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A long-term evaluation of osteoinductive HA/β-TCP ceramics in vivo: 4.5 years study in pigs

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Abstract It has been proved that some material-dependent calcium phosphate ceramics have intrinsic potentials to induce osteogenesis. But there is little literature concerning about the tissue response in long-term. The aim of this study is to evaluate the safety of the osteoinductive biocreamics and the stability of the newly formed bone after long-term tissue response. Porous calcium phosphate ceramics rods which contain hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) were implanted in the dorsal muscles of Banna Minipig Inbreding Line. After 4.5 years, all the implanted rods with surrounding tissues were harvested and stained with hematoxylin and eosin for histological observation. The 7 months' rods were also harvested as short-term comparison. The histological results showed that compared with the short-term rods, amount of bone tissue formed after 4.5 years. And the newly formed bone in this bioceramics neither disappeared nor gave rise to uncontrolled growth. The bone growth in this bioceramics seemed to be self-confined. The surrounding soft tissues were normal and no tumor cell was found. We conclude that instead of disappearing or giving rise to out of control, the induced bone tissue trends to be further matured. And this bioceramics thus might have potentials in future clinical use.

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Introduction

To promote and take advantage of body's own native ability to overtake disease or regenerate the function of defected tissue or organ is the dream of medical research. In 1967, Winnter and Simpson firstly discovered bone formation at heterotopic site induced by biomaterials [1]. And in last two decades, the conception of osteoinduction attracted so many scientists. With the increasing number of osteoinductive biomaterials, the conception of osteoinduction was gradually accepted by scientists in this area. In 1988, Heughebaert reported a bone-like substance formed in porous hydroxyapatite (HA) ceramics implanted in soft tissue of hamsters [2]; in 1990, Yamasake found bone formation in porous HA ceramics implanted in the subcutis of dogs [3]; in 1991, Xingdong Zhang and Ripamonti documented non-osseous bone formation in porous calcium phosphate ceramics implanted in dogs and baboons respectively [4–6]; in 1992, Toth, Clein, Vargerik and Yamasake reported osteoinceddtion of calcium phosphate [6–9]. Since then series bioceramics based on calcium phosphate were reported to be osteoinductive. Synthetic hydroxyapatite ceramics, biphasic calcium phosphate ceramics, tricalcium phosphate ceramics, calcium pyrophosphate ceramics, and coral-derived hydroxyapatite have been developed to be intrinsicly osteoinductive [10–17]. These biomatrials were thus expected to be used in orthopaedic and dental surgery. However, the prerequisite of that is safety, which demands a long-term observation in big animal model.

It is known that bone tissues are the most important reservoir of calcium and phosphate. Actually, there are upper 99% calcium and 85% phosphate stored in bone in the form of hydroxyapatite. More importantly, instead of stably maintained in bone, calcium and phosphate play important roles in homeostasis of microenvironment and in bone's

self-physiology [18]. The degradable calcium phosphate biomaterials may break their own balance in the body fluid in vivo. Whether this broken equilibrium status will cause chronic harm to the newly formed bone or the nearby tissue needs long-term observation of in vivo tissue response in big animals. It was suggested that these kinds of materials could adsorb signal molecules, such as bone growth factors in the implanted ceramics [15]. Whether these optimal geometry designed biomaterials would continuously adsorb signal molecules or continuously stimulate bone formation is considerable. Previous investigations in pigs for 4 months revealed that the bone was formed from both inside and outside of this biomaterial. It was hard to confirm whether it would give rise to uncontrolled growth even to osteoma due to the limited investigating time [19].

Herein our research focus on long-term in vivo tissue response. The purposes of this long-term observation was to clarify (1) the stability of the newly formed bone, i.e., whether it disappeared or gave rise to uncontrolled growth; (2) the stability of the bioceramics and its effects to their surrounding soft tissues.

Materials and methods

Biphasic bioceramics

The biphasic calcium phosphate ceramics were provided by our colaborator (National Engineering Research Center for Biomaterials, Sichuan, P.R. China). The preparation procedure of the ceramics was described as following. Porous calcium phosphate ceramics were made from wet-synthetic calcium phosphate powder with the HA/β-TCP ratio of 70:30. After initial preparation, the green bodies were foamed by 5–10% H_2O_2 at temperature of 70–80 °C and dried. Then the green bodies were sintered at 1,100 °C for 3 h. Its chemical characteristics were confirmed by X-ray diffraction (XRD) as is shown in Fig. 1. Its surface structure was analyzed with scanning electron microscopy (SEM). It has pores with interconnect macropores (picture not shown) and the micropores present on the surface of the macropores . The porosity of this bioceramics was about 50–60% with main pore dimension ranging from 300 to 500 μm (Fig. 2). Ceramics rods of $\varphi 5 \times 10$ mm were obtained from the porous calcium phosphate ceramics for implantation. The osteoinductivity of this bioceramics have been confirmed by our previous study [20].

Kiel bone

Soft tissues and periosteum were peeled by scalpel from the fresh pig bone. This bone was then immersed in 20% hydrogen peroxide solution for 72 h with the changing of fresh hydrogen peroxide solution for every 24 h. Then it was immersed in 100% ethanol for 24 h to defat, and then acetone was used for dewatering. Several rods ($\varphi 5 \times 10$ mm) were obtained from this Kiel bone.

Animal experiments

The animal experiment was approved by the animal care and use committee of Sichuan University. Several biphasic calcium phosphate bioceramics rods ($\varphi 5 \times 10$ mm) and natural inorganic bone rods (Kiel bone) were steam sterilized at 121 °C for 30 min before implantation. Three

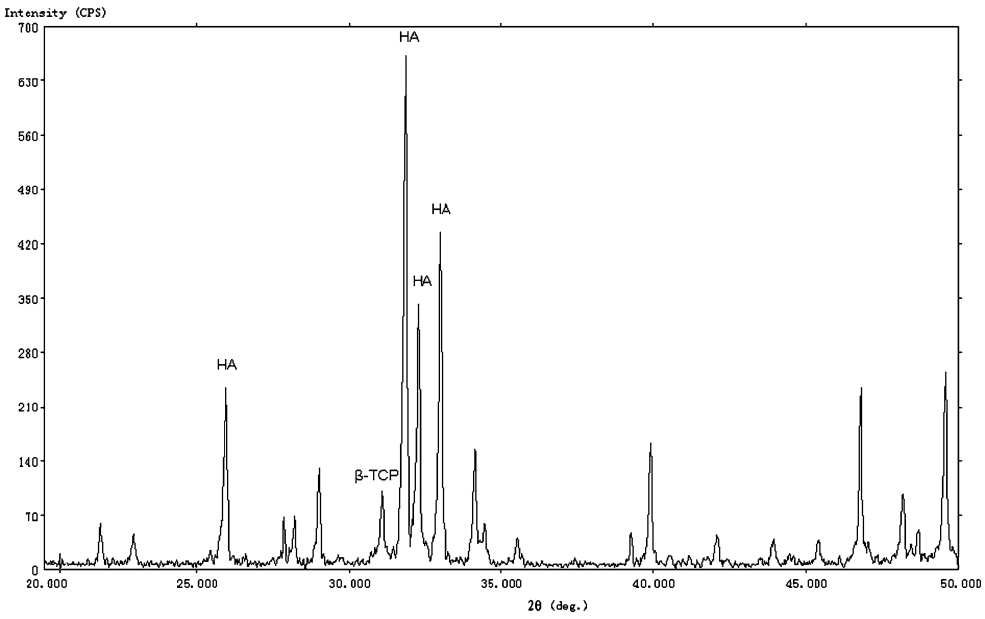

Fig. 1 X-ray diffraction patterns of the HA/β-TCP bioceramics. Main peaks of HA and β-TCP are marked

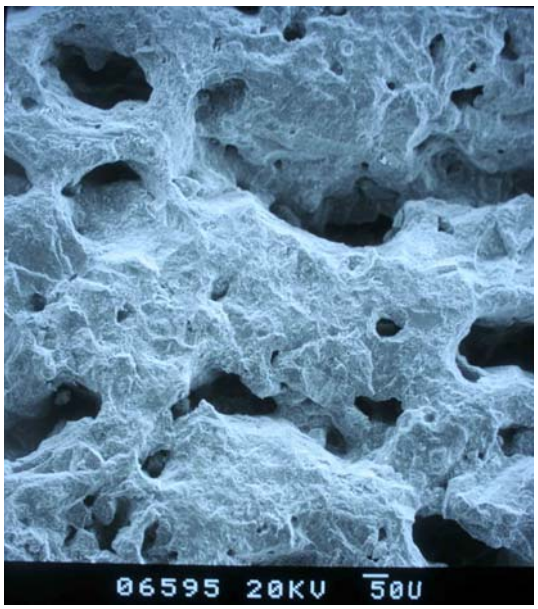

Fig. 2 Scanning electron microscopy picture of HA/β-TCP (70:30) before implantation, 100×

2-year-old healthy pigs (Banna Minipig Inbreding Line, body weight about 65 kg) were anesthetized with ketamine hydrochloride (4 mg kg^{-1}) via intravenous injection. An 8 cm incision was made by scalpel at the center of the animal's back skin, and both sides of the erector muscles were exposed by blunt separation. Two bioceramics were implanted in the muscle pouch at each side, at the same time, Kiel bone rods were also implanted in the muscle pouch to serve as control. In 1, 3, 6, and 7 months Kiel bone rods were harvested as comparisons. In 7 months, some biomaterials rods were harvested as short-term comparison. After 4.5 years from implantation, the Banna Minipigs were sacrificed with overdose of pentobarbital sodium. The bioceramics rods were harvested with surrounding soft tissues and fixed in 10% formaldehyde (pH 7.4).

Histological preparation

The fixed implants were decalcified in decalcifying fluid (hydrochloric acid, 8 mL; methanoic acid, 8 mL; and distilled water, 184 mL) for 24 h. Then the implants were washed with phosphate buffer solution (PBS), dehydrated with gradient of ethanol solutions at 70, 80, 90, 95 and 100% concentration and embedded in paraffin wax. Continuous 5 μm sections were made and mounted on slides. The samples were then stained with hematoxylin and eosin (H&E) for histological observation.

Results

Bone

Compared with short-term tissue response (Fig. 3A), it was indicated that more area of the bioceramics was substituted by the newly formed bone after 4.5 years in vivo. And the nearby bone tissues integrated to form a whole mature bone

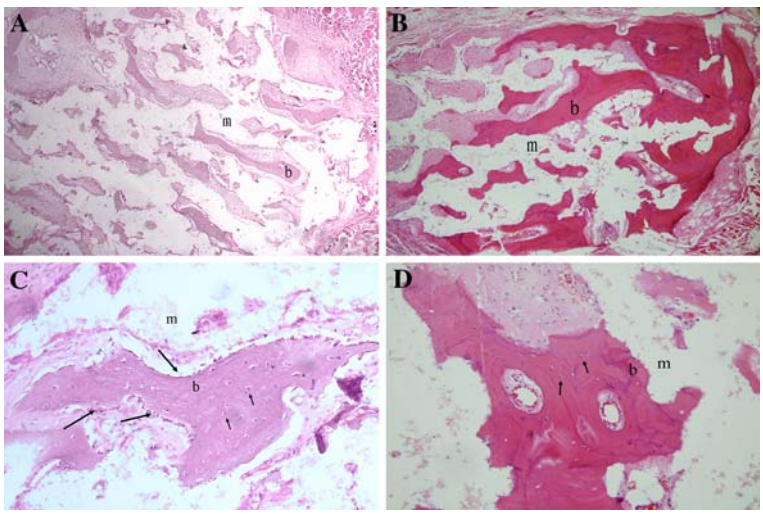

Fig. 3 After 4.5 years' tissue response in dorsal muscle, the implanted rods were harvested. H. E. staining was made for observation. 7 months' rods were also harvested as short-term comparison. (**A**) In the short-term group, the bone tissue was formed but not integrated as a whole (40×); (**B**) Amount of bone tissue was found, and the area of the bioceramics was substituted by the newly formed bone. The nearby bone tissues integrated to form a whole mature bone (40×); (**C**) the bone tissue in short-term group (Big arrows: linearly presented osteoblasts; small arrows: the irregular arranged osteocytes) (200×); (**D**) the Haverian-like structure: central canal, concentric circular bone lamella, interstitial bone lamella and lacuna (arrows: the regular arranged osteocytes, 200×). m, bioceramics; b, bone

(Fig. 3B). The osteoblasts presented linearly on the interface between the bone tissue and the bioceramics (Fig. 3C), which did not present in the long-term group. Instead, all the bone tissue after long-term in vivo response was lamellar bone with Haverian-like structure including central canal, concentric circular bone lamella, interstitial bone lamella and lacuna (Fig. 3D). Compared with the short-term group (Fig. 3C), the arrangement of the osteocytes in the long-term group was much more regular and the amount of osteocytes declined (Fig. 3D). All these differences suggested that the bone in long-term group was remodeled and highly matured. In the Kiel bone control group, instead of inducing new bone, the Kiel bone rods were totally absorbed in about 3–6 months (not shown).

Bone marrow

Mature yellow bone marrows were found in the medullary cavity of bone (Fig. 4A and B). The reticular connective tissue, adipose tissue and blood cells could be seen in the cavity of bone marrow. Amount of granulocytes and some erythroid cells such as erythrocytoblasts, presented in the reticular connective tissue, and the latter existed only in bone marrow. No atypical cell could be seen in the reticular connective tissue (Fig. 4A and B).

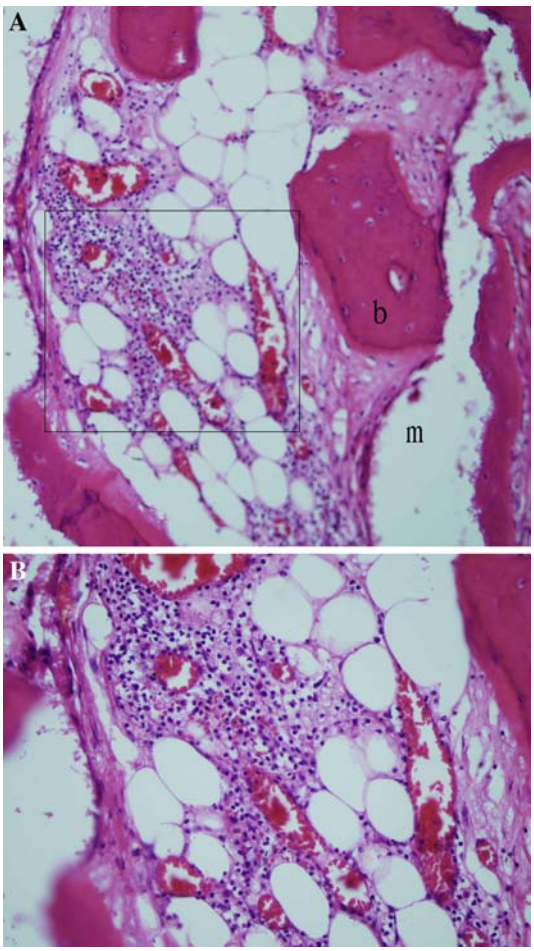

Fig. 4 The mature yellow bone marrow was found in the medullary cavity of bones. (**A**) The reticular connective tissue, adipose tissue and blood cells presented in the cavity of bone marrow, 200×; (**B**) granulocytes and some erythroid cells in the bone marrow, 400×

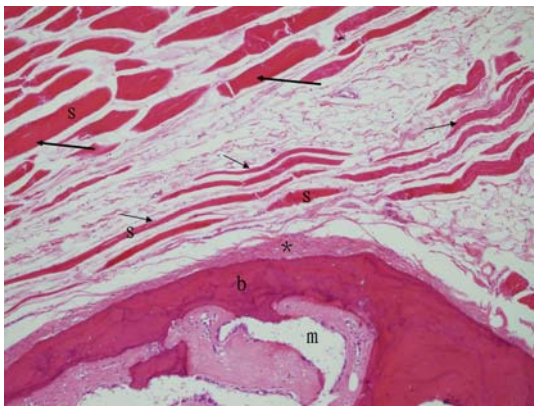

Fig. 5 After 4.5 years' tissue response, the soft tissues around the bioceramics presented to be bland. Dense connective tissue could be observed tightly around the newly formed bone, and none bone tissue was found outside this layer. Skeletal muscles which were near the dense connective tissue presented to be slender compared with the nearby normal skeletal muscles. These skeletal muscles seemed to be a little atrophy. (big arrow: the normal skeletal muscles; small arrow: the muscles near the dense connective tissue. m, HA/β-TCP; b, bone; s, muscle; *, dense connective tissue, 200×)

Surrounding soft tissue

Dense connective tissue tightly surrounded the newly formed bone, and no bone tissue was found outside this layer. Collagen in the connective tissue seemed slightly dense. Skeletal muscle layer was found near to the connective tissue layer. But their muscle bundles were slender compared with the nearby normal skeletal muscles. The nucleus of the fibrocyte was short-fusiform shaped. These skeletal muscles were a little atrophy (Fig. 5), but all of them seemed to be bland and none was atypical. The soft tissue around the bioceramics was normal and no tumor cells in surrounding soft tissues were found. Like Fig. 3D, the induced bone in the edge of the bioceramics was highly matured and had no tendency to generate new bones.

Discussion

According to our knowledge, this is the first report on long-term tissue response to HA/TCP implanted into pigs and is the longest study on osteoinductive biomaterials in all kinds of animal models. It suggests that instead of

disappearing or giving rise to uncontrolled growth, the newly formed bone further matured with typical bone structure and seemed to be self-confined. And this bioceramics also seemed harmless to the surrounding soft tissues.

After 4.5 years' tissue response, Haversian-like structure and mature bone marrow were found. All these bone induced by the bioceramics seemed to be normal bone tissue and was significantly different from pathologic calcification in histological observation. Although this bioceramics were partially degraded and substituted by newly formed bone, the released ions, such as Ca^{2+} and PO_4^{3-}, seemed to be harmless to the neighboring tissues. No necrosis, inflammation, pathologic calcification or neoplasm was found in our investigation. The skeletal muscles tightly near to the dense connective tissue were a little atrophy, but we deemed this could be considered as normal phenomenon because these skeletal muscles did not tractate this bioceramics for functioning.

Previous study in pigs for 4 months revealed that the bone was formed from both inside and outside of the bioceramics. It was hard to confirm whether the new bone was normal or would give rise to uncontrolled growth due to the limited investigating time [19]. In this study, we could draw a conclusion that with prolonged response time in vivo, the new bone was still limited inside the dense connective tissue and its growth was under a fine control.

It is encouraging that this bioceramics was partially degraded but did not have chronic detriments to the newly formed bone and the surrounding soft tissues. Previous reports have revealed that high concentration of ions, i.e., Ca^{2+} and PO_4^{3-} , were detrimental for the bone and the surrounding tissues in that microenvironment [21]. In 2001, the effect of high local ions on the bone formation were compared by Yuan. The result showed that high concentration of Ca^{2+} and PO_4^{3-} was harmful in that microenvironment. Bone tissue could not form in α-TCP group which eluted more ions, while in β-TCP group which had lower concentration of ions, the earlier newly formed bone reversed to bone-like tissue [17]. The problem of this bioceramics is that the HA/β-TCP bioceramics might be also slightly and chronically harmful to the newly formed bone and the surrounding tissues. Our long-term in vivo observation revealed that there was no detrimental effect to the newly formed bone and surrounding tissues in this biphasic HA/β-TCP bioceramics up to 4.5 years. Instead, the bone further matured with bone marrow and the surrounding tissues around this bioceramics was normal.

One possibility is that the slowly dissolved Ca^{2+} and PO_4^{3-} ions from HA/β-TCP bioceramics were up taken and reused for metabolism. These ions could not break the equilibrium status in the homeostasis due to low concentration. Bone is the most important reservoir of Ca^{2+} and PO_4^{3-}. These Ca^{2+} and PO_4^{3-} ions do not stay in the bone steadily but play an important role in the homeostasis. All these released extra ions might be taken away to be used in Ca^{2+} and PO_4^{3-} metabolism, and thus no Ca^{2+} and PO_4^{3-} could be accumulated in the local site. This proposal could be supported by plenty of blood vessels found in the newly formed bone induced by this bioceramics (Fig. 4A and B). Without high local concentration of these ions, the bone induced by this biomaterial did not disappear and the growth of both the bone and the surrounding soft tissues were normal. It suggested that this HA/β-TCP biphosphat bioceramics did not harm the bone and the surrounding tissues even in a slight or chronic way.

Selecting animal model for evaluating the safety is a combined consideration which involves several critical factors, i.e., the animal's relativity to human, the osteoinductive rate of this species, and the background of its inheritance. Pigs are so similar to human that the focus of xenotransplantation animal model is now moving from primates to pigs. The results from the pigs are more credible compared with that from other animal models. Besides, the osteoinduction of these calcium and phosphate based bioceramics is animal-dependent. Previous studies have revealed that these kinds of bioceramics could not induce bone formation in rat [22], but bone tissue could be found in rabbit about 6 months [23], in goats about 3 months [21], in dogs about 2 months [24], and in pigs about 45 days [19] after implantation. The purpose of this investigation is to evaluate the safety especially the control of the growth of this newly formed bone induced by this kind of biomaterials. To better estimate the safety, the best animal model should have a very fast osteoinductive rate. Combined these considerations, the Banna Minipig Inbreding Line was believed as a perfect big animal model because it has a clearer inheritance background for further investigation.

Although it was found that this kind of osteoinductive bioceramics seemed to be harmless to the surrounding soft tissues and the newly formed bone in this long-term evaluation, many questions still need to be clarified before these kinds of materials are used in clinic. As the osteoinductivity of these kinds of biomaterials is animal-dependent, the tissue response, especially the safety in human body is hard to be predicted. Future studies should be directed towards a better understanding of the safety of these biomaterials at molecular level.

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