

The effect of loading icariin on biocompatibility and bioactivity of porous β -TCP ceramic

X. Zhang · Y. Guo · D. X. Li · R. Wang ·
H. S. Fan · Y. M. Xiao · L. Zhang ·
X. D. Zhang

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Abstract In order to enhance the ability of calcium phosphate-based biomaterials for bone defect repair, icariin (Ica), one natural product with ability of promoting osteoblasts differentiation in vitro and enhancing bone formation in vivo, was loaded into porous β -tricalcium phosphate ceramic (β -TCP) disks. The obtained Ica-loaded porous β -TCP ceramic (Ica/ β -TCP) disks were characterized by SEM. The SEM photos indicated that the disks had porous structure and the surface morphology of the porous β -TCP ceramic (β -PTCP) disks had no obvious difference from the Ica/ β -TCP disks. The Ica release curve of Ica/ β -TCP disks showed a burst release during the first 1 day and the concentration of released Ica during the first 3 days had low cytotoxicity. The loading Ica in Ica/ β -TCP disks hardly affected the attachment and morphology of Ros17/28 cells, however, the Ica/ β -TCP disks were favorable to supporting the proliferation and differentiation of Ros17/28 cells better compared with the β -PTCP disks. There was plenty of bone-like apatite formed on the surface of Ica/ β -TCP disks soaked in SBF solution for three days. After back intramuscular implantation of rats for three months, no obvious osteogenic evidence was detected in β -PTCP disks, but

new bone formation was observed in Ica/ β -TCP disks. Fibrous tissues and slight inflammatory reaction was also found in the Ica/ β -TCP disks and β -TCP disks. Therefore, the loading Ica did not change the biocompatibility of β -TCP ceramic, but enhanced the bioactivity of β -TCP ceramic in vivo. The Ica/ β -TCP ceramic had potential to be used for bone defect repair.

1 Introduction

With good biocompatibility and bioactivity, calcium phosphate-based bioactive materials such as calcium phosphate ceramics and bone cement have been used extensively as bone defect repair or substitution material in clinic [1–5], especially for the repair of periodontal defects [1], certain orthopedic applications [2], and maxillofacial surgery [5]. Although the osteoinductivity of calcium phosphate-based materials in vivo had also been largely reported [6–10], their osteoinductivity needed to enhance further in order to meet the demand of bone repair [11]. To enhance the osteogenesis rate and therapeutic effects of calcium phosphate-based materials for bone repair, the usually adopted method was adding growth factors such as bone morphogenetic proteins (BMPs) into materials except improving the surface morphology and constitutions of materials [11–13]. Many researches had shown that BMPs could promote bone regeneration [11–13]. Hoshino et al. [11] reported that the β -TCP implants were ineffective in promoting rib repair when the periosteum was absent, but the rhBMP-2/ β -TCP composite implants were able to promote rib bone regeneration in the presence or absence of the periosteum. Jansen et al. [12] proved that bone formation occurred only in rhBMP-2 loaded porous Ca–P cement discs after 10 weeks of subcutaneous implantation.

X. Zhang · Y. Guo · D. X. Li · H. S. Fan ·
Y. M. Xiao (✉) · X. D. Zhang
Engineering Research Center in Biomaterials,
Sichuan University, 610064 Chengdu, China
e-mail: xymzl2000@126.com

X. Zhang · R. Wang
College of Chemical Engineering,
Sichuan University, 610065 Chengdu, China

D. X. Li · L. Zhang
Sichuan Academy of Chinese Medicine Science,
Institute of Pharmacology & Toxicology,
610041 Chengdu, China

However, the high cost, rapid degradation and easy to lose activity of growth factors limit their clinical use. Therefore, we attempted to adopt some natural medicaments with the proven effect of promoting osteogenesis and lower costs than BMPs to enhance the osteoinductivity of biomaterials.

Icariin (Ica) was a natural product isolated from Herb *Epimedium* (HEP) and a major pharmacological active constituent of HEP which was a potent enhancer of bone healing [14–16] and applied in many Chinese formulas for anti-osteoporosis [17]. HEP extract has been proven to have therapeutic effect on animal models of osteoporosis induced by ovariectomy [18–23], increase *cbfa1* expression in the bone of ovariectomized rat [21], and stimulate the proliferation of osteoblast and osteoblast-like UMR 106 cells [24, 25]. Further studies demonstrated that Ica was able to induce osteogenic differentiation and increase alkaline phosphatase (ALP) activity in MC3T3-E1 cells and mouse primary osteoblasts [26–28], up-regulate the mRNA expression of the osteoblast marker genes runt-related transcription factor 2 (*Runx2*) [27], induce the mRNA expression of BMP-4 [27], and enhance TGF-1 level in serum of beagle [29]. Recent studies demonstrated that CPC scaffolds loaded with Ica accelerated bone regeneration at 4 and 6 weeks after implantation in the mouse calvarial defect model, which proved that Ica was able to enhance bone formation in vivo and be used as an osteoinductive drug for regenerative medicine [28].

Then considering the characteristics of Ica and calcium phosphate-based materials, we hypothesized that composites of calcium phosphate-based biomaterials and Ica could promote better osteoblast differentiation in vitro and osteoinductivity in vivo compared with calcium phosphate-based biomaterials due to the effect of Ica. In the present study, Ica/ β -TCP composite was prepared by loading Ica into porous β -tricalcium phosphate ceramic (β -PTCP) and its biocompatibility, bioactivity and effect on osteoblast proliferation and differentiation were evaluated.

2 Materials and methods

2.1 Cytotoxicity of Ica

The cytotoxicity of Ica to Ros17/28 cells was evaluated. Ros17/28 cells were provided by West China Hospital, Sichuan University. The cells were routinely grown and maintained in RPMI-1640 medium containing 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were seeded on 24-well plates and fresh media containing different concentrations of Ica (purity $\geq 98\%$, purchased from Nanjing TCM institute of Chinese materia medica) were added. The cell density of every well was 2×10^4 /ml. Cells were incubated under static conditions in

a humidified atmosphere with 5% CO₂ at 37°C. Culture media containing different concentrations of Ica were changed every three days.

The proliferation of cells was measured by MTT method. Cells were harvested at 2, 4, and 6 days. 100 μ l MTT solution (5 mg/ml) was added to every well and cultured for 4 h at 37°C. After removal of the medium, the formazan pigment by reducing MTT was dissolved with dimethyl sulphoxide (DMSO). To a 96-well plate, 150 μ l pigment solution was added and read in an ELISA reader at 490 nm.

2.2 Preparation and characterizations of the Ica/ β -TCP disks

Tricalcium phosphate (TCP) powders were provided by Engineering Research Center in Biomaterials, Sichuan University. Porous tricalcium phosphate ceramic (PTCP) was prepared by foaming TCP powder with H₂O₂ porogen and sintering at 1100°C. The porosity of PTCP was about 70%. PTCP disks with a thickness of 2 mm and a diameter of 12 mm were obtained by cutting with a slicer, grinding with whetstone, washing with deionized water and drying at 60°C. Phase compositions for PTCP disks fabricated were measured with X-ray diffraction (XRD). The XRD spectra of the fabricated disks shown in Fig. 1 agreed well with the standard XRD card of β -TCP (card No. 09-0169), which indicated that the component of the disks was β -TCP with high crystalline degree. β -PTCP disks were divided three groups and soaked respectively in 600 μ l Ica solution with concentration of 0.1 mg/ml for 1 h, then dried at 50°C under vacuum condition, washed with deionized water for three times and dried. By this way, the Ica-loaded β -PTCP ceramic (Ica/ β -TCP) disks were obtained. The surface morphology of the disks was observed by scanning electron microscopy (SEM, JSM5900).

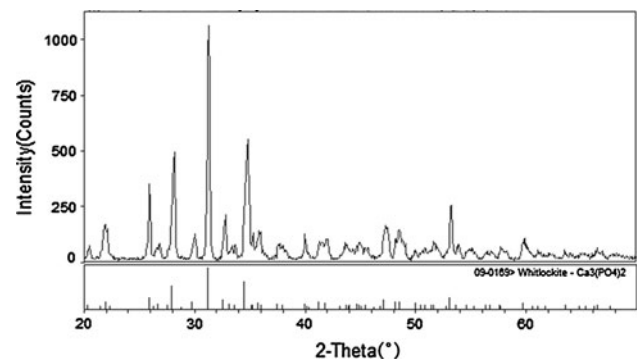


Fig. 1 XRD pattern of ceramic disk

2.3 Release behavior of Ica from Ica/ β -TCP disks in vitro

The in vitro release of Ica from Ica/ β -TCP disks was performed in PBS at 37°C. The disks were placed in a 24-well plate and 1 ml PBS solution was added in each well. The plate was incubated at 37°C, and 0.9 ml PBS solution in each well was decanted and replenished with fresh PBS on 3 h, 1, 2, 3, 5, 7, and 10 days. The concentration of Ica in PBS solution was measured by UV detection at a wavelength of 270 nm. All experiments were carried out in triplicate.

2.4 The effect of loading Ica in Ica/ β -TCP disks on cell proliferation, differentiation and morphology

The cytocompatibility of Ica/ β -TCP disks to Ros17/28 cells was evaluated. The β -PTCP disks and Ica/ β -TCP disks sterilized by autoclave prior to use. Ros17/28 cells were seeded on β -PTCP disks and Ica/ β -TCP disks in a 24-well plate with the density of 2×10^4 /ml and maintained in a humidified atmosphere with 5% CO₂ at 37°C. Culture medium was changed every three days. Cells were harvested at 2, 4, and 6 days. In the same way, MTT method was used to measure cell proliferation.

The cell-seeded ceramic disks were rinsed with phosphate buffer solution for two times after cultured for 2 and 4 days. The cells seeded on the disks were treated by repeatedly freezing and thawing method. Then the alkaline phosphatase (ALP) activity was quantitatively measured at 490 nm on an ELISA reader using the alkaline phosphatase detection kit (a product of Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions. The total content of protein was determined with a micro bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce Protein Research Products) according to the manufacturer's specifications. ALP activity was normalized to total protein content of the cells. All experiments were carried out in quintuplicate.

After cultured for 2, 4, and 6 days, samples were fixed by 2.5% glutaraldehyde and treated in a series of ethanol solution. Then, critical point drying and coating with gold were performed, respectively. Cell morphology on the surfaces of samples was observed under SEM.

2.5 SBF incubating experiment

Simulated body fluid (SBF) was prepared according to Kokubo's method [30]. SBF incubating experiment was carried out to compare the ability for forming bone apatite on surface of Ica/ β -TCP disks with that of β -PTCP disks. Samples were incubated in SBF solution (10 ml) at 37°C for 3 days, washed with deionized water and dried at 50°C.

Then the surface morphology of samples was observed under SEM after coating the samples with gold.

2.6 Biocompatibility and bioactivity evaluation in vivo

To evaluate the biocompatibility of Ica/ β -TCP disks, the Ica/ β -TCP and β -PTCP disks were implanted into back muscle of 18 Wistar Albino rats. All animals were anesthetized by intravenous injection of nembutal and the operation were finished under standard aseptic conditions. Two disks were implanted into back muscle of every rat. One and three months post implantation, the rats were killed by pentobarbital overdose. For histological examinations, the disks were fixed in 10% phosphate-buffered formalin. Then the disks were decalcified, dehydrated, embedded in paraffin and stained with toluidine blue (TB) according to standard procedures.

2.7 Statistical analysis

All data were expressed as means \pm standard deviation. Significance level between two groups was determined by the Student's *t*-test. $P < 0.05$ was considered statistically significant.

3 Results and discussion

3.1 Cytotoxicity of Ica

We examined the cytotoxicity of Ica with different concentrations to Ros17/28 cells for six days. Figure 2 showed the cell proliferation at different concentrations of Ica. It could be seen that the concentration of Ica ranging from 1×10^{-5} to 1×10^{-7} M had low cytotoxicity toward Ros17/28 cells. However, Ica at concentration of 5×10^{-5} M exhibited a strong inhibition on the proliferation of Ros17/28 cells. Zhao et al. [27] examined the cytotoxicity of Ica with different concentrations to MC3T3-E1 cells for 72 h and found that the concentration of Ica ranging from 10^{-5} to 10^{-10} M had low cytotoxicity toward MC3T3-E1 cells and in contrast, Ica with concentration of 10^{-5} M had the highest cytotoxicity, which was consistent with our results. However, many reports demonstrated that Ica with concentration of 10^{-5} M produced promoting effect on UMR106 cell and human osteoblast proliferation [24, 25, 32]. Therefore, the optimal concentration of Ica with low cytotoxicity toward osteoblasts was equal or less than 10^{-5} M (6.8 μ g/ml).

3.2 Surface microstructures

The SEM photos displayed in Fig. 3 showed the surface morphology of disks. These photos illustrated that there

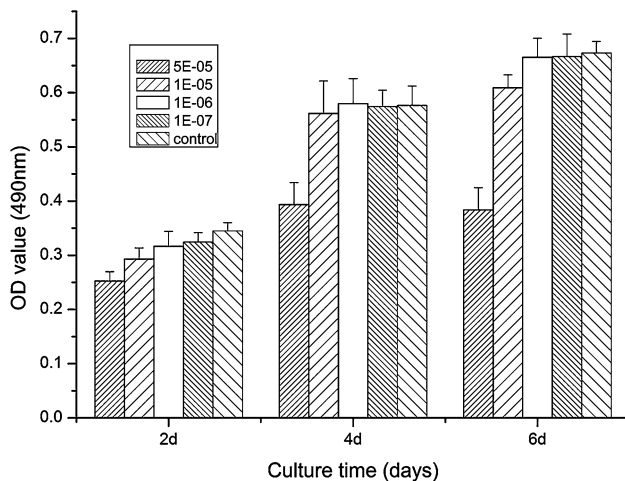


Fig. 2 Toxicologies of different concentrations of icariin toward Ros17/28 cells (means \pm SD, $n = 3$)

were no obvious differences about the surface microstructures between β -PTCP disks and Ica/ β -TCP disks, besides there were some Ica precipitate on the surfaces of Ica/ β -TCP disks. Because the microstructure of biomaterials would affect cell attachment and proliferation in vitro and their osteoinductivity in vivo, the differences of biocompatibility, cytocompatibility, bioactivity and osteoinductivity between Ica/ β -TCP and β -PTCP disks should arise from Ica. Compared with Fig. 3c, it could be seen in Fig. 3d that the tiny Ica particles distributed uniformly on the surfaces and inner of Ica/ β -TCP disks. For instance, some tiny particles seen within rectangles of Fig. 3d were the precipitate of Ica. Therefore, the Ica/ β -TCP disks in which Ica distributed uniformly in β -TCP disks could be obtained by the method of soaking and vacuum drying. Two kinds of disks had porous structure and the diameter of the pores was about 200–500 μ m (Fig. 3a and 3b). There were many micropores about 1–4 μ m in the macroporous walls (Fig. 3c and 3d) so that the ceramic disks had good connectivity. The porosity distribution and interconnectivity of ceramics were beneficial for tissue in-growth and nutrient transport [31]. So the Ica/ β -TCP and β -PTCP disks should make for tissue in-growth when they were used to repair for defect bone tissue in vivo.

3.3 In vitro Ica release study

To ensure the biocompatibility of Ica/ β -TCP disks in vitro and in vivo and verify the utilization of β -TCP disks as Ica carriers, the time course of Ica release from Ica/ β -TCP disks was examined. Figure 4 showed the release curve of Ica from Ica/ β -TCP disks in vitro for 10 days, which revealed a sustained release of Ica. A burst release during the first 1 day was observed for samples. Then a period of continued moderate to slow release ensued. In the study of

cytocompatibility of Ica/ β -TCP disks, the culture medium was changed every three days. Therefore, the Ica concentration released from Ica/ β -TCP disks during the first 3 days was the most important. As shown in Fig. 4, the concentration of Ica released from Ica/ β -TCP disks in PBS solution during the first 3 days was around 10^{-5} M (7.3 μ g/ml). The optimal concentration of Ica for promoting differentiation of osteoblasts was around 10^{-5} M [27, 28], so the obtained Ica/ β -TCP disks should be favorable to supporting cell differentiation.

3.4 Cell proliferation, differentiation and morphology

MTT assay was used to evaluate cell proliferation on Ica/ β -TCP disks. Figure 5 showed the results of proliferation of the Ros17/28 cells cultured on Ica/ β -TCP disks and β -PTCP disks, respectively. From Fig. 5 it could be seen that no significant difference of cell proliferation was detected on disk surfaces of Ica/ β -TCP and β -PTCP ($P > 0.05$) after cultured for 2 and 4 days. Only after 6 days culture, the proliferation of cells was higher ($P < 0.05$) on Ica/ β -TCP disk surfaces than that of β -PTCP disk surfaces. These results indicated that the obtained Ica/ β -TCP disks loading Ica possessed good cytocompatibility to Ros17/28 cells. Many reports had proved that Ica with concentration of 10^{-5} M produced promoting effect on UMR106 cells and human osteoblasts proliferation [24, 25, 32], and Ica concentrations ranging from 10^{-5} M to 10^{-10} M had low cytotoxicity toward MC3T3-E1 [27] and Ros17/28 cells. Therefore, in the present study, the results of Ros17/28 cell proliferation on Ica/ β -TCP disks proved that the highest Ica concentration released from Ica/ β -TCP disks in culture media during the first 3 days was around 10^{-5} M, which was consistent with the results of Ica release study. The results of cell proliferation also showed that the loading Ica influenced the response of β -TCP ceramic to Ros17/28 cells. Then, we speculated that the loading Ica in vivo might change the biological response of β -TCP ceramic and then promote bone regeneration.

In addition, Ica/ β -TCP disks enhanced ALP activity of cultured Ros17/28 cells compared to the β -PTCP disks (Fig. 6). ALP is one of the marker enzymes for functional osteoblasts. It had been reported that Ica could induce osteogenic differentiation and increase ALP activity of MC3T3-E1 cells and mouse primary osteoblasts [26, 27]. And Ica exerted its potent osteogenic effect through induction of Runx2 expression, production of BMP-4 and activation of BMP signaling [27]. In our study, the enhancing ALP activity of Ica/ β -TCP disks might also be exerted by the effect of loaded Ica. So the Ica/ β -TCP disks might be a favorable biomaterial applied to bone defect repair or substitution due to its ability of promoting osteoblastic differentiation.

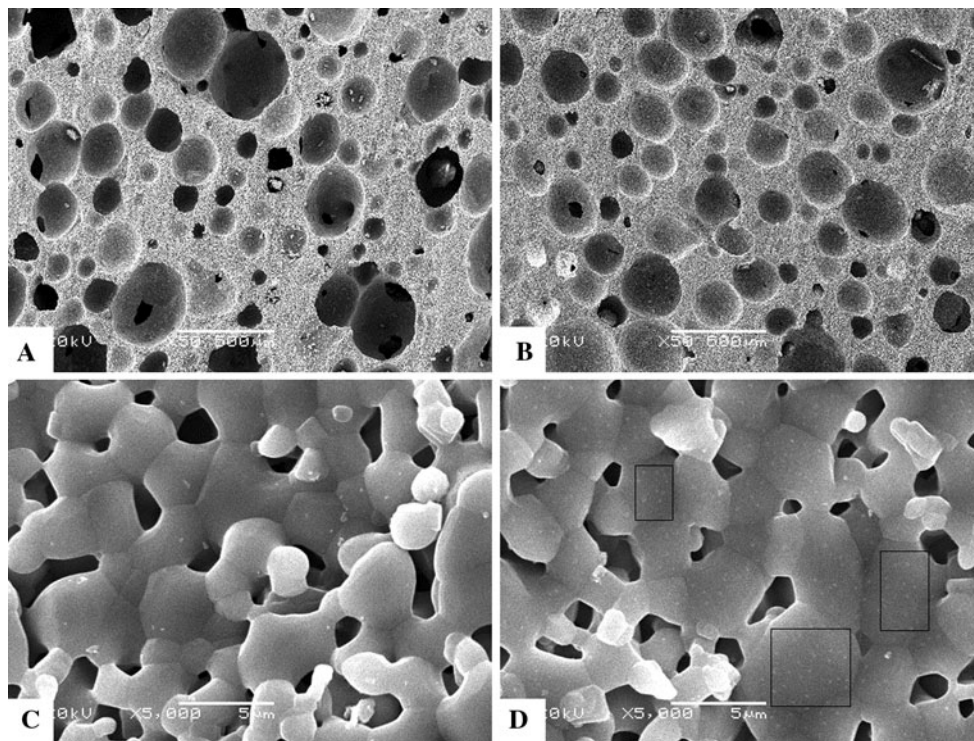


Fig. 3 Surface morphology of (a) and (c) β -PTCP disks, (b) and (d) Ica/ β -TCP disks

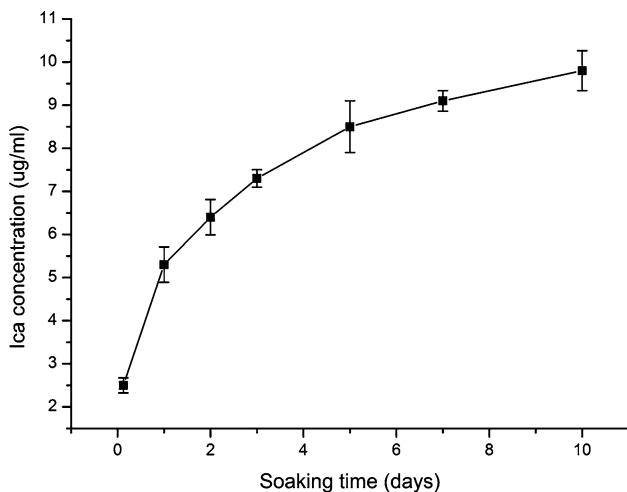


Fig. 4 The in vitro release curve of icariin from Ica/ β -TCP disks. The values represent means \pm SD ($n = 3$)

Figure 7 showed the surface morphology of Ros17/28 cells on disk surfaces. Although the results of cell proliferation were different on two kinds of disk surfaces, there were no obvious differences of cell morphology. The cells on the disk surfaces spread very well and many cells took on spindly structure, stretched out fibrillar pseudopods and closely catch hold of the surfaces of disks or others cells after two days culture. There were no obvious changes of cell morphology on disk surfaces after four days culture

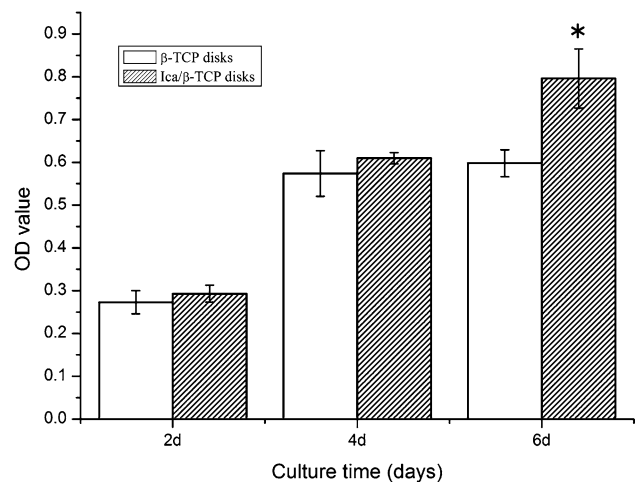


Fig. 5 The proliferation of Ros17/28 cells on Ica/ β -TCP disks and β -PTCP disks at culture time of 2, 4, 6 days. Cell seeding density was 2×10^4 /ml (means of 15 readings \pm SD, $n = 5$). * $P < 0.05$

compared to that of two days culture besides increasing cell number. Then the whole surfaces of two kinds of disks were almost covered by cells after six days culture. Therefore, although Ica was able to promote Ros17/28 cell proliferation and enhance ALP activity, Ica had no effect on cell morphology. The reason might be that attachment of cells depended on the surface properties of materials and Ica was unable to change the surface properties of β -PTCP ceramic disks.

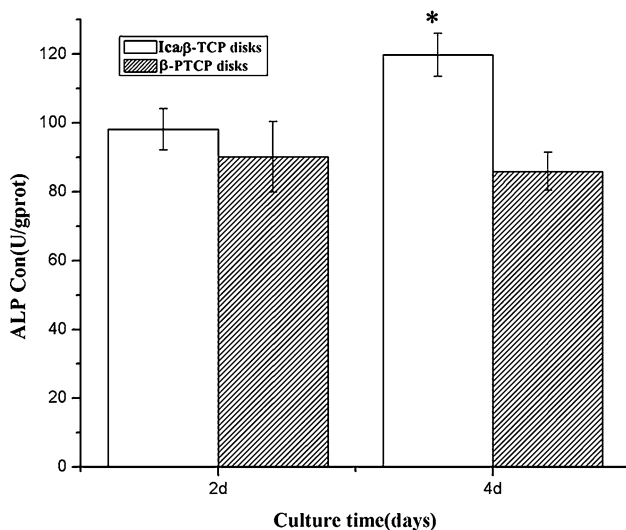


Fig. 6 ALP activity of Ros17/28 cells on Ica/β-TCP and β-PTCP disks (means of 15 readings \pm SD, $n = 5$). * $P < 0.01$

3.5 Bioactivity in vitro

Simulation in vitro was a kind of usual method of evaluating bioactivity of implanted materials. All the reported bioactive materials were able to induce a bone-like apatite layer on their surfaces in vitro biomineralization. The prerequisite for a biomaterial to be bioactive after implanted in vivo was its ability to induce bone-like apatite growth on its surface in SBF [33, 34]. Therefore, bone-like apatite formed on the surface of Ica/β-TCP disks in SBF solution was an important characterization of Ica-loaded β-TCP ceramics which possessed good bioactivity in vitro. The SEM photos in Fig. 8 showed the surface morphology of two kinds of disks incubated in SBF for three days. From the photos, it could be seen that plenty of bone-like apatite formed on the surface of Ica/β-TCP disks and β-PTCP disks after incubated in SBF, and there were no obvious differences between them. Many studies have proven that local ion concentration and microporous structure of pore wall were key factors affecting the formation of bone-like apatite on calcium phosphate ceramic [35]. Because the Ica loaded β-PTCP disks was unable to change the main component and microstructure of the β-PTCP disks, the Ica/β-TCP disks had similar bioactivity as the β-PTCP disks in vitro. So these ceramic disks might possess good ability of osteoinductive formation of new bone when they were implanted in vivo.

3.6 Biocompatibility and bioactivity in vivo

The TB staining images of the samples harvested from 1 to 3 months implantation in vivo were shown in Fig. 9. At one month post-implantation, some inflammatory cells,

such as neutrophils and lymphocytes were seen in two kinds of disks. Fibrous tissues were observed growth into the pores of and some bone-like tissue formed around pore walls and in pores of two kinds of disks. By comparing Fig. 9a, b, the amount of bone-like tissue formed in Ica/β-TCP disk was more than that of β-PTCP disk. After back intramuscular implantation for three months, degradation profile of the disks was observed obviously and the diameter of pores enlarged. Macrophages appeared around pore walls and slight inflammatory reaction was still found. Fibrous tissues continued growth into the pores of disks. After implantation for three months, no obvious osteogenic evidence was detected in β-PTCP disks (Fig. 9d). However, new bone formation was observed in Ica/β-TCP disks (Fig. 9c). These results indicated that Ica/β-TCP disks had good biocompatibility and bioactivity in vivo. At the same time, chondrocyte-like was also observed in pores of Ica/β-TCP disks (Fig. 9c), which implied that some new bone formation in Ica/β-TCP disks might pass through the process of endochondral ossification.

In osteoinductivity study of materials, materials were usually implanted in muscle of animals to eliminate the effect of bone tissue on osteogenesis in materials. Many studies have proven that porous calcium phosphate ceramics could induce bone formation in soft tissue of dogs, monkeys and goats [9, 10, 36, 37]. But it was difficult to observe the osteoinductivity of calcium phosphate ceramic implanted in soft tissue of rats and mice [37, 38]. Kondo et al. [10] and Yuan et al. [36] reported that β-TCP has osteoinductivity after implantation in dog dorsal muscles without use of bone marrow cells or osteoinductive cytokines. However, Uemura et al. [38] reported that β-TCP/BMO composite (BMO, bone marrow-derived osteoprogenitor cells) with or without osteogenic medium were implanted into subcutaneous sites of syngeneic rats for 24 weeks, mature bone and lots of blood vessels were observed only in the β-TCP/BMO composite with osteogenic medium. Therefore, the same bioactive biomaterials showed different osteoinductivity after implanted in different kinds of animals [7] and it was difficult to observe the osteoinductivity of β-TCP in soft tissue of rats and mice.

Zhao et al. [28] reported that Ica was able to enhance bone formation in a mouse calvarial defect model and might be effective as an osteoinductive drug for bone regeneration. In order to verify further the osteoinductive effect of Ica, rat was chosen as a animal model. We hypothesized that the loading Ica could enhance osteoinductivity of β-TCP ceramic and the enhanced effect could be reflected in muscles of rats. In the present study, new bone formation was observed in Ica/β-TCP disks after intramuscular implantation of rats for three months, whereas no evidence of bone formation in β-TCP disks,

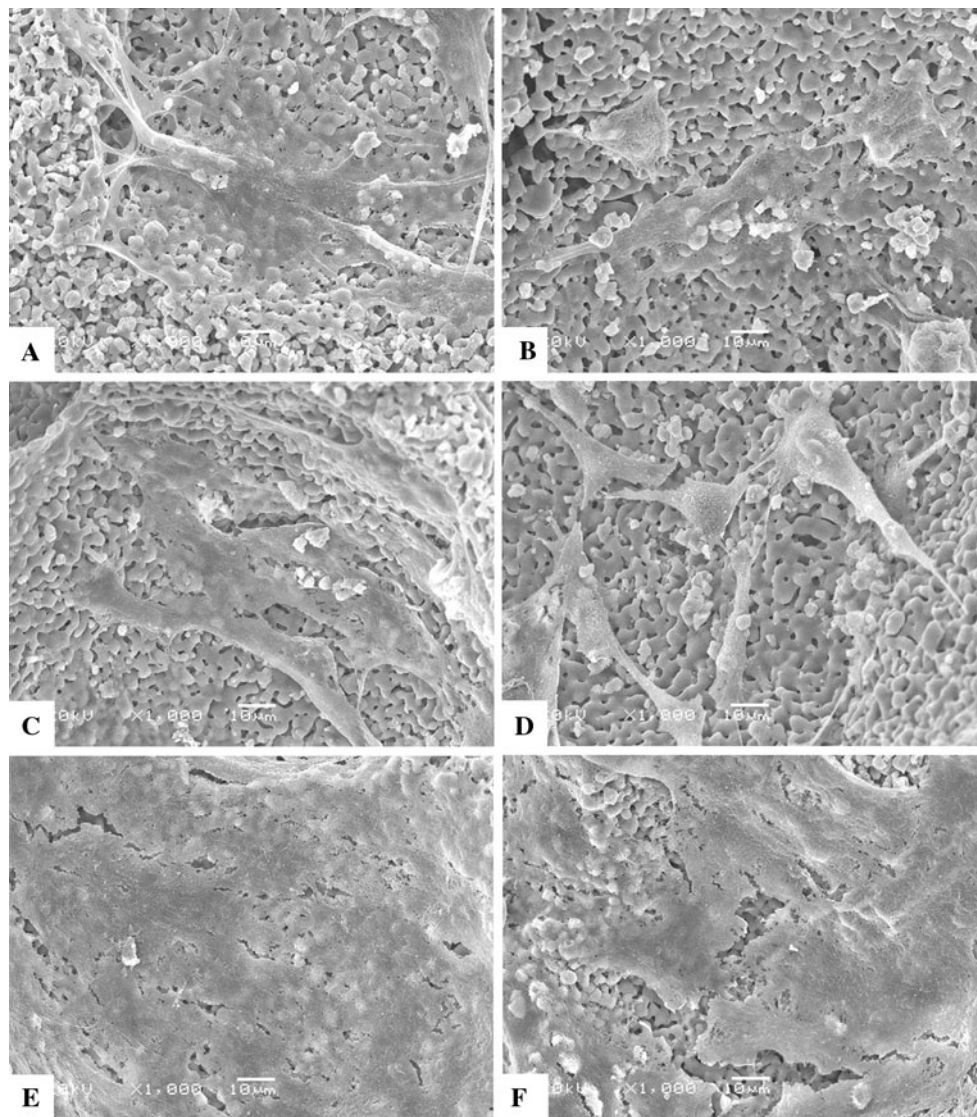


Fig. 7 Surface morphology of cells culture for: (a) and (b) 2 days; (c) and (d) 4 days; (e) and (f) 6 days. (a), (c) and (e) Ica/β-PTCP disks; (b), (d) and (f) β-PTCP disks

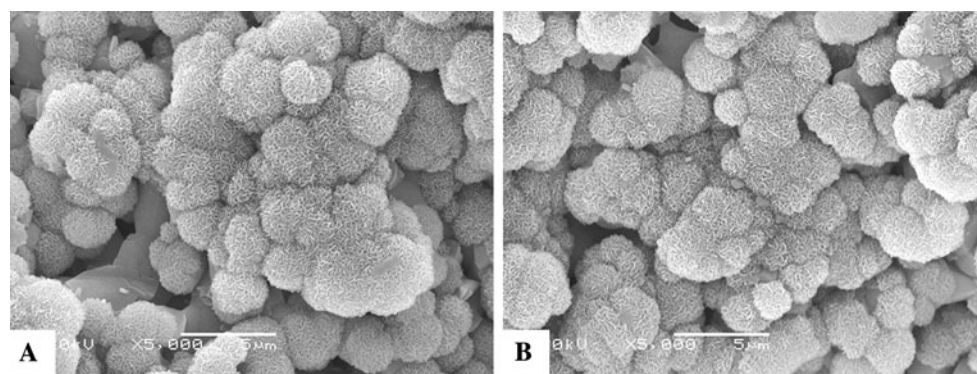


Fig. 8 Surface morphology of disks incubated in SBF for 3 days (a) β-PTCP disks, (b) Ica/β-TCP disks

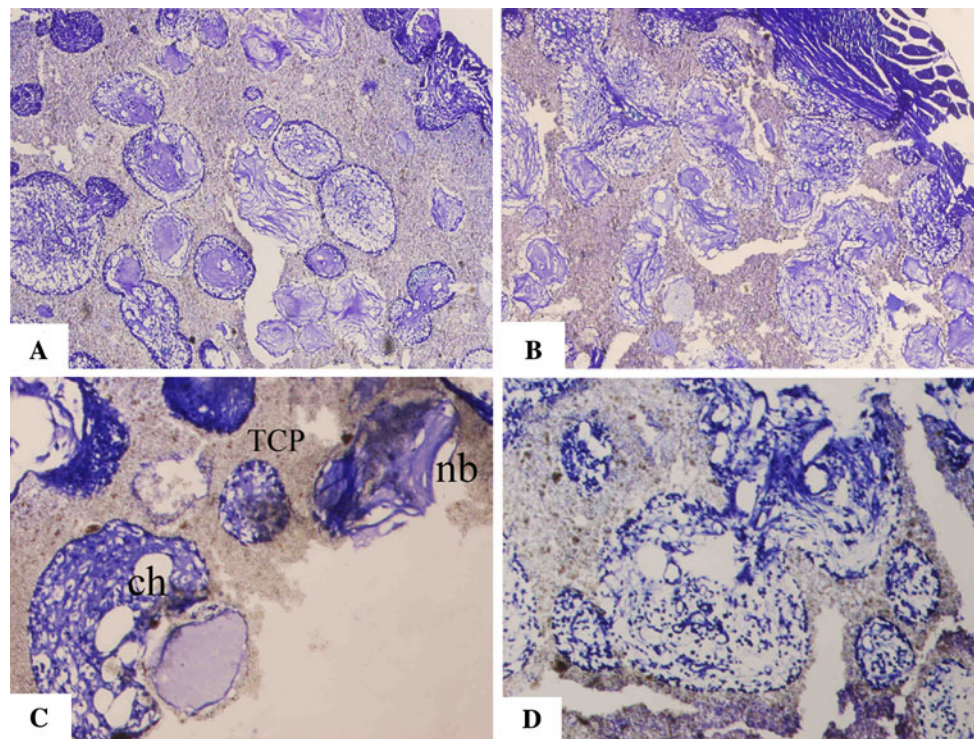


Fig. 9 The TB staining images of the samples harvested from 1 to 3 months after back intramuscular implantation of rats. (a) and (c) Ica/ β -TCP disks, (b) and (d) β -PTCP disks. (a) and (b) 1 month,

(c) and (d) 3 months. TCP Ica/ β -TCP, nb newly formed bone, ch chondrocyte-like. Original magnification $\times 100$

which proved that the loading Ica in Ica/ β -TCP disks could enhance the osteoinductivity of β -TCP ceramic.

Although the Ica/ β -TCP disks promoted new bone formation compared to β -TCP ceramic, a stronger effect was expected. In vivo the released Ica would be removed from the site of the implant and the level of 10^{-5} M was probably not achieved. Maybe the optimization of loading of Ica and delivery system would better promote bone regeneration [28]. However, the present findings further verified Zhao's research [28] that Ica should be effective as an osteoinductive drug for bone regeneration. Therefore, the Ica combined calcium phosphate ceramics was a good choice for bone regeneration.

4 Conclusions

The Ica-loaded β -PTCP ceramic disks were obtained by soaking method. There was a burst release during the first 1 day for Ica/ β -TCP disks and the concentration of released Ica during the first 3 days was around 10^{-5} M. The loading Ica in Ica/ β -TCP disks hardly changed the surface microstructure and biocompatibility of β -TCP disks and affected the attachment and morphology of Ros17/28 cells, however, the loading Ica promoted the proliferation

and differentiation of Ros17/28 cells. The loading Ica enhanced the osteoinductivity of β -TCP ceramic in vivo and the Ica combined β -TCP ceramic might be a good choice for bone tissue engineering.

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