 4℃ for UPLC examination) and replaced by adding the same amount of fresh PBS. For analysis of icariin concentration, all the samples were centrifuged at 3000 $r\cdot min^{-1}$ for 10 min and 0.5 mL supernatants were isolated, and after adding 0.5 mL of acetonitrile, the mixed solution were re-centrifuged at 6000 $r\cdot min^{-1}$ for 10 min and then 20 μL supernatants were applied for UPLC analysis. Data of peak area integration was done using Empower 2 software. Icariin released from scaffolds was calculated according to standard curve and the percentage of icariin released was accumulated.

1.6 Bone repair capability *in vivo*

(i) Surgery procedures. 60 Male white New Zealand rabbits (clean grade, 2.0±0.2 kg, Nanfang Hospital Laboratory of Animal, Guangzhou, China) were allocated into groups of icariin-CS/HA, CS/HA and control (no treatment) randomly (n=12). After anesthetized by pentobarbital sodium (30 mg/kg), a 1.5 cm segment defect was made in the right radius of the animals using a small saw. All bone tissue and interosseous membrane at the defect site was cleaned away. The bone defect areas were filled with icariin-CS/HA with different icariin dosages (the icariin-CS/HA groups), CS/HA scaffolds alone (the CS/HA group) or no scaffolds (the control group). All animals received a prophylactic dose of cefazolin for 3 days following the surgery.

(ii) Emission computed tomography (ECT). Four weeks after the surgery, four rabbits in every group were selected randomly for ECT examination. 3 h after administration of ^{99m}Tc-MDP (5 MBq/kg), the right forelimb was scanned with a single photon emission computed tomography (GE Co., Milwaukee, USA). Data collection parameters: static plane, matrix of 256×256, magnification 2 and 500 K counts per planar view. Thereafter, region of interesting (ROI) of the same size was chosen and quantitative counting was performed, the mean of ROI = value/ $\text{area}_{\text{selected}}$.

(iii) X-ray. X-ray images (Siemens Co., München, Germany) of right forelimb were taken 4, 8 and 12 weeks after implantation. Radiograph conditions: voltage 40 kV, electric current 50 mA and exposure time 0.2 s.

(iv) Bone mineral density (BMD). 12 weeks after the surgery, specimens of radius were used for BMD examination with dual energy X-ray densitometry (DEXA, Norland Co., Fort Atkinson, USA). Bone mineral content (BMC) of defect site was analyzed by computer and BMD was calculated according to the equation: BMD = BMC/ $\text{area}_{\text{selected}}$.

(v) Histological observation. All of radius specimens were fixed in buffered formalin, and decalcified in EDTA solution. Following routine histological processing 5-μm-thick tissue slices were obtained and stained with haematoxylin and eosin (H&E) and observed under a light microscope (Olympus Co., Tokyo, Japan).

1.7 Statistical analysis

All the experiments were performed in triplicate，and representative experiments are shown. Values were expressed as mean ± S.D. Statistical analysis between two samples was performed using Student's t-test or one way ANOVA. In all cases, P<0.05 was considered as significant.

2 Results

2.1 Morphology and mechanical properties of icariin-CS/HA scaffolds

As we expected, icariin-CS/HA composite had abundant homogeneous pores with the diameter around 110 μm (Figure 1), which provided appropriate 3-demensional micro-structure for cells. Icariin loading did not change the physical structure of CS/HA composite significantly, but decreased mechanical properties of CS/HA composite with higher dosage. As shown in Table 1, 10^{-5} and 10^{-6} mol icariin-CS/HA have lower fracture strength and elastic modulus; 10^{-5} mol icariin-CA/HA has decreased elastic modulus (P<0.05 vs. blank CS/HA scaffolds).

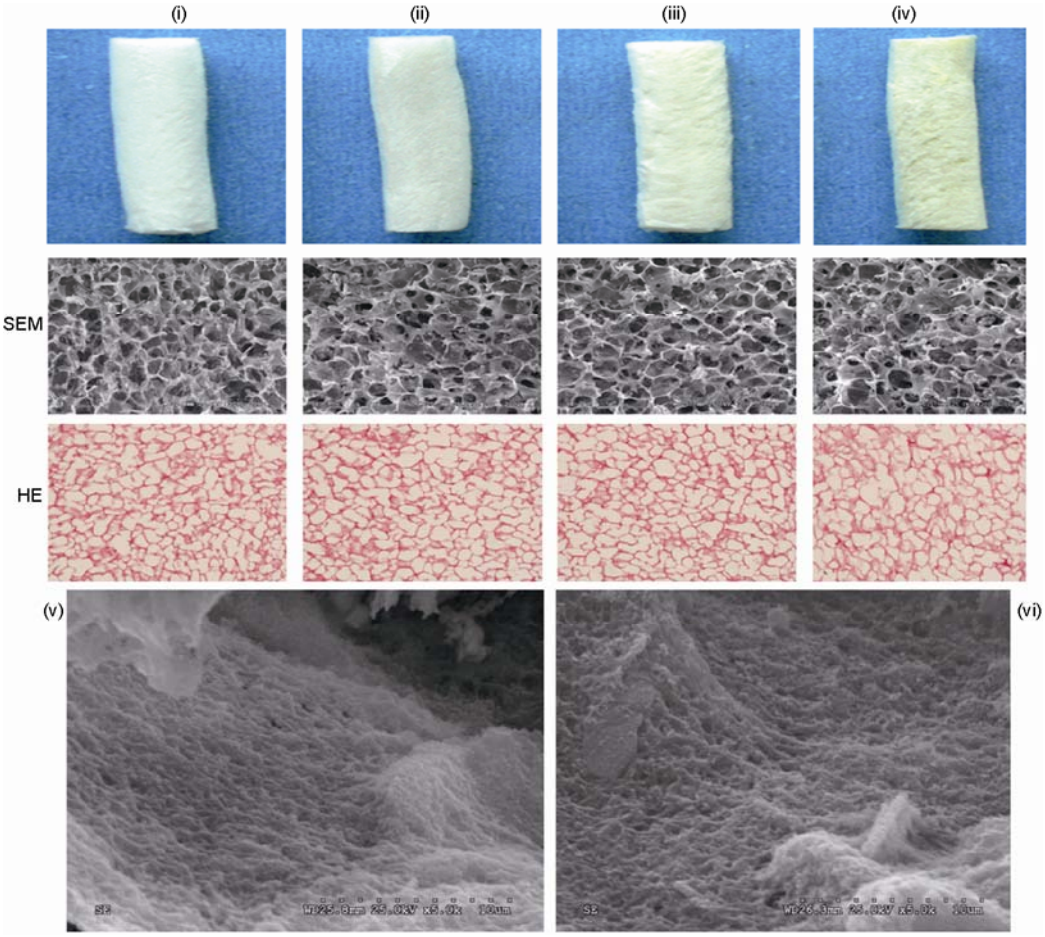

Figure 1 Icariin-CS/HA scaffolds. SEM, 200×; HE, 200×. (i) CS/HA scaffold, (ii), (iii) and (iv) icariin-CS/HA scaffolds with 10^{-7}, 10^{-6}, 10^{-5} mol loading icariin, respectively, (v) the surface-micro structure of CS/HA (SEM) 5000×, (vi) the surface-micro structure of 10^{-5} mol icariin-CS/HA (SEM) 5000×.

Table 1 Morphology and mechanical properties of icariin-CS/HA scaffolds[a)]

Scaffolds	*n*	Porosity (%)	Pore diameter (μm)	Density (kg/m^3)	Fracture strength (MPa)	Elastic modulus (kPa)
CS/HA	5	88.7±2.3	112.6±20.5	71.5±2.6	1.5±0.2	37.5±3.4
10^{-7} mol icariin-CS/HA	5	87.1±3.2	118.9±21.6	70.1±2.8	1.4±0.2	34.6±4.1
10^{-6} mol icariin-CS/HA	5	88.8±3.9	116.6±24.6	73.0±3.7	1.2±0.2	31.0±4.4*
10^{-5} mol icariin-CS/HA	5	85.4±3.9	124.1±28.5	67.8±3.8	1.1±0.2*	28.7±4.9*
F		1.057	0.199	2.256	3.902	4.217
P		0.395	0.895	0.121	0.029	0.022

a) Compared with CS/HA, $P<0.05$.

2.2 Cell biocompatibility of icariin-CS/HA scaffolds

As in Figure 2(a), CS/HA conditioned media did not change the proliferation of hBMSCs after cultured for 48 h, indicating that CS/HA composite had undetectable (if any) cytotoxicity. However, icariin-CS/HA conditioned media demonstrated the capacity to inhibit cell proliferation significantly when icariin dosages were 10^{-6} and 10^{-5} mol (Figure 2(b)), suggesting that high concentration of icariin could inhibit cell proliferation, and this result was consistent with that in previous reports[1]. However, after cells were cultured at high density, the cell proliferation rate was very low, and after they were cultured with conditioned media for 10 d, the ALP levels of hBMSCs in three icariin-CS/HA groups increased 7~9 folds compared with the CS/HA and control group ($P<0.001$, Figure 2(b)), suggesting that hBMSCs had been differentiated into osteoblasts. Meanwhile, hBMSCs on CS/HA scaffolds were scattering with smooth surface and little extracellular matrix

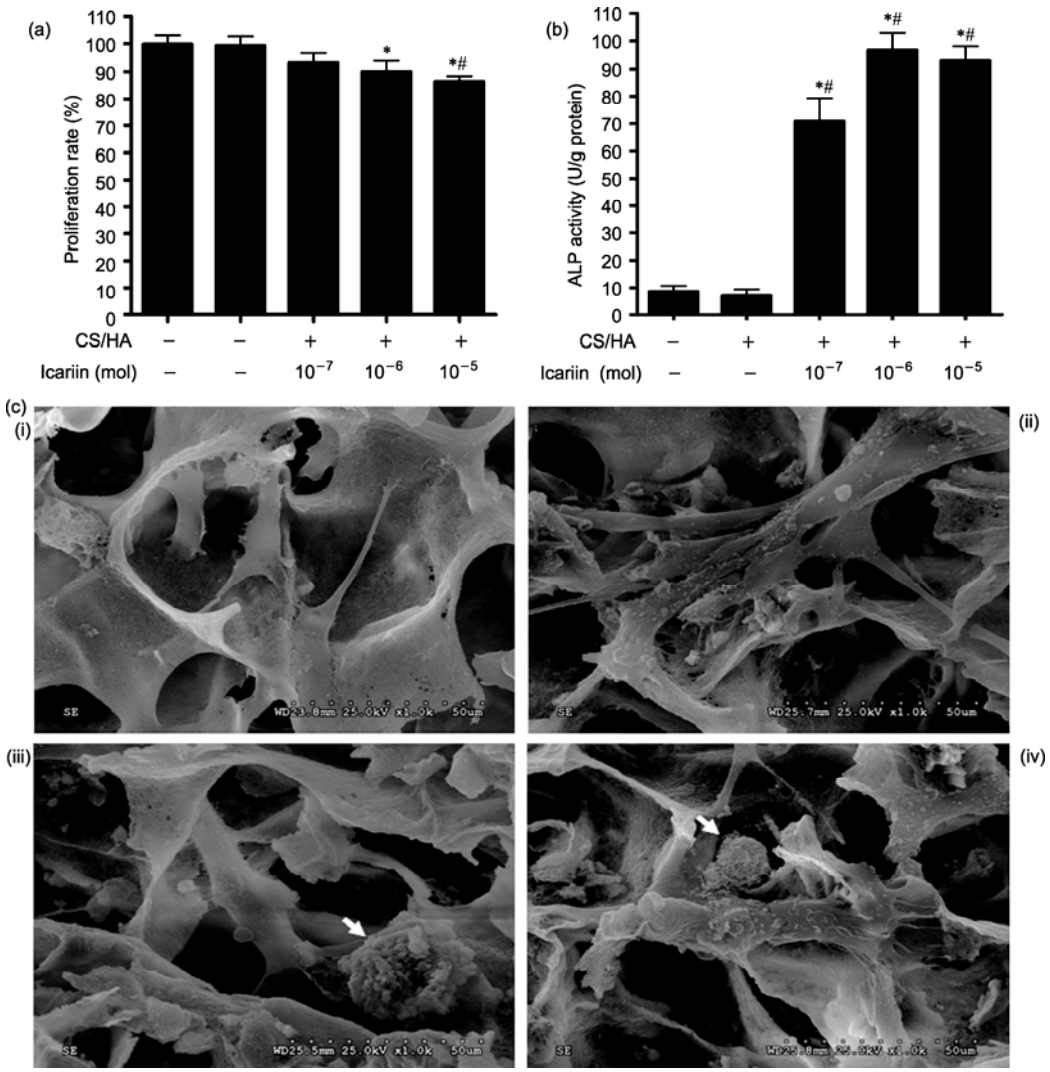

Figure 2 Cell biocompatibility of icariin-CS/HA scaffolds. (a) Effects of conditioned media of CS/HA or icariin-CS/HA scaffolds on proliferation of hBMSCs (n=6, F=3.172, P=0.031), $^{*}P$<0.05 vs. control, $^{\#}P$<0.05 vs CS/HA; (b) effects of conditioned media of CS/HA and icariin-CS/HA scaffolds on osteogenic differentiation of hBMSCs (n=4, F=68.116, P=0.000), $^{*}P$<0.001 vs. control, $^{\#}P$<0.001 vs. CS/HA; (c) cells on the surface of CS/HA and icariin-CS/HA scaffolds on 10 d (SEM, 1000×). (i) CS/HA scaffold; (ii), (iii) and (iv) icariin-CS/HA scaffolds with the loading icariin dosage of 10^{-7}, 10^{-6} and 10^{-5} mol respectively, arrow indicateds the calcium nodules-like structure.

secretion, but hBMSCs on icariin-CS/HA scaffolds were in overlapping growth with abundant fine granules secretion on the cell surface and calcium nodule-like structure formation between cells (Figure 2(c)).

2.3 Drug release behavior of icariin-CS/HA scaffolds *in vitro*

The release behavior of icariin-CS/HA composite *in vitro* was investigated by UPLC examination (Figure 3(a)). The absorption peak of CS/HA is at 0.56 min, while the absorption peak of icariin is at 1.30 min. Icariin releasing from scaffolds was calculated based on standard curve and demonstrated as the accumulated percentage of icariin (Figure 3(b)). On day 1 to day 3, approximately 25% icariin was released; then the speed decreased and about 40%—60% icariin was released by day 20. 90 d later, there was still a certain amount of icariin remaining in CS/HA scaffolds.

2.4 Bone repair capability of icariin-CS/HA *in vivo*

Rabbit radius defect model has been widely accepted as animal model for the bone repair research[10,11]. As reported before, the self-repair ability of bone defect control was low, therefore, medullary cavity at both ends of defect site was closed during 4—8 w, and the defect was obviously visible 12 weeks postoperatively (Figure 4(a)—(d)). Investigated by ECT, a sensitive index of bone formation at early stage, osteogenesis *in situ* could

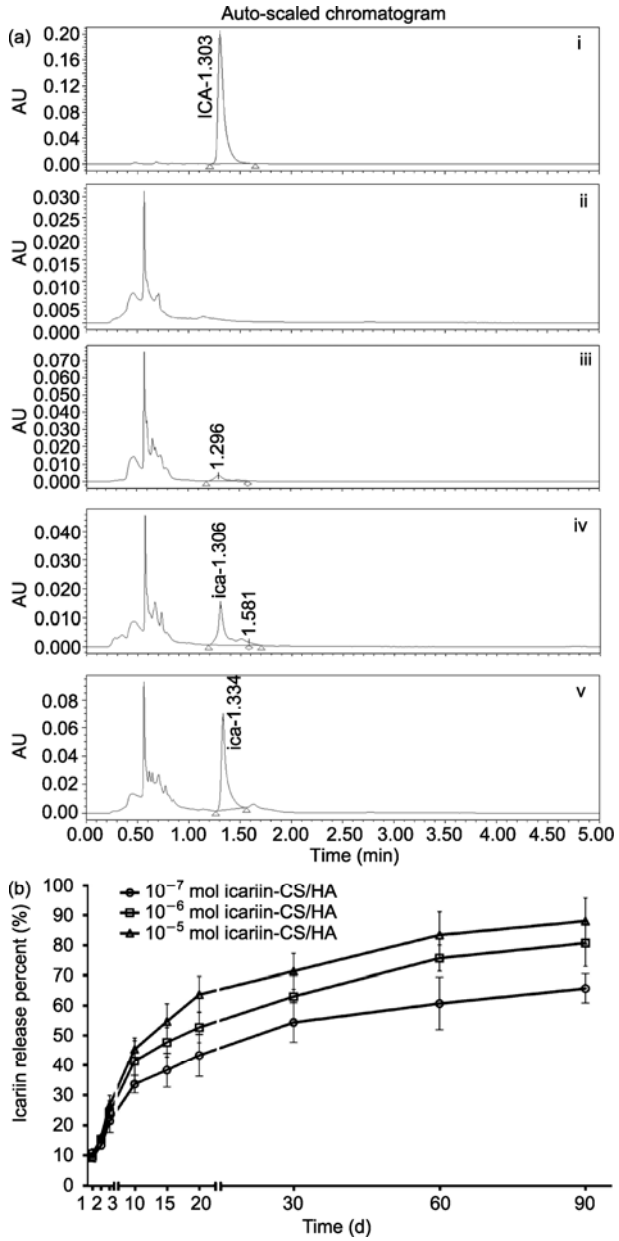

Figure 3 Release behavior of icariin from icariin-CS/HA scaffolds *in vitro*. (a) The UPLC maps of icariin-CS/HA. (i) icariin, (ii) CS/HA, (iii), (iv) and (v) icariin-CS/HA with the loading icariin dosage of 10^{-7}, 10^{-6}, 10^{-5} mol, respectively. (b) Drug release curves of icariin-CS/HA (n=3).

be monitored by ^{99m}Tc-MDP density. At week 4, ROI values of three icariin-CS/HA groups (icariin dosages were 10^{-7}, 10^{-6} and 10^{-5} mol each) and CS/HA group were higher than that of the bone defect control group (P<0.001). Furthermore, ROIs of the 10^{-6} and 10^{-5} mol icariin- CS/HA groups were higher than that of the CS/HA group (P<0.001, Figure 4(a)). At the same time, obvious bony callus at defect sites with icariin-CS/HA scaffolds could be seen by X-ray. Considering both ECT and X-ray results, icariin-CS/HA composites had osteoinduction functions at early stage. The X-ray examination thereafter showed a large amount of bony callus formed and the healing of defects at week 8 and bone marrow cavity reappeared at week 12, in icariin-CS/HA groups (Figure 4(b)).

The quantization of bone formation content at week 12 of each group was carried out by DEXA. The bone mass density (BMD) values of three icariin-CS/HA groups and the CS/HA group are higher than that of the bone defect control group (P<0.001), and that of the 10^{-6} and 10^{-5} mol icariin-CS/HA groups is higher than that of CS/HA group (P<0.001, Figure 4(c)). Synthesis results of imaging examination (ECT, X-ray and BMD) indicate that bone repair abilities in three icariin-CS/HA groups were better than the CS/HA group.

Histological observations (Figure 4(d)) at different intervals show that after implanted in defect site for 4 w, CS/HA composite was degraded partially with microporous structure loss and inflammatory cell infiltration. Scaffolds kept on degrading at week 8, and newborn fibrous and cartilage tissues crept along scaffolds. Residual CS/HA scaffolds were segmented and encapsulated by newborn tissues and bone defect site was substituted by cartilage and bone tissues at week 12. As icariin dosage increased, the degradation speed of icariin-CS/HA composite increased and the disintegration and fragmentation could be seen earlier at week 4, and there were large amounts of newborn cartilage around the scaffolds. At week 8, scaffolds degraded mostly and some of the cartilage tissues transformed into bone tissues. At week 12, icariin-CS/HA scaffolds were completed and cartilage tissues were substituted by bone tissues which were arranged in disorder, and small medullary cavities reformed in the center.

3 Discussion

It has always been of interest to find repairing materials with osteoinduction and bone-mimic structure. With the progress in understanding *in situ* tissue's regeneration, composite materials loaded with osteoinductive active factor have attracted much attention, and the so-called "growth factors + scaffolds" , with the functions of osteoinduction and bone supporting, has been an attractive strategy for bone tissue reconstruction[12–14]. Although bone morphogenetic protein (BMP), one of typical growth factors, has showed favorable bone induction

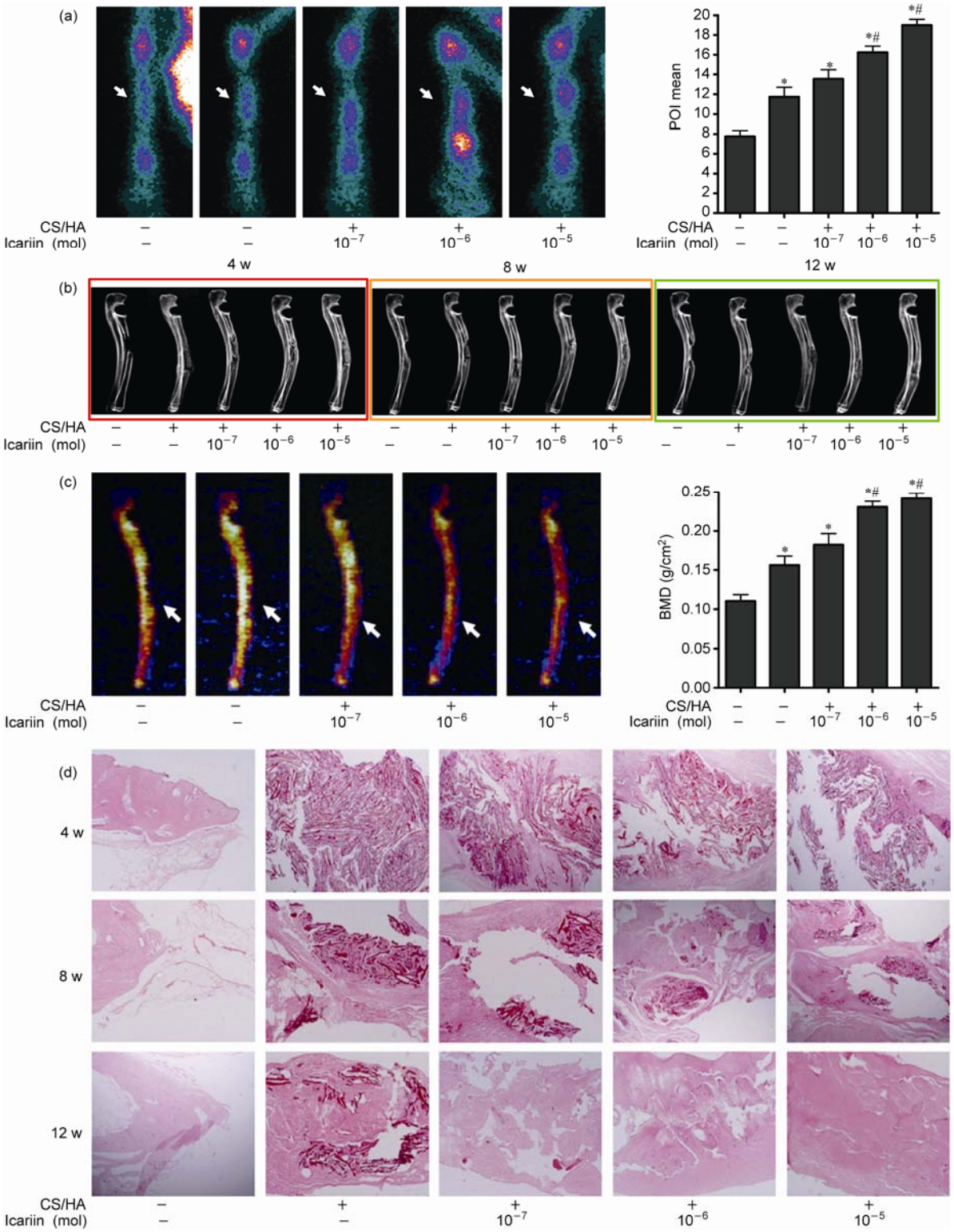

Figure 4 Bone repair capability of icariin-CS/HA scaffolds *in vivo*. (a) ECT examination and ROI quantitative analysis at 4 w (n=4, F=32. 010, P=0.000, $^{*}P$<0.001 vs. control, $^{\#}P$<0.001 vs. CS/HA); (b) X-ray; (c) BMD examination and quantitative analysis at 12 w (n=4, F=28.720, P=0.000, $^{*}P$<0.001 vs. control, $^{\#}P$<0.001 vs. CS/HA). The arrow indicates position of quantitative analysis; (d) histological observation (H&E staining, 40×).

activity, its natural "fragile" properties require high-standard scaffolds with no damage and with excellent sustained release ability after having been loaded. Moreover, in order to avoid inactivation during disinfection, aseptic manipulation is strictly demanded during the whole process from preparation *in vitro* to application *in vivo*, and so on. These disadvantages of BMP, therefore, hinder the preparation of new-type bone repair material made with "growth factors + scaffolds". Trying to solve this problem, we studied icariin, a type of plant-derived flavonoid, which has been confirmed to be a bone inductive factor in our previous study, to have high melting point (231℃), to be stored easily, and to tolerate radiation sterilization. All the advantages mentioned above lead us to study the feasibility for icariin-CS/HA scaffolds.

In the beginning, icariin-CS/HA compound was prepared in a bionic way. In this way, both CS, a natural polysaccharide possessing good biocompatibility and plasticity, and HA, an essential component of natural bone, had already been widely applied to the construction of materials in tissue engineering[15–17]. *In situ* composition with $Ca(NO_3)_2$ and KH_2PO_4 could get homogeneous dispersed nanometer HA (nano-HA), avoiding using traditional methods, which is easily aggregated and hard scattered. Using water as a pore-forming agent, freeze-drying technique can produce satisfactory micropores and decrease toxicity of scaffolds. In the view of microstructure, in CS/HA composite, the combination of nano-HA and CS polymer, which conjugate with electrostatic interaction, van der waals, hydrogen-bond and hydrophobic bond, load is transferred between mineral phase and organic phase, thus improving mechanical properties of scaffolds. Besides, as nano-HA can be used by cells directly and nontoxic CS can be degraded by tissues, CS/HA scaffolds have excellent bioactivities.

There were two major problems in the preparation of icariin-CS/HA scaffolds. One was keeping pharmacological activity of icariin and the other was keeping physical properties of scaffolds. Liu et al.[18] showed that physiochemical characteristics of icariin were stable, and even incubated with artificial gastric juice, the glycoside structure of icariin still kept normal. In our study, though icariin was added into scaffold precursor solution at early stage of preparation and treated with acid, alkaline and radiation, the results of icariin characteristic absorption peak examined by UPLC, and the results of cells ALP activities and bone repair tests all indicated that icariin kept good pharmacological activity. These data revealed that the preparation procedure of icariin-CS/HA scaffold did not damage pharmacological activity of icariin. From the perspectives of scaffolds, icariin encapsulated by CS or absorbed by HA might affect physicochemical properties of CS/HA composite. In our preliminary tests, the maximum icariin loading dosage of a 4.0 mm×15.0 mm CS/HA scaffold was 3.0×10^{-5} mol, which did not interfere with micropores structure significantly. However, the maximum icariin loading dosage in this study was 1.0×10^{-5} mol, and the data of SEM and scaffolds section observation showed that icariin loading procedure did not affect density, porosity, and the micro surface structure of scaffold. Although fracture strength and elastic modulus decreased in icariin with high dosage, the mechanical strength of CS/ HA composite basically met the requirements of bone repair at non-weight bearing sites. Thus, icariin-CS/HA scaffolds with 1.0×10^{-5} mol icariin is acceptable.

In this study, conditioned media from CS/HA composite did not affect the proliferation of hBMSCs, suggesting that CS/HA composite was safe and nontoxic. Conditioned media from icariin-CS/HA, however, inhibited cell proliferation partially with high doses. As we all know that proliferation and differentiation are contradictive aspects of cell life activity, proliferation activity is inhibited when cells differentiate vigorously, and vice versa[19–21]. Therefore, high concentration icariin might compromise the proliferation of hBMSCs with improving osteodifferentiation, because we found that conditioned media from icariin-CS/HA increased ALP expression in hBMSCs and more extracellular matrix secreted by hBMSCs grew on icariin-CS/HA scaffolds than those on CS/HA scaffolds. These data indicate that icariin-CS/HA scaffolds have good biocompatibility and osteoinductive activity.

Controlled/sustained release is a necessary index of scaffolds with drug loading[22,23]. Because of its instability, growth factors (such as BMP) can only be loaded on scaffolds by soaking, absorbing or encapsulation, and accordingly, the controlled release effects are unsatisfying. The differences between icariin-CS/HA scaffold and traditional physical absorption scaffold in drug loading methods are obvious. Icariin was dissolved in ethanol first and then added into CS/HA precursor solution and homogeneous distribution of icariin was obtained by stirring. Therefore, after having been freeze-dried, icariin was absorbed by nano-HA, encapsulated

by CS or stored in micro interspaces of the scaffold. When contacted with water, CS expanded with water immersion, then the icariin encapsulated in superficial layer was released, and a quick release could be seen at the initial stage. As time went on, the swelling reached equilibrium, and icariin could only be released by slow diffusion or scaffold degradation, thus icariin release rate was decreased. In addition, nano-HA particles may affect icariin release behavior partially, because there are adsorption-desorption processes between icariin and surfaces of nano-particles. If the surface area of HA becomes larger, icariin cannot diffuse until greater intermolecular forces are surmounted, and therefore icariin releasing time is extended.

Osteoconduction and osteoinduction are important aims of bone tissue engineering scaffolds, because broad spatial structure provides necessary carrier for creeping substitution of newborn tissues (osteoconduction) and osteoinduction promotes ossification of newborn tissues at early stage and improves loading functions of newborn bone. The histology of this study shows that CS/HA scaffolds have excellent histocompatibility and exert osteoconduction function by substitution with newborn cartilage and bone tissues. Besides the above-mentioned advantages, icariin-CS/HA scaffolds can promote endochondral ossification and accelerate bone repair and reconstruction. Combined with results of imaging observation, data of bone repair tests *in vivo* suggest that icariin-CS/HA scaffolds have favorable abilities of bone induction and repair.

In conclusion, this study proves that icariin-CS/HA scaffolds have the capacity of bone repair, and the advantages in both osteoconduction and osteoinduction. Considering its low price, simple preparation and sterilization procedure, icariin-CS/HA scaffolds can be used in research and applications of bone tissue engineering.

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