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Osteoinduction by Ca-P biomaterials implanted into the muscles of mice*

Rui-na YANG[1], Feng YE[1], Li-jia CHENG[1], Jin-jing WANG[1], Xiao-feng LU[1], Yu-jun SHI[1], Hong-song FAN[2], Xing-dong ZHANG[2], Hong BU†‡[1,3]

([1]*Key Laboratory of Transplant Engineering and Immunology, Ministry of Health, West China Hospital, Sichuan University, Chengdu 610041, China*)

([2]*National Engineering Research Center for Biomaterials, Sichuan University, Chengdu 610065, China*)

([3]*Department of Pathology, West China Hospital, Sichuan University, Chengdu 610041, China*)

†E-mail: hongbu@scu.edu.cn

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Abstract: The osteoinduction of porous biphasic calcium phosphate ceramics (BCP) has been widely reported and documented, but little research has been performed on rodent animals, e.g., mice. In this study, we report osteoinduction in a mouse model. Thirty mice were divided into two groups. BCP materials (Sample A) and control ceramics (Sample B) were implanted into the leg muscle, respectively. Five mice in each group were killed at 15, 30, and 45 d after surgery. Sample A and Sample B were harvested and used for hematoxylin and eosin (HE) staining, immunohistochemistry (IHC) staining, and Alizarin Red S staining to check bone formation in the biomaterials. Histological analysis showed that no bone tissue was formed 15 d after implantation (0/5) in either of the two groups. Newly-formed bone tissues were observed in Sample A at 30 d (5/5) and 45 d (5/5) after implantation; the average amounts of newly-formed bone tissues were approximately 5.2% and 8.6%, respectively. However, we did not see any bone tissue in Sample B until 45 d after implantation. Bone-related molecular makers such as bone morphogenesis protein-2 (BMP-2), collagen type I, and osteopontin were detected by IHC staining in Sample A 30 d after implantation. In addition, the newly-formed bone was also confirmed by Alizarin Red S staining. Because this is the report of osteoinduction in the rodent animal on which all the biotechnologies were available, our results may contribute to further mechanism research.

Key words: Osteoinduction, Hydroxyapatite/β-tricalcium phosphate, Biphasic calcium phosphate, Osteogenesis
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1 Introduction

In the past few decades, osteoinduction has been widely reported and documented. Ripamonti *et al*. (1989) reported bone induction in a composite allogeneic bone/alloplastic implant; Zhang *et al*. (1991), Osborn (1991), and Ripamonti (1991a) discovered bone formation at ectopic sites after implantation of porous calcium phosphate ceramic materials in dogs and baboons. Later, osteoinductive bioceramics began to be reported by different labs in pigs, sheep, rabbits, and other large animal models all over the world (Damien and Parsons, 1991; Toth *et al*., 1993; Li *et al*., 1994; Yuan *et al*., 2000; 2001a; Nihouannen *et al*., 2005; 2008; Ye *et al*., 2007; Fellah *et al*., 2008). Since then, generous attention was paid to these kinds of biomaterials, such as synthetic hydroxyapatite ceramics (HA), porous biphasic calcium phosphate ceramics (BCP), tricalcium phosphate ceramics (TCP), calcium pyrophosphate ceramics, and coral-derived hydroxyapatite. These ceramic materials have

‡ Corresponding author

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been shown to have intrinsic osteoinduction (Ducheyne and Cuckler, 1992; Toth *et al*., 1993; Yang *et al*., 1997; Yuan *et al*., 1998a; 1998b; Nihouannen *et al*., 2005; 2008; Ye *et al*., 2007; Fellah *et al*., 2008). However, the main evidence of bone formation in these biomaterials was based on histological analysis and there were few reports focusing on the molecular mechanism.

Based on the extensive research work surrounding these biomaterials, the intriguing phenomena have been categorized into two aspects: material factors and biological factors (Zhang *et al*., 2000; Yuan *et al*., 2002; Habibovic *et al*., 2005; Fan *et al*., 2007; Fellah *et al*., 2008; Nihouannen *et al*., 2008; Ripamonti *et al*., 2009). The majority of research performed on these types of bioceramic materials was focused on the material factors including porosity, porous structure, phase composition, crystallinity, and sintering temperature, etc. (Yuan *et al*., 1999; Zhang *et al*., 2000). The osteoinduction of these biomaterials was described as material-dependent. The quantity and quality of the newly-formed bone tissue were affected by a series of material factors. Fellah *et al*. (2008) found that high micro-porosity and a small crystal size were essential for the adhesion, proliferation, and differentiation of the osteogenic cells which produced the bone extracellular matrix. Fan *et al*. (2007) and Kasten *et al*. (2008) reported that the biological function of osteoinduction depended on the micro/nano structural surface characteristics of the biomaterials. Habibovic *et al*. (2005) investigated a 3D microenvironment of osteoinductive biomaterials and discovered that the presence of micropores within macropore walls was an essential prerequisite for osteoinduction. On the other hand, for the biological factors, these osteoinductive Ca-P biomaterials were found to be animal-species dependent (Ripamonti, 1991c; Ducheyne and Cuckler, 1992; Yamasaki and Sakai, 1992; Green *et al*., 1995; Nihouannen *et al*., 2005; 2008; Fellah *et al*., 2008). Based on these studies, osteogenesis was reported in baboons, monkeys, pigs, dogs, goats, and rabbits after undergoing different types of implantation (Ripamonti, 1991b; 1996; Yang *et al*., 1997; Yuan *et al*., 2002; 2006; Ye *et al*., 2007; Nihouannen *et al*., 2008). Overall, the majority of the animals were quite large but included few rodent animals (rats or mice).

However, further mechanism research was difficult to perform on large animals, because most of the molecular biotechniques were specifically based for rodent animals. Owing to this restriction, the mechanism of osteoinduction was not well explored; e.g., the source of stem cells, the types of signalling molecules and transcription factors, as well as the regulatory mechanism remained unknown. Thus, osteoinduction in the mouse model was considered to be the prerequisite for the above mentioned studies.

Our study was designed to investigate osteoinduction in a mouse model with the use of two types of bioceramic materials. Immunohistochemistry (IHC) staining and Alizarin Red S staining (a bone chelating fluorescent markers for calcium) were used to confirm the newly-formed bone at the ectopic sites in the muscles of mice.

2 Materials and methods

2.1 Porous BCP biomaterials

Two different BCP biomaterials (Sample A and Sample B) were provided by the National Engineering Research Center for Biomaterials (Sichuan University, China). Both of them were composed of HA and β-TCP with a 70/30 ratio. The starting apatite powers with a Ca/P ratio of 1.50 were wet-synthesized. Porous green bodies were foamed by 5%–10% H_2O_2 under 70–80 °C. Sample A was sintered at 1100 °C and Sample B was sintered at 1200 °C for 2 h, respectively, then cooled naturally. Microporosities of Sample A and Sample B were approximately 50%–60% and 20%–30%, respectively, both with pore dimensions ranging from 100 to 500 μm. The total porosity, including macropores and micropores, in Sample A was considerably more than that in Sample B. The chemical characteristics and surface structures of the two samples were measured by X-ray diffraction (XRD) and scanning electron microscopy (SEM). The ceramics were fabricated into Φ3 mm×5 mm cylinders and steam-sterilized at 121 °C for 30 min before implantation.

2.2 Animals

Thirty mice were obtained from the Sichuan University Laboratory Animal Center (strain name: Balb/C; body weight: 28–32 g; gender: male), and were maintained in ventilated air-filtered cages in a

temperature and light-controlled environment. All of the experiments were approved by the Animal Care and Use Committee of Sichuan University. All of the animals were anaesthetized by intraperitoneal injection of 0.02 g/ml pentobarbital sodium (40 mg/kg body weight; Sigma Chemical, St. Louis, MO, USA) before implantation and injected with penicillin at 2.5 U/time twice a day for 3 d continuously post-operation to prevent infection. All procedures were performed according to the Guideline for the Care and Use of Laboratory Animals of the National Institutes of Health (Publication No. 85-23, Revision, 1985) under the supervision of a licensed veterinarian.

2.3 Surgical procedure

Thirty mice were divided into two groups. Each animal was implanted with different ceramics in the muscles of both hind legs (Sample A: Group 1; Sample B: Group 2). X-ray pictures were taken to confirm the positions of the samples in the muscles (Fig. 1a). Five mice of each group were sacrificed with overdose of pentobarbital sodium at 15, 30, and 45 d after implantation. All of the implants were traced at the implanted sites (Fig. 1b). The implants were harvested with surrounding soft tissues together and then fixed in 10% neutral formalin (pH=7.2) for 24–48 h at room temperature.

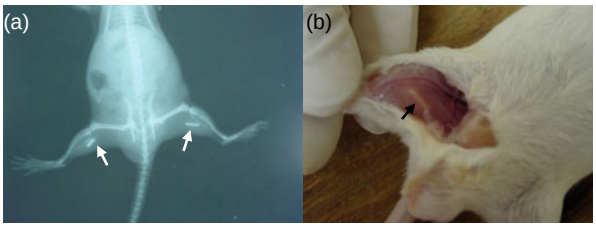

Fig. 1 Detection of implanted sites of BCP biomaterials
(a) X-ray picture was taken to show the position of the implanted site; (b) All the implants were traced in the implanted site. Arrows: the implanted materials

2.4 Histological preparation

The fixed samples were decalcified in a fast-decalcifying fluid (hydrochloric acid 8 ml, methanoic acid 8 ml, and distilled water 184 ml) for 24 h, washed with phosphate buffer solution (PBS), dehydrated with gradient of ethanol solutions at 70%, 80%, 90%, 95% and 100%, and then embedded in paraffin (melting point 56–58 °C). Continuous 5-μm sections were made, and transferred onto slides. The sections were then stained with hematoxylin and eosin (HE) for histological analysis. Sequential sections were also prepared for IHC staining and Alizarin Red S staining.

2.5 IHC staining

The sections of 30 d in Group 1 were deparaffinized, and rehydrated in water. The endogenous peroxidase was blocked with 3% H_2O_2 and epitope was retrieved under pressure sterilizer. Then, the sections were further incubated with primary antibody of anti-mouse collagen type I (1:500; Santa Cruz, CA, USA) or anti-mouse osteopontin (1:500; Santa Cruz) or anti-mouse bone morphogenesis protein-2 (BMP-2) (1:500; Santa Cruz) over night at 4 °C. After being washed with PBS five times, the sections were incubated with proper horseradish peroxidase (HRP)-labeled second antibodies for 1 h at 37 °C. Subsequently, the sections were developed with 3,3′-diaminobenzidine (DAB; Pierce Biotechnology, USA) and counterstained with hematoxylin.

2.6 Alizarin Red S staining

Alizarin Red S staining was conducted under the description by Sontag (1980), which was applied by Qu *et al.* (2004). Briefly, the serial sections were deparaffinized, and rehydrated in water. Immersion solutions (0.01 g/ml) were prepared by dissolving 1 g of Alizarin S (Sigma Chemical, St. Louis, MO, USA) in 90 ml deionized water plus 10 ml of 0.01 g/ml NH_4OH. The sections were then immersed in the solutions for 10 min. After the immersion, the sections were rinsed in fresh water for several minutes, and then allowed to stand for 10 min to allow the unbound Alizarin S to diffuse out of the tissues. After being quickly immersed in 0.05 g/ml acetic acid, the sections were dehydrated in ethanol. Observations were carried out at 400× magnification by using an Olympus IX 71 microscope with green and blue fluorescence rays.

3 Results

3.1 Characterization of BCP biomaterials

As shown in Fig. 2, there was significant difference in the surface structure and morphology

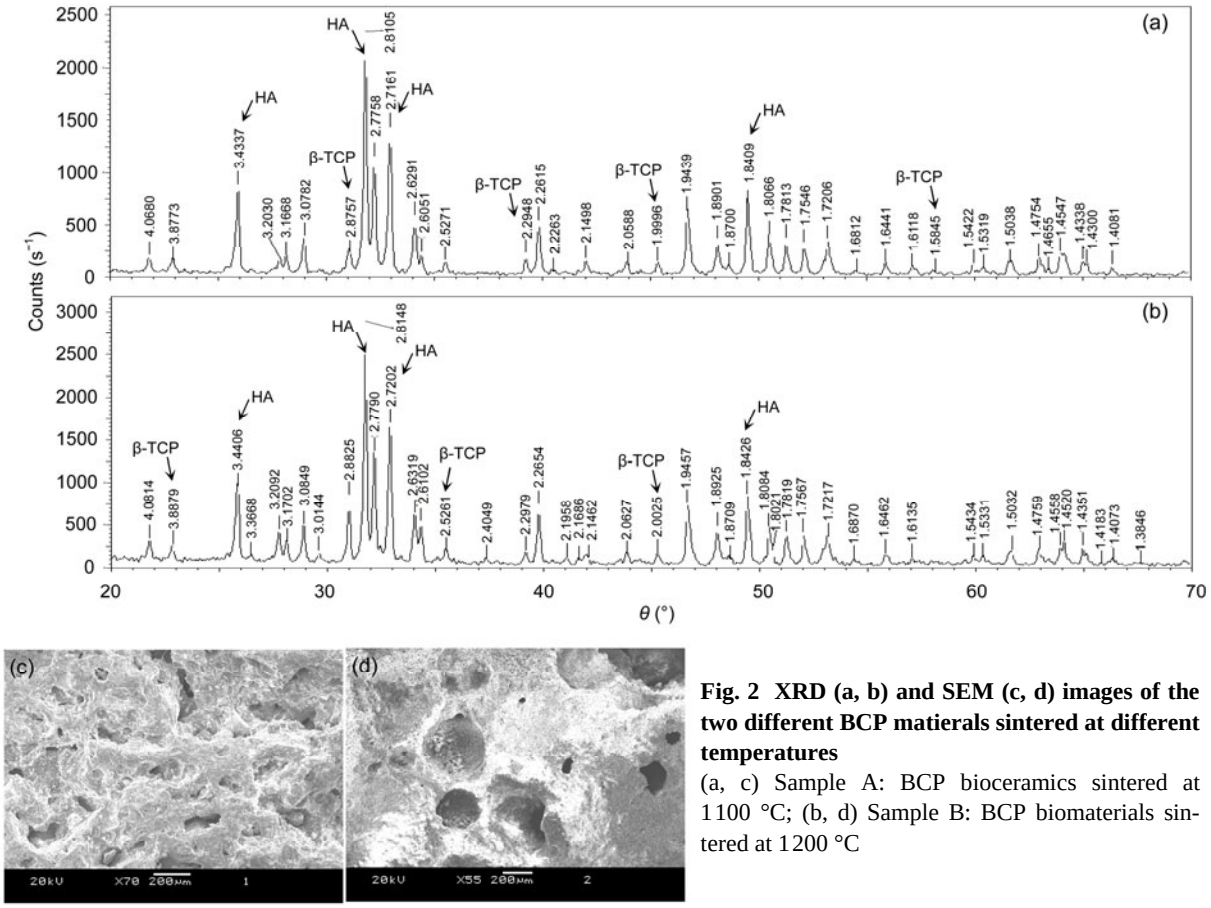

Fig. 2 XRD (a, b) and SEM (c, d) images of the two different BCP matierals sintered at different temperatures

(a, c) Sample A: BCP bioceramics sintered at 1100 °C; (b, d) Sample B: BCP biomaterials sintered at 1200 °C

between the two types of BCP biomateials. All of them contained 70% HA and 30% β-TCP, but the porosity of each group was obviously different. Microporosity of Sample A was approximately 50%–60%, while that of Sample B was approximately 20%–30%. The total porosity in Sample A was more than that in Sample B. For SEM observations, the architectures of the two biomaterials possess a trabeculae-like structure and interconnected micropores. But, Sample A had a rough surface with small crystals and uniform distribution macropores, while the surfaces of the Sample B had less pores (Figs. 2c and 2d).

3.2 Histology observation

In Group 1, the bioceramic rods were observed to be encapsulated with a layer of dense connective tissues 15 d after implantation (Fig. 3a). Monocytes and lymphocytes were found infiltrated in these dense connective tissues. A large amount of active mesenchymal tissues were found outside of these porous BCP biomaterials. Macrophages were also observed on the interface between the biomaterials and mesenchymal tissues (Fig. 3a). Thirty days after implantation, the bioceramic rods were also encapsulated with a layer of dense connective tissues. A large number of irregular arrangements of bone tissues were found outer of the collected biomaterials in all animals (5/5) (Figs. 3b and 3c). Some osteoblasts were observed to be excreting bone matrix and embedding themselves to form bone lacuna (Fig. 3b). The osteoblasts were observed linearly on the interface between the induced bone and the mesenchymal tissues (Fig. 3c). Forty-five days after implantation, more bone tissues were observed in the micropores of the bioceramics in all animals (5/5), the arrangement was more regular compared with the bone tissues harvested at 30 d. Some calcification lines could be observed in the bone matrix (Fig. 3d). Overall, after implantation of Sample A, the average amounts of newly-formed bone tissues were about 5.2% and 8.6% in 30 and 45 d, respectively. However,

bone tissue was not found until 45 d after implantation in Sample B (Figs. 3e–3h). Only inflammatory cells, such as monocytes, macrophages and lymphocytes, were observed in the implanted biomaterials (Figs. 3g and 3h).

3.3 Immunohistochemistry

Thirty days after implantation, serial sections of Sample A were used for HE and IHC stainings. The brown color indicated the expression of the specific antigen BMP-2. As shown in microscopic pictures, BMP-2 was positive within all the osteocytes in the lacunae (Fig. 4d), and collagen type I was located within the osteocytes and in the bone matrix of the harvested bone tissue (Fig. 4e). The linearly-arranged osteoblasts were positively identified with the presence of collagen type I. Also, the collagen type I in the surrounding connective tissues was in brown color. Osteopontin was also tested to be positive within the osteocytes and in the bone matrix (Fig. 4f).

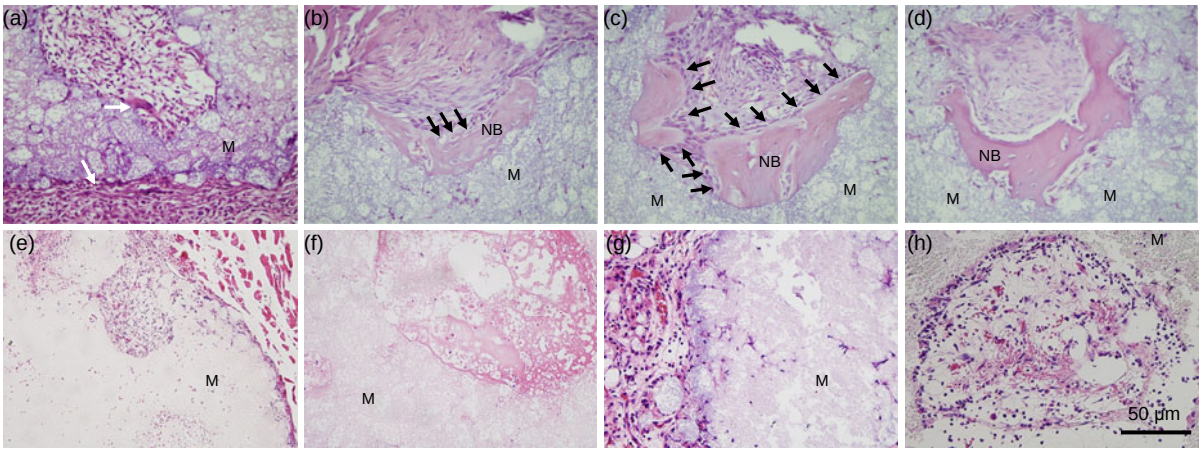

Fig. 3 HE staining of two different types of BCP biomaterials harvested from mice

Histological features of Sample A (a–d) and Sample B (e–h) were observed after 15, 30, and 45 d. (a, e) Histological features of BCP biomaterials implanted in the muscles of mice after 15 d. (b, c, f, g) Histological features of BCP biomaterials implanted in the muscles of different mice after 30 d; (d, h) Histological features of BCP biomaterials implanted in the muscles of mice after 45 d. M: decalcified biomaterials; NB: induced bone. White arrow: dense connective layer; Black arrow: induced bone

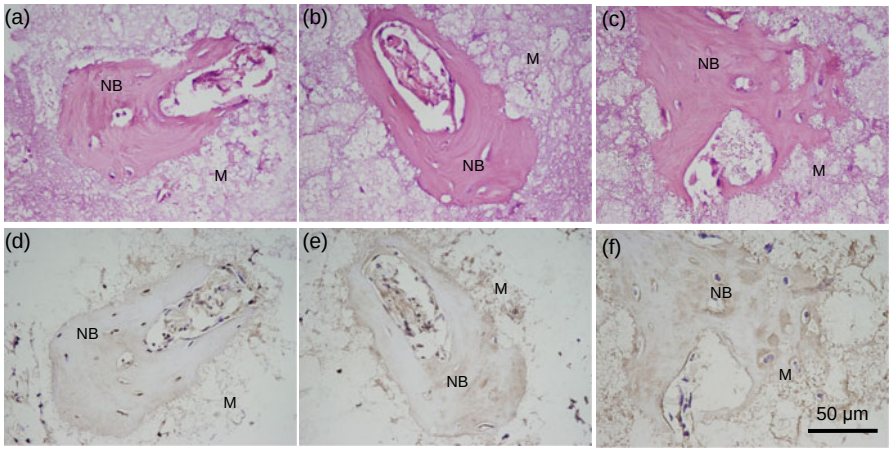

Fig. 4 HE staining (a–c) and IHC staining (d–f) of serial sections of Sample A after 30-d implantation

(a, d) Primary antibody of anti-mouse BMP-2; (b, e) Primary antibody of anti-mouse collagen type I; (c, f) Primary antibody of anti-mouse osteopontin. M: decalcified biomaterials; NB: induced bone. Brown colored area: positive region

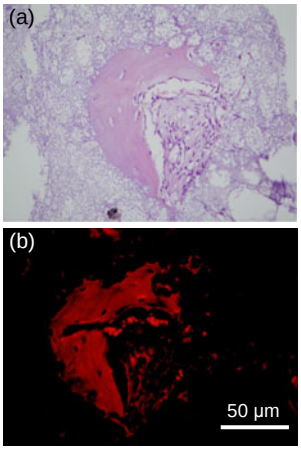

Fig. 5 Induced bone of Sample A after 30-d implantation

(a) HE staining; (b) Alizarin Red S staining. The fluorescence marker chelated to calcium emitted red ray

3.4 Alizarin Red S staining

Serial sections were used for HE staining and Alizarin S staining after 30 d of implantation in Group 1. For the Alizarin Red S section, the observation was conducted under the excitations of green and blue fluorescence rays. The fluorescent marker chelated to the induced bone was excited to emit red ray under the excitation of green fluorescence ray (Fig. 5b), but no signal was detected under the excitation of blue ray (data not shown).

4 Discussion

This research addressed the bone formation at ectopic sites in a mouse model by the specific bioceramic materials. Previously, a large number of studies have reported osteoinduction of bioceramic materials mainly in dogs, pigs, monkeys, baboons, goats, and rabbits (Ripamonti, 1991a; Ripamonti, 1996; Yang *et al*., 1997; Yuan *et al*., 2002; 2006; Ye *et al*., 2007; Nihouannen *et al*., 2008; Kasten *et al*., 2008). As indicated in the reports of Fellah's team, osteoinduction by bioceramic materials is a complex biological phenomenon which is not fully understood, but has shown that ectopic bone formation is both material and animal dependant (Nihouannen *et al*., 2005; 2008; Fellah *et al*., 2008). Cheng *et al*. (2010) reported osteoinduction in the fractured fibula of mice by certain calcium phosphate ceramics containing 60% HA and 40% β-TCP. Consistently, we found bone tissue could be induced in mice muscles without any specific treatment on the animals. More importantly, the common mouse was available for all of the modern biotechniques. Based on our results, the achievements in molecular biology could be used for the further research in this area.

In this study, we implanted two different BCP biomaterials into the leg muscles of mice. A large number of newly-formed bone tissues were found outer of the collected biomaterials after implantation with BCP materials sintered at 1100 °C in 30 and 45 d. However, only a large amount of monocytes and lymphocytes were observed after implantation with BCP materials sintered at 1200 °C within 45 d. These results were confirmed by labelling bone-related molecular makers, such as BMP-2, collagen type I, and osteopontin, as well as Alizarin Red S staining. Previous research indicated that bone formation was observed in contact with the BCP granules in ectopic sites after six weeks, but this research was performed on a large-bodied animal model (Fellah *et al*., 2008). In addition, the preparation of the bioceramic materials was not very identical (Cheng *et al*., 2010). It has been reported that the sintering temperature, microstructure, and porosity of the materials seemed to play a critical role in the bone-induction mechanism in ectopic sites, and ectopic bone formation was firstly observed not only inside the macropores, but also between the ceramic particles (Nihouannen *et al*., 2005; 2008; Fellah *et al*., 2008). Our results also showed that specific BCP ceramics were osteoinductive in mice, and this osteoinduction was closely related to the properties of the biomaterials. Therefore, the distributions and sizes of the macro- and micro-pores, sintering temperature, as well as the surface structure, might play an important role in osteogenic differentiation in vivo (Habibovic *et al*., 2005; Fellah *et al*., 2007; 2010; Kasten *et al*., 2008).

Previous reports on the bone formation at ectopic sites induced by these biomaterials were mainly based on histological analysis. Decalcified and undecalcified sections were made for histological observation under ordinary optical microscopes and back-scattered scanning electron microscopy (BSEM) (Nihouannen *et al*., 2005; Yuan *et al*., 2006; Fan *et al*., 2007; Fellah *et al*., 2008). Fellah *et al*. (2007; 2010) recently reported that the inflammatory cytokines, e.g., IL-6 and TNF-α, released by macrophages stimulated by BCP microparticles, may have both positive and negative effects on new bone formation. However, the very little related research that labelled the marker molecules expressed by the osteocytes or osteoblasts has been performed and was most likely due to the limitation of animal models to some extent (Hennessy *et al*., 2009). In this study, we observed bone formation in the mice model and our results may contribute to further mechanism studies based on the modern biotechnologies developed in the mice model.

This type of biomaterial was expected to be used in orthopaedic, maxillofacial surgery and dental devices as bone substitute, but the prerequisite is safety. Wang *et al*. (2004a) compared the bone-related gene expression of human primary osteogenic sarcoma cell

line SaOS-2 cultured on calcium phosphate ceramics with different phase compositions and different sintering temperatures in vitro. The gene expression patterns from his and other researches supported the biocompatibility and bioactivity potentials of calcium phosphate ceramics (Wang C. *et al*., 2004b; Wang H. *et al*., 2007; Wang J.J. *et al*., 2009; Guo *et al*., 2009). Previous research on safety was mainly focused on histological analysis of long-term tissue response after the biomaterials were implanted in the animals. Yuan *et al*. (2001b) reported that the quality and quantity of the induced bone by BCP bioceramics were normal with bone marrow after 2.5-year tissue response in dogs. Ye *et al*. (2007) reported that based on observation of 4.5-year tissue response in pigs, the newly-formed bone after implantation of BCP bioceramics neither disappeared nor gave rise to autonomous growth, and the surrounding soft tissues were normal with no presence of tumor cells. Owing to all, the results regarding osteoinduction were based on histological analysis and we thought that further research should be directed towards a better understanding at molecular levels. In addition, the research on the osteoinduction was still limited, and many questions needed to be clarified, e.g., the origin of the osteoblasts, signaling molecules, and the transcriptional factors involved in the process of the stem-cell differentiation.

5 Conclusions

In this study we reported osteoinduction with the specific BCP biomaterials in mice. Our research indicated that bone tissues could be induced in the muscles of mice in 30 d after implantation of specific BCP biomaterials sintered at 1100 °C at a 70/30 ratio of HA and β-TCP. The average amounts of newly-formed bone tissues were approximately 5.2% and 8.6% in 30 and 45 d, respectively. The results were confirmed by bone-related molecular makers, such as BMP-2, collagen type I, and osteopontin, and also confirmed by Alizarin Red S staining. Based on these data, we concluded that specific BCP biomaterials could induce bone formation in the mice model. Our results may contribute to further mechanism research benefiting from the modern biotechnologies developed on a mouse model.

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