# Bone morphogenetic protein and ceramic-induced osteogenesis

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To investigate the relationship between bone morphogenetic proteins (BMP) and calcium phosphate ceramic-induced osteogenesis in soft tissues, *in vitro* and *in vivo* experiments were performed. In an *in vitro* study, the ability of different calcium phosphate ceramics to absorb bovine BMP (bBMP) from a bBMP solution was tested. *In vivo* studies included immunohistochemical BMP staining before bone formation in the ceramics was detected, and the enhancement of bone formation in calcium phosphate ceramics by bBMP. The results were: (1) calcium phosphate ceramics have a strong ability to absorb bBMP; (2) a high BMP concentration reaches inside the ceramic implants before bone formation in soft tissues of domestic pig occurs; (3) by 56% at 50 d and by 23% at 100 d, bBMP enhances bone formation in calcium phosphate ceramics implanted in soft tissues of dogs. The results indicate the BMP plays an important role in calcium phosphate ceramic-induced osteogenesis and that adsorption of native BMP from the body fluids to ceramic implants may be a key step in osteoinduction by calcium phosphate ceramics 

## 1. Introduction

In 1965, Urist introduced "osteoinduction" which means bone formation in extraskeletal sites and suggested soft tissue implantation as the laboratory model for osteoinduction studies [1]. Subsequently, a protein named bone morphogenetic protein (BMP), which can induce bone formation in soft tissues, was purified [2] and since 1988 several kinds of human BMP subtypes have been cloned and recombined [3]. Osteoinductive capacity has been found in demineralized bone and dentine and other materials containing BMPs [4].

In 1991, Ripamonti reported ectopic bone formation induced by coral-derived hydroxyapatite ceramic in baboon [5], thereafter ectopic bone formation has been found in several other types of calcium phosphate ceramics in different animals [6–15]. Osteoinduction seemed to be a property of certain calcium phosphate biomaterials [15], but the mechanism was not clear. A possible reason for this could be the involvement of certain biochemical factors in calcium phosphate-induced osteogenesis. Owing to the fact that BMP can induce bone formation in soft tissues, one suggestion for the mechanism of calcium phosphate-induced osteogenesis is the adsorption of native BMPs from body fluids to calcium phosphate biomaterials in the process of osteoinduction [10, 11]. However, direct evidence of BMP adsorption to calcium phosphate ceramics has not yet been shown. In this study, bovine BMP (bBMP) adsorption to calcium phosphate ceramics, both *in vitro* and *in vivo* were tested, and the enhancement of calcium phosphate ceramic-induced osteogenesis by bBMP was investigated.

## 2. Materials and methods

### 2.1. Preparation of the calcium phosphate ceramics

Apatite powders with different Ca/P ratio were wet-synthesized, green bodies were foamed by 5%–7% $H_2O_2$ solution and dried; after sintering the green bodies at 1200 °C for 3 h, ceramic bodies were obtained [9, 11]. The chemical composition was analyzed by XRD. Ceramic rods with suitable size for different experiments were machined from the ceramic

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bodies and cleaned with distilled water. For implantation, the ceramic rods were sterilized autoclavely at 121 °C for 30 min.

### 2.2. *In vitro* study

Calcium phosphate ceramics with the same porosity (50%) but different chemical constitution (Cl, HA; C2, 22TCP/78HA; C3, 37TCP/63HA) were selected. Ceramic rods with the same size (diameter 12 mm × 8 mm) were used. Partially purified bovine BMP used in this study was prepared and provided by Professor Lianjia Yang [16, 17]. During the experiment, a ceramic rod was placed in a rubber tube (diameter 10 mm) and connected in a cycle system in which 10 ml bovine BMP solution (300 μg bBMP/ml 4 M Gu-HCl) circulated at a rate of 50 ml $h^{-1}$. The bBMP concentration in the circulating solution was measured by fluorescene specto-photometry (scanning: 150–600 nm) at different time periods. It was tested that there was linearity between fluorescence intensity and bBMP concentration between 0 and 300 μg $m^{-1}$ 4 M Gu-HCl. After 2 h, the ceramic column with the absorbed bBMP was taken out and washed in the cycle system with 10 ml distilled water or 0.1% $CaCl_2$ or phosphate buffer for 1 h. The bBMP in the evaluate was measured by UV-spectrophotometry (scanning: 220–320 nm).

### 2.3. *In vivo* study

Four TCP/HA (30TCP/70HA, porosity, 50%–70%, average pore size 400 μm) rods (diameter 5 mm × 6 mm) were implanted in one side of dorsal muscles of one domestic pig, 15 d later, a second operation was performed on the same animal and another four ceramic rods were implanted in the other side of the dorsal muscles. The samples were harvested 30 d after the second operation and fixed in 10% buffered formalin, declacified in acid compound solution (8.5 g sodium chloride, 100 ml Formalin, 70 ml 37% hydrochloric acid, 80 ml formic acid, 40 g aluminum chloride, 25 ml glacial acetic acid in 1000 ml) for 72 h, dehydrated in a series of ethanol and embedded in paraffin. Semi-thin sections (5–7 μm) were made and stained with HE. Some 30 d sections were stained immunohistochemically by a monoclonal antibody against bBMP (bBMP-McAb), normal goat serum instead of bBMP-McAb as the negative control [16–18].

### 2.4. Bovine BMP enhanced bone formation

bBMP gel (10 mg) was minced and put into 50 ml 0.9% NaCl, kept at 37 °C. After 3 d, the solution was filtered through a 0.22 μm filter and was indicated as supersaturate bBMP solution, because only a small amount of bBMP can be dissolved in salt solution. One sterile TCP/HA (30TCP/70HA, porosity 50%–70%, average pore size 400 μm) ceramic rod (diameter 5 mm × 6mm) was aseptically soaked in 5 ml supersaturated bBMP solution for 48 h under negative pressure and control implants (diameter 5 mm × 6 mm) were incubated in 0.9% NaCl. A total of 16 implants were implanted in dorsal muscles of four dogs (each animal was loaded with four implants, two impregnated with bBMP and two as control) and harvested after 50 and 100 d. After embedding in MMA, non-decalcified sections (20 μm) were made and stained with methylene blue and basic fuchsin. Image analysis was performed on five sections of each implant with regard to the amount of bone formation.

## 3. Results

### 3.1. *In vitro* study

Calcium phosphate ceramics had a strong ability to absorb bBMP from the solution. At 30 min, a large amount of bBMP was absorbed by ceramic rods (Fig. 1a). In 2 h, most of bBMP in the solution was absorbed by the ceramic rods (Fig. 1b). Among the three kinds of calcium phosphate ceramics, HA has the stronger adsorption ability than 22TCP/78HA than 37/68HA (Fig. 1a, b). Only a small amount of bBMP could be washed out by distilled water (Fig. 2a) and $CaCl_2$ solution (Fig. 2b), while a large amount of bBMP was washed out by phosphate buffer (Fig. 2b).

### 3.2. *In vivo* study

Bone formation could be detected inside the ceramics implanted in the dorsal muscle of the domestic pig at 45 d (Fig. 3b), while no bone formation could be observed at 30 d (Fig. 3a). At 30 d, however, the organic matrix and polymorphic cells on the pore surface of the implants and some cells in the pores stained positively by bBMP-McAb (Fig. 3c) compared to a negative control (Fig. 3d). These results indicate that calcium phosphate ceramic could induce bone formation in domestic pigs and a high BMP concentration had been reached inside the ceramic implants before bone formation occurred.

### 3.3. Bovine BMP enhanced bone formation

Bone was found in all samples implanted in dorsal muscles of dogs at both 50 and 100 d post-operatively (Fig. 4). More bone was found in samples pre-treated with bBMP. Compared to the controls, bone formation in the bBMP treated implants increased 56% at day 50 and 23% at day 100 (Table I). These results indicated that bBMP enhanced calcium phosphate ceramic-induced osteogenesis.

## 4. Discussion

Biocompatibility and osteoconductivity are two important properties of calcium phosphate ceramics as bone substitutes [19–24]. Although it was generally thought that calcium phosphate biomaterials are not osteoinductive [19–24], osteoinduction of calcium phosphate biomaterials has been paid more attention in recent years [5–15]. It has been shown to be both material-dependent [7–9] and animal-dependent [9–11], although the mechanism is not well known.

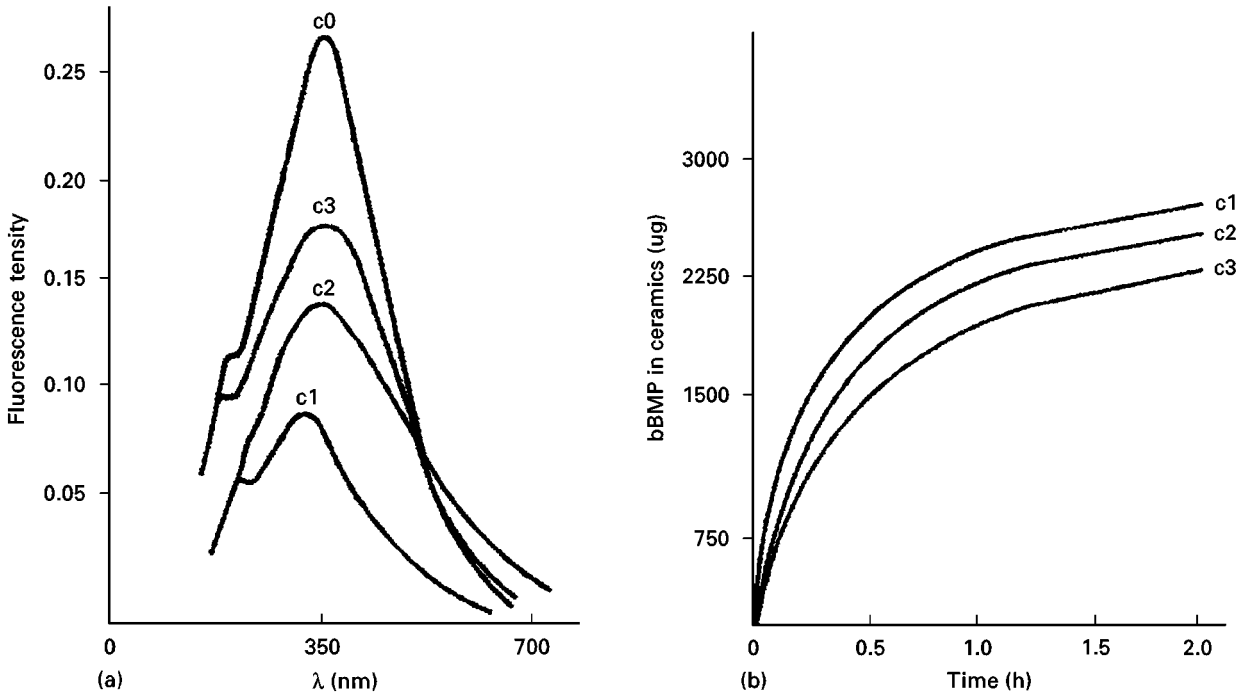


*Figure 1* Adsorption of bBMP to calcium phosphate ceramics. (a) Fluorescence spectro-photometry of bBMP solution circulating for 30 min. (b) bBMP absorbed to calcium phosphate ceramics in 2 h c1, HA; c2, 22TCP/78HA; c3, 37TCP/63HA; c0, 300 μg bBMP/ml 4M Gu-HCl.

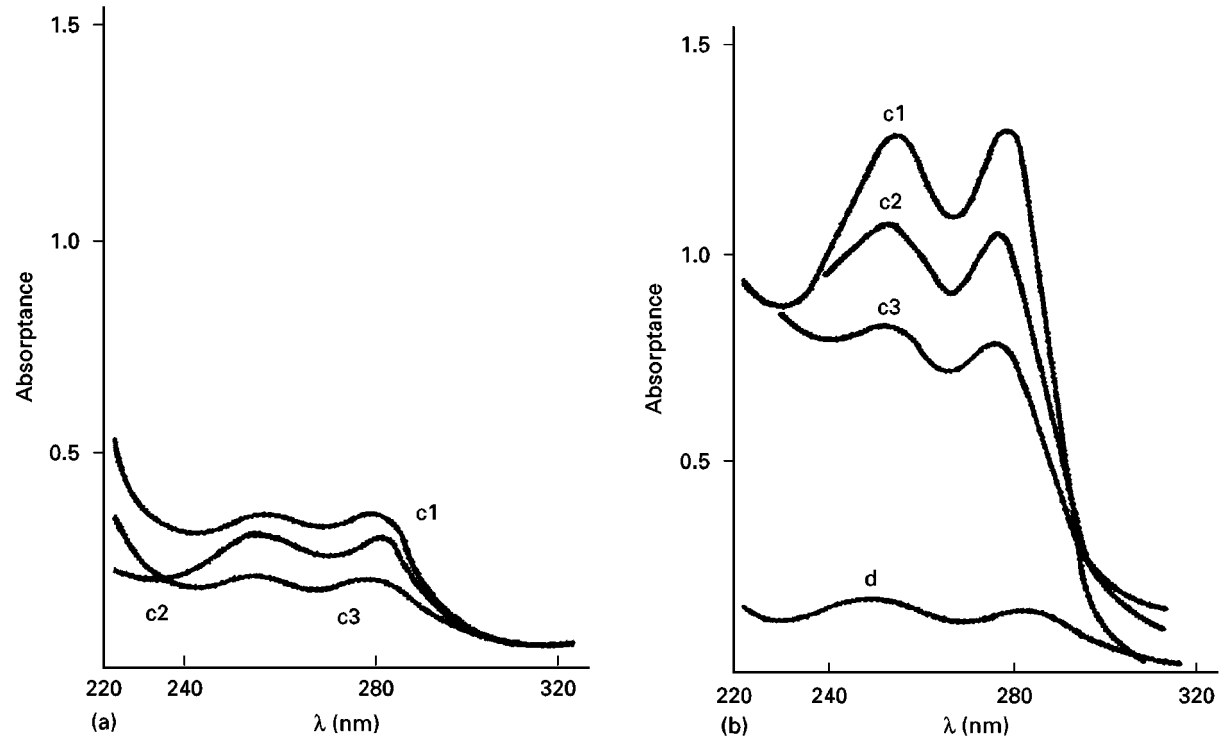


*Figure 2* Washing out of bBMP from calcium phosphate ceramics. (a) UV-spectrophotometry of eluate when washed by distilled water (cl–c3). (b) UV-spectrophotometry of eluate when washed by phosphate buffer (cl–c3) and $CaCl_2$ solution (d). c1, HA; c2, 22TCP/78HA; c3, 37TCP/63HA; d, bBMP washed out from HA by $CaCl_2$ solution.

The present results suggest that BMP can have an important role and adsorption of BMP from body fluids to calcium phosphate ceramic was an important step in osteoinduction.

The importance of BMP in calcium phosphate ceramic-induced osteogenesis has always been hypothesized [5, 10–14]. One reason was that purification of BMP by hydroxyapatite gel, indicated that calcium phosphate can absorb BMP [25]; the second was that BMP or materials cotaining BMP can induce bone formation in soft tissues [4]; the third reason is that when combined with BMP, calcium phosphate ceramics can induce rapid bone formation in soft tissues [26–28], and that bone formation in the composites was more than that in BMP alone [26]. The *in vitro* and *in vivo* experiments in this study provided some direct evidence for this hypothesis. Calcium phosphate ceramic not only absorbed bBMP from

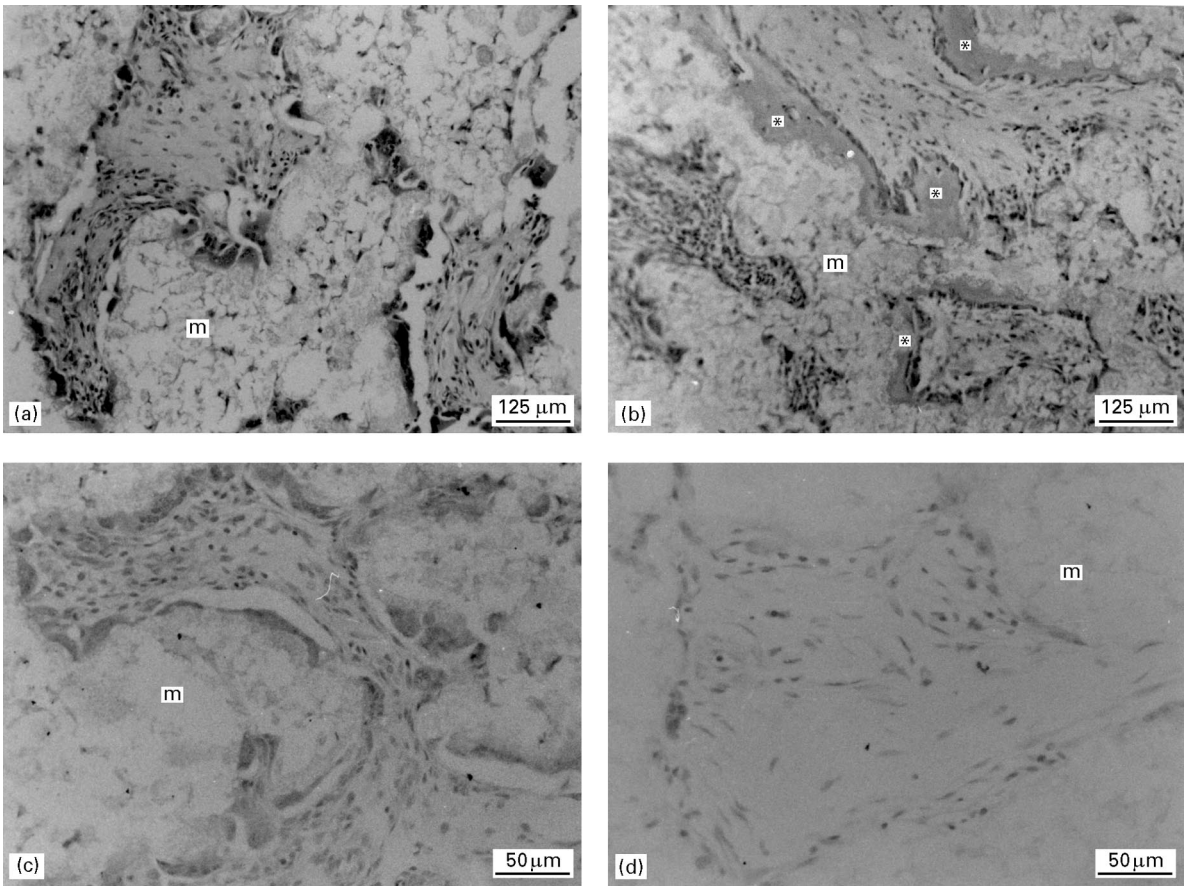


*Figure 3* Histological and immunohistochemical staining of decalcified sections made from the samples harvested from domestic pigs. (a, b) HE staining; (c. d) immunohistochemical staining, Haematoxylin counterstaining; (c) positive staining with bBMP-McAb; (d) negative control; (a, c, d) 30 d (b) 45 d. *, bone; m, ceramic ghost.

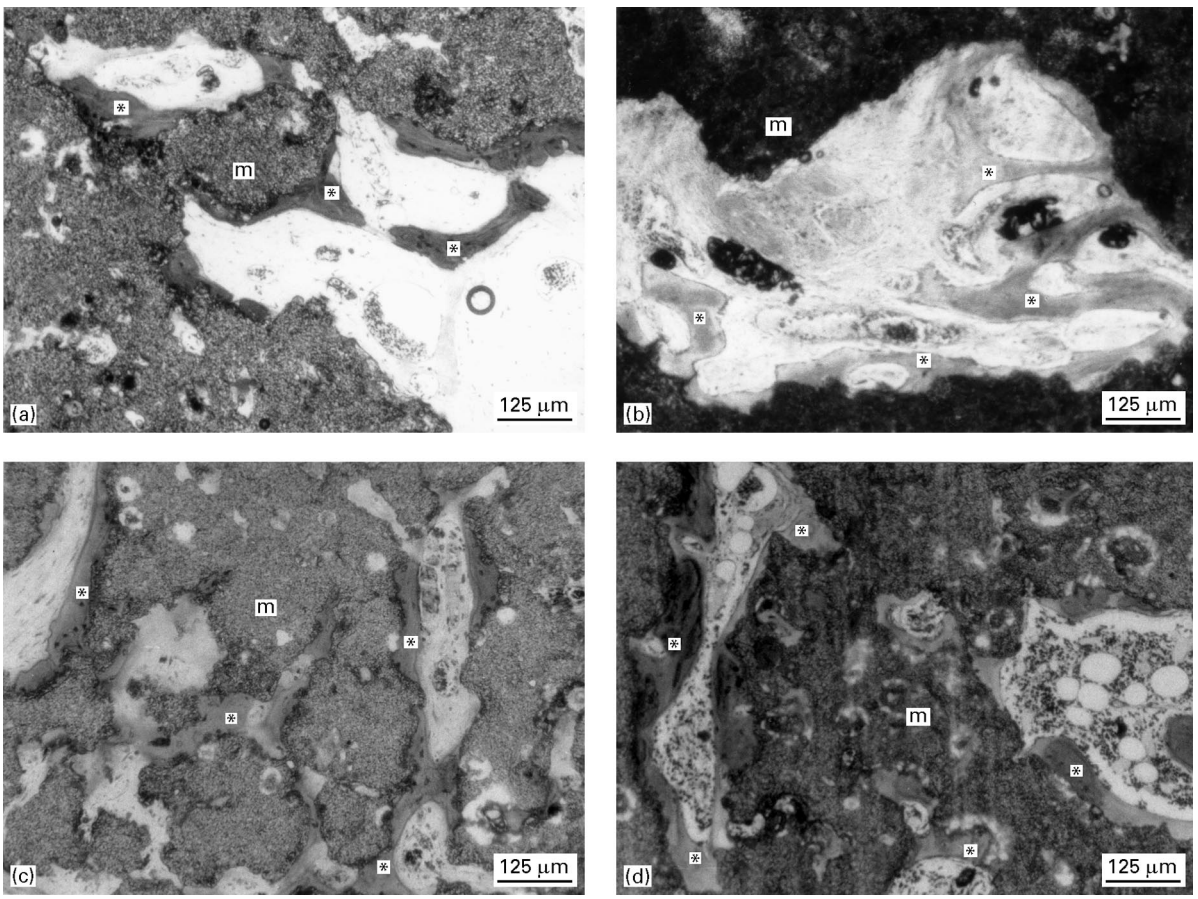


*Figure 4* Bone formation in ceramics with or without bBMP in dorsal muscles of dogs. (a, c) Without bBMP; (b, d) with bBMP; (a, b) 50 d; (c, d) 100 d. m, ceramic; *, bone. (Un-decalcified section; methylene blue and basic Fuchsin staining.)

TABLE I Ratio of bone area to pore area

| | Time | |
|---|---|---|
| | 50 d | 100 d |
| Control | 20.0 ± 5.2 | 36.3 ± 7.4 |
| bBMP-treated | 31.2 ± 8.1 | 44.7 ± 6.3 |
| Ratio[a] | 1.56 | 1.23 |

[a]Ratio = bBMP-treated/control.

a simple bBMP solution *in vitro* but also accumulated native BMP from body fluids *in vivo*; furthermore, when pre-treated by bBMP, more bone occurred in calcium phosphate ceramic.

Previous studies indicated that, as shown in this study, protein adsorption to calcium phosphate ceramics was material-related: the higher Ca/P ratio, the more protein can be absorbed [29, 30]. Other factors affecting protein adsorption include sintering temperature [29]: the higher the sintering temperature, the less protein is absorbed. This could be the reason why, in some cases, when sintered at 1300 °C, hydroxyapatite could not induce bone formation even combined with BMP, and collagen was needed as a BMP carrier to induce bone formation [27]. It was suggested that protein is bound to $Ca^{2+}$ in calcium phosphates [31]. That only a little amount of bBMP could be washed out by distilled water and a large amount of bBMP was washed out by phosphate buffer indicates that BMP was absorbed to calcium phosphate ceramic mainly by chemical way. Moreover, the reason that bBMP could not be washed out by $CaCl_2$ indicates that BMP might bind to $Ca^{2+}$ of calcium phosphate ceramic. It should be noted that *in vitro* adsorption of bBMP to calcium phosphate ceramic might not indicate that this also occurs *in vivo*, because, in body fluids, many proteins other than BMP may compete for the binding site and the ions may also affect protein adsorption. However, the positive BMP immunohistochemical staining indicated that calcium phosphate ceramic could accumulate BMP from the body fluids.

Osteoinduction of calcium phosphate ceramics was a complex process. BMP may play important roles in it, but many other factors may also be involved. Previous studies have shown that tricalcium phosphate/hydroxyapatite ceramic (TCP/HA) can induce bone formation easier than hydroxyapatite ceramic [9], while a stronger BMP adsorption by hydroxyaptite ceramic than by TCP/HA ceramic was found in this study. It is possible that with mild dissolution, the easily formed bone-like apatite surface in TCP/HA can provide an ideal environment for bone formation.

## 5. Conclusion

The results of the studies described herein indicate that calcium phosphate ceramics have the ability to absorb BMP from body fluids, while it is suggested that BMP absorption to calcium phosphate ceramics is an important step in calcium phosphate ceramic-induced osteogenesis.


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