Biphasic calcium phosphate–casein bone graft fortified with *Cassia occidentalis* for bone tissue engineering and regeneration

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Abstract. Research on traditional herbs is gaining momentum owing to their potent medical properties, among which *Cassia occidentalis* (CO) is a promising herb, with osteogenic potential. The study investigates the efficacy of CO extract incorporated biphasic calcium phosphate as an osteoinductive material. Prepared bone implants were characterized physico-chemically using FT-IR, TGA, XRD, SEM and EDX. The implants were analysed further for mechanical and biological properties. The results revealed that CO extract-incorporated bone implants possessed better compression strength and it was able to induce proliferation and enhance alkaline phosphatase activity in SaOS-2 cells. The implant proves to be promising for bone tissue engineering, and hence it demands further *in vivo* evaluation.

Keywords. Biphasic calcium phosphate; bone graft; *Cassia occidentalis*; simulated body fluid; SaOS-2 cell line.

1. Introduction

Bone can be seen as a composite of collagen fibre matrix strengthened by hydroxyapatite (HA) ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) crystal fillers, which constitute about 70% of total weight of the bone.¹ Cells concerned with modifying bone growth are osteoblasts, osteoclasts, osteocytes and bone lining cells. Bone-forming cells are osteoblasts, which are responsible for the production of bone matrix. In addition to production of collagens and other non-collagenous proteins, they also control the mineralization of the matrix.²

Bioceramic materials have greater potential in bone repair due to their inherent ability to interact with the living bone, so as to stimulate growth of latter. Hence, they find use in filling the defects and healing bone fractures. In the different classes of bioceramics, calcium phosphates and their derivatives—HA and β -tricalcium phosphate (β -TCP)—are extensively used components of bioactive formulations. They play a major role in replacing hard tissue as well as in creating chemical attachment between the bone tissue and the ceramic material.^{3,4} Synthetic bone graft substitutes should be biocompatible, show fibrotic reaction, as well as it should undergo resorption and support the formation of new bone. They should possess the elasticity and toughness corresponding to that of bone. Synthetic materials that exhibit these properties are mostly composed of silicon, calcium or aluminium.⁵ HA and β -TCP are the two main delegate compounds of calcium phosphate family having

potential applications in spinal surgery, dental implants and in orthopaedics.⁶ Even though there are various organic and synthetic materials to replace bone or encourage osteogenesis, there is no such material at present that fulfills all the expected requirements. Currently, used bone substitutes include calcium phosphate, calcium sulphate and bioglass, which are used alone or in combination with polymeric materials. The main drawback in using these materials is difficulty in initial tissue interaction due to their non-biological nature. Hence, it is necessary to formulate a material that has better initial cell interaction.

Over the years HA has attained the significant consideration due to its compositional resemblance to human bone.⁷ HA is promisingly bioactive and biocompatible. It becomes directly and strongly bonded to the bone when it is implanted into bone defects.⁸ Several synthetic techniques are in practice for the preparation of HA, which include mechano-chemical synthesis,⁹ combustion preparation,¹⁰ wet chemistry techniques, such as direct precipitation from aqueous solutions,¹¹ electrochemical deposition,¹² sol–gel procedures,¹³ hydrothermal synthesis¹⁴ and emulsion or micro-emulsion routes.¹⁵

β -TCP is one of the most primitive calcium phosphate compounds to be used as a bone graft substitute. It increases the rate of bone union when injected into the gap of a segmental bone defect.¹⁶ β -TCP possesses good biodegradability and osteoconductivity.^{17–20} Owing to its excellent osteoconductive properties and resorbable feature, it has been found to be extensively used in bone cements and as an implant material.²¹ The use of pure β -TCP as bone substitute is critical because any existence of a second phase could induce weakening of its property.⁶ Thus, biphasic

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calcium phosphate (BCP) ceramics are friendly mixtures of HA and β -TCP crystals of varying phase compositions. They have been established for various bio-medical applications, generally as bone graft substitute in stomatology and orthopaedics.^{22,23}

Casein is the main proteineous component of milk and has grabbed our attention for use in tissue engineering field, due to its non-toxicity, cost effectiveness and easy availability. Frequently, casein has been used as a carrier in drug delivery.^{24,25} Casein is poorly soluble in water, exhibiting hydrophobic properties, and appears in milk as a suspension of particles. It is used in the manufacture of adhesives, binders, protective coatings and in food additives.^{26,27} Proteins have the capability of readily adsorbing on the surface of HA.²⁸ Owing to favourable interaction between the HA and proteins, resultant composite materials have noticeably different mechanical properties from those of HA.²⁹ It is reported that casein phosphopeptides are bioactive and biocompatible, concerned with numerous physiological regulation processes. On the surface of casein phosphopeptides (CPP), phosphate groups are present which generate a polar and acidic domain encouraging binding of divalent calcium ions.³⁰ Hence, it favours the nucleation sites for binding calcium ions and promoting HA formation.³¹ Sodium caseinate-stabilized olive oil-in-water emulsions have been prepared in order to act as templates for the adsorption of artificial HA.³²

Natural compounds have traditionally played a major role in the prevention and treatment of diseases and disorders. Plant-derived natural compounds have been found to stimulate osteoblast differentiation and bone formation. These phytochemicals include flavonoids, polyphenol, lignins, coumarins, terpenoids, carotenoids, anthraquinone (emodin) and alkaloids.³³ Plant extracts containing anthraquinone (emodin) are being increasingly used in cosmetic, food, dye and pharmaceutical industries due to their wide therapeutic and pharmacological properties.³⁴ Anthraquinones are group of functionally diverse aromatic chemicals, structurally related to anthracene, with parent structure 9,10-dioxoanthracene.³³ *Cassia occidentalis* (CO) is a plant that contains anthraquinone (emodin). It is used for stimulating osteoblastic differentiation and mineralization through activated PI3K-Akt/MAPKs, which led to the expression of BMP-2 in mouse MC3T3-E1 cells. When BMP-2 gene is triggered using the PI3K-Akt/MAPKs-NF κ B signalling pathways, it ultimately mediates the anabolic activity of emodin.³⁵

In order to formulate a bone graft material with good initial cell interaction and enhanced osteoproliferative properties, a new material is proposed in this study which investigates the possibility of using BCP–Casein–CO extract as a bone graft material.

2. Materials and methods

Casein was purchased from Sigma-Aldrich, India. $\text{Ca}(\text{OH})_2$, H_3PO_4 , NaCl, NaHCO_3 , KCl, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$,

CaCl_2 , Na_2SO_4 and Tris buffer (hydroxylmethyl) aminomethane ($((\text{CH}_2\text{OH})_3\text{CNH}_2)$) were of analytical grade (Merck, India). All other reagents used in this study were of analytical grade.

2.1 Synthesis of HA

The HA powder used in this study was prepared by following the procedure established by Bouyer *et al.*³⁶ In brief, $\text{Ca}(\text{OH})_2$ and H_3PO_4 were starting materials of this reaction. A quantity of 1.9 ml (0.5 M) $\text{Ca}(\text{OH})_2$ was dissolved in 100 ml of distilled water; subsequently 3.7 g (0.3 M) H_3PO_4 was dissolved in 100 ml distilled water. To 100 ml of 0.5 M $\text{Ca}(\text{OH})_2$, 0.3 M H_3PO_4 was added drop by drop and at pH 12.3 the reaction was ended to obtain Ca/P molar ratio of 1.67. H_3PO_4 was added at the rate of 1 ml min^{-1} under constant stirring for 2 h. The reaction is stopped after 24 h of vigorous stirring and the precipitate formed was washed 2–3 times and filtered to remove unreacted precursors. The precipitate was dried at 100°C for 2 h, which resulted in synthetic HA.

HA particles achieved by this reaction are very sensitive to the orthophosphoric acid addition rate and the reaction temperature. The orthophosphoric acid addition rate is strongly linked to the pH obtained at the end of the synthesis.

2.2 Synthesis of β -TCP

β -TCP was synthesized according to the procedure followed in our laboratory.³⁷ In brief, $(\text{CH}_3\text{COO})_2\text{Ca} \cdot x\text{H}_2\text{O}$ and H_3PO_4 were used as calcium and phosphorus source, respectively. Methanol was used as solvent and 7 ml of H_3PO_4 was dissolved in methanol. Subsequently, 23.6 g $(\text{CH}_3\text{COO})_2\text{Ca} \cdot x\text{H}_2\text{O}$ was added slowly along with vigorous stirring and kept for 8 h for precipitation. Later, the precipitate was sintered at $800 \pm 10^\circ\text{C}$ for 5 h using muffle furnace.

2.3 Preparation of CO extract

The aerial part of CO used in this experiment was procured locally and authenticated with the help of plant taxonomist of University of Madras, Chennai, India. Fresh aerial parts of CO were washed with double distilled water, dried under shade and powdered. The dried powder (500 g) was successively extracted using 95% ethanol using Soxhlet apparatus. The last trace of the solvent was removed under reduced pressure by rotary evaporator. The dried crude ethanolic extract has been used for further study.

2.4 Preparation of BCP–casein–CO and BCP implant

HAp and β -TCP powders in the ratio of 60:40 were adopted for preparing BCP–casein–CO implant. A quantity of 10 g of BCP was taken in a mortar and finely powdered, to that

1 g of calcium hydroxide, 2 g casein and, finally, 5 mg of CO extract was added and mixed uniformly. A volume of 2 ml of distilled water was added to the mixture and triturated until it formed dough by adjusting the pH to 7. This dough was filled in a glass tube, diameter of 10 mm and extruded out with a glass rod. The cylindrical implants obtained were cut into required length and allowed to cure at room temperature for 2–3 h. The cured samples were dried initially at 55°C overnight and finally at 100°C for 4 h. The dried samples were sealed in polythene covers and sterilized by gamma-irradiation at 2 Mrads. Subsequently, cylindrical implant was prepared without the addition of CO extract (BCP), dried initially at 55°C overnight and finally at 100°C for 4 h. The dried samples were sealed in polythene covers and sterilized by gamma-irradiation at 2 Mrads.

2.5 In vitro biomineralization test

The biomineralization of the specimen was evaluated by its apatite-forming abilities in simulated body fluid (SBF). It has an ionic concentration almost like that of human blood plasma. The SBF is prepared according to the procedure established by Kokubo³⁸ (Kokubo method). NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, CaCl₂, Na₂SO₄, (CH₂OH)₃CNH₂ and Tris buffer were dissolved in 1 litre of double distilled water, reagents are added one after another, ensuring complete dissolution of earlier reagent in the order given in table 1. The temperature of the solution is maintained at 37°C. To maintain the pH 7.4 of the solution, HCl is added. The prepared cylindrical implant BCP and BCP–casein–CO was soaked in SBF for 21 days. The immersed specimens were removed from the SBF, then abundantly rinsed using deionized water and dried for SEM investigations to show the formation of bone-like apatite layer on their composite surfaces.

2.6 Characterization

The IR spectrum is performed with a Nicolet Impact 400 Fourier-Transform Infrared (FT-IR) spectrophotometer to confirm the surface functional groups of the HA, β -TCP, BCP, casein, CO and BCP–casein–CO. Powder samples were

mixed with anhydrous potassium bromide (KBr), pelletized, and used for FT-IR analysis. The TGA of the prepared samples HA, β -TCP, BCP, BCP–casein–CO was carried out using a Seiko SSC 5200 H in nitrogen atmosphere (80 ml min⁻¹) at a heating rate of 10°C min⁻¹. Primary weight loss of these materials as function of temperature was recorded. The X-ray powder diffraction (XRD) patterns of the prepared samples HA, β -TCP and BCP were done with Seifert JSO Debyelex 2002, an X-ray diffractometer (30 mA, 40 kV) using CuK α radiation ($k = 0.154056$ nm). The surface structure analysis of the samples HA, β -TCP was visualized by scanning electron microscopy (Hitachi S-4800) attached with Phonix energy dispersive X-ray spectroscopy (EDX), which also carried out the elemental analysis. The dried and powdered samples were coated with gold ions using ion coater under the following conditions: 0.1 Torr pressure, 20 mA current and 70 s coating time, using a 15-kV accelerated voltage.

2.7 Porosity and density measurements

Liquid displacement method was performed for measuring the porosity and density of implant using ethanol as the displacement liquid. As ethanol easily penetrates through pores of the implant and does not cause shrinkage or swelling, it is used as non-solvent of the implant.³⁹ A known weight (W) of the sample was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The sample was kept in ethanol for 5 min, and then a series of brief evacuation–repressurization cycles were conducted to force the ethanol into the pores of the scaffold. The process was repeated until the air bubbles stopped. The total volume of the ethanol and the ethanol-impregnated scaffolds was then recorded as V_2 . The difference in the volume was calculated by ($V_2 - V_1$). The scaffolds impregnated in ethanol were removed from the cylinder, and the residual ethanol volume was recorded as V_3 . Thus, total volume of the composite scaffolds was

$$V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3.$$

The density of the scaffold (not the real density), ρ , was expressed as

$$\rho = \frac{W}{(V_2 - V_3)}.$$

Porosity of the scaffold was obtained with, ε

$$\varepsilon = \frac{V_1 - V_3}{V_2 - V_3}.$$

2.8 Tensile strength

To measure the compressive strength, the samples BCP, BCP–casein and BCP–casein–CO implants were loaded along the cylindrical axis in between the platens of the

Table 1. Reagents for preparing SBF for 1 litre at pH 7.4.

Order	Reagent	Quantity
1	NaCl	7.996 g
2	NaHCO ₃	0.350 g
3	KCl	0.224 g
4	K ₂ HPO ₄ ·3H ₂ O	0.228 g
5	MgCl ₂ ·6H ₂ O	0.305 g
6	1 M-HCl	40 ml
7	CaCl ₂	0.278 g
8	Na ₂ SO ₄	0.071 g
9	(CH ₂ OH) ₃ CNH ₂	6.057 g

Figure 1. FT-IR spectra of (a) HA, (b) β -TCP, (c) BCP, (d) casein, (e) CO and (f) BCP-casein-CO.

Figure 2. TGA of HA, β -TCP, BCP and BCP-casein-CO.

machine at a crosshead speed of 1 mm min^{-1} , using Instron 4501 model. The compressive strength was calculated from the break load and dimensions of the pellets.

2.9 Cell culture studies

In vitro assay was performed on human osteosarcoma cell line (SaOS-2) obtained from the National Centre for Cell Science, Pune, India. The cells were grown in McCoy's 5A medium (HiMedia) supplemented with 15% fetal bovine serum (Gibco Laboratories), antibiotics (streptomycin, penicillin and amphotericin B (Sigma)), β -glycerophosphate (100 mM), ascorbic acid (100 nM) and dexamethasone (10 nM) and incubated under standard conditions (37°C , humidified, 5% CO_2 , 95% air).

2.9a Viability assay: Biocompatibility was assessed using alamar blue viability assay.⁴⁰ SaOS-2 cells grown on 24-well plate was exposed to various concentrations (25, 50, 100, 150 and $200 \mu\text{g ml}^{-1}$) of BCP-casein-CO and BCP. The

cell viability was measured after 24, 48 and 72 h. Alamar blue ($100 \mu\text{l}$) was added at the end of incubation period and the plates were incubated for 4 h at 37°C . Following incubation, medium was aspirated and the absorbance was read at 570 and 600 nm using Tecan (infinite M200). The percentage reduction of each well was calculated using standard formula for alamar blue. The experiment was done in triplicates.

2.9b Alkaline phosphatase activity: Alkaline phosphatase (ALP) activity was measured in SaOS-2 cells grown on 24-well plates. Cells were treated with various concentrations (25, 50, 100, 150 and $200 \mu\text{g ml}^{-1}$) of BCP-casein-CO and BCP, and ALP activity was measured after different periods of incubation. After incubation, cells were thoroughly washed with phosphate-buffered saline (PBS) and then lysed using ice-cold lysis buffer (20 mM Tris-HCl—pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100), followed by centrifugation at 3300 g, at 4°C for 5 min. The supernatant was carefully retrieved and stored at 4°C until further use. Working solution of ALP kit was prepared as per the manufacturer's guidelines. Lysis buffer was used as reference for absorbance. Then, absorbance was measured at 405 nm after 10 min. The enzyme activity was calculated according to the manufacturer's formula.⁴¹ The experiment was done in triplicates.

2.9c Statistics: The results of the cell culture studies are given as mean $\pm$ standard deviation of three individual experiments ($n = 3$). The comparison between means was done by one-way analysis of variance ($P < 0.05$) followed by Duncan's multiple range analysis using statistical software package SPSS, version 13.0.

3. Results and discussion

In figure 1a the band at 1040 cm^{-1} has been attributed to $\nu_3 \text{ PO}_4$, and the band at 601 and 562 cm^{-1} to $\nu_4 \text{ PO}_4$. All the absorption bands of HA were found in the FT-IR analysis.

Figure 3. SEM images of (a) HA, (b) β -TCP, (c) energy (EDX) profile of HA and (d) Energy (EDX) profile of β -TCP.

Figure 4. XRD of (a) BCP, (b) β -TCP and (c) HA.

Carbonate peaks were also seen, this may be due to the absorption of carbondioxide from the atmosphere.⁴² The sharp peaks at $560, 607\text{ cm}^{-1}$ correspond to vibration peaks of PO_4^{3-} , while peaks at 966 and 1028 cm^{-1} correspond to PO_4^{3-} ion in β -TCP (figure 1b).⁴³ The FT-IR spectra of BCP (figure 1c) revealed the characteristic bands of phosphate and hydroxyl groups. The ν_1 phosphate bands were present at 962 cm^{-1} and ν_4 absorption bands at 567 cm^{-1} . The band at 606 cm^{-1} can be assigned to the antisymmetric

bending motion of phosphate group in HA. The $-\text{OH}$ stretching bands of hydroxyl groups were also seen at 3500 cm^{-1} . Carbonate peaks found may be due to absorption of carbondioxide from the atmosphere. FT-IR spectrum of casein (figure 1d) showed the characteristic peak of amide I, II, III bands $1650, 1540$ and 1242 cm^{-1} , respectively. Spectrum of $-\text{CO}$ (figure 1e) exhibited anthraquinone characteristic absorption band at 1448 cm^{-1} .⁴⁴ IR spectrum of BCP-casein-CO (figure 1f) indicates the presence of all the components. There is no shifting of peaks and it is assumed that the composite is a physical mixture. The residues of HA, β -TCP and BCP at 800°C was found to be 92, 98 and 91% (figure 2), respectively. This shows that the materials are purely inorganic in nature. However, in the case of BCP-casein-CO, there is a three-step weight loss. Initial weight loss up to 268°C is due to the loss of water and bound water. The second weight loss between 268 and 400°C is due to the degradation of protein.⁴⁵ Third weight loss between 400 and 650°C is due to the decomposition of degraded products of protein and the CO extract. At 800°C , 58% of residue was observed in the BCP-casein-CO implant. This shows the organic and inorganic composite nature of the implant. Figure 3a and b shows the SEM micrographs, while figure 3c and d shows EDX details of HA and β -TCP. In figure 3a, it is possible to observe needle-like crystal morphology of particles in the size range of $50\text{--}100\text{ nm}$. From EDX of HA (figure 3c), the Ca/P ratio was found to be 1:6. The surface morphology of the β -TCP powders precipitated at pH 12.3 (figure 3b). The sintered β -TCP

Figure 5. (a) BCP after treating in SBF and (b) BCP-casein-CO after treating in SBF.

Figure 6. Porosity measurement.

at 800°C precursors was almost spherical and highly agglomerated, and the observed particle size was of 200 nm.

From EDX of β -TCP (Figure 3d), the Ca/P ratio was found to be 1:5. X-ray diffraction (XRD) is routinely employed to characterize the phase composition and percentage crystallinity. It can be seen that the main component of HA and β -TCP (figure 4b and c) is in pure phase. The obtained peaks were compared with the standard data (JCPDS 09-0432), and the diffraction pattern of β -TCP showed the characteristic peaks (JCPDS 09-0169).⁴⁶ The final sample BCP (figure 4a) powder corresponded to a 60/40 wt% HA/ β -TCP. Besides, it is observed in BCP that the characteristic peaks of HA are much more clear than those of β -TCP. This indicates that the ratio of the HA is higher than that of β -TCP in BCP. The mineralization of samples BCP (figure 5a) and BCP-casein-CO (figure 5b) after treatment in the SBF for 21 days is shown in figure 5. A comparatively smooth surface is seen in the case of BCP and less amount of mineralization is obvious. However, mineralized crystals are seen on the surface of BCP-casein-CO sample. Figure 5b indicates the nucleation and growth of HA crystals on the implant.

Table 2. Mechanical properties of BCP, BCP-casein and BCP-casein-CO.

Sl. no.	Sample	Compressive strength (MPa)
1.	BCP	8.34 ± 0.83
2.	BCP-casein	10.44 ± 1.75
3.	BCP-casein-CO	15.23 ± 2.19

The porosity of HA and BCP was found to be more than 70% but, in the case of incorporation of casein with HA (65%), casein with BCP (60%) and casein-CO with BCP (60%), the porosity has comparatively reduced (figure 6). Also, the density values for HA-Casein ($63.45 \times 10^3 \text{ g cm}^{-3}$), BCP-casein ($65.13 \times 10^3 \text{ g cm}^{-3}$) and BCP-casein-CO ($105.2 \times 10^3 \text{ g cm}^{-3}$) were comparatively higher than those of HA ($45.3 \times 10^3 \text{ g cm}^{-3}$) and BCP ($52.42 \times 10^3 \text{ g cm}^{-3}$).

Compressive strength is an important mechanical property of a bone graft material. Factors concerned with strength of a bone repairing material include particle size of individual components, nature of adhesives and percentage of moisture present in the sample. The variation in the compression strengths of various compositions of BCP, BCP-casein, BCP-casein-CO is given in table 2. Casein (insoluble protein of milk) is rendered soluble by the alkali, and then acts as the binder.⁴⁷ Casein could have considerably decreased the porosity of HA, thereby increased its compressive strength. Casein gets firmly adsorbed on the HA surface and it has the ability to interact and stabilize the crystal surface.^{48,49} CPP stabilizes the amorphous phase of calcium phosphate (ACP) in solution and form CPP-ACP complexes.⁵⁰ BCP was in powder form and without casein it was difficult to prepare cylindrical block out of BCP. Casein with BCP has shown improved compression strength ($10.44 \pm 1.75 \text{ MPa}$). However, the compression strength of BCP-casein was less than that of BCP-casein-CO. The reason for increase in compression strength could possibly be due to increase in density and decrease in porosity of the implant (figure 6). This compressive strength

Figure 7. Alamar blue viability (a) 24 h, (b) 48 h and (c) 72 h.

Figure 8. Alkaline phosphatase (a) 4th day and (b) 7th day.

is less when compared with those of natural bone (165 MPa). Hence, these implants cannot be used in defects of weight-bearing bones, however, they may be used in the

defects of non-weight-bearing bones, e.g., maxillofacial bones, spinal cord bone defects, etc.

Viability studies using SaOS-2 cell line, clearly indicate that BCP-casein-CO is highly biocompatible in nature. Cells exhibited more than 80% viability at different time periods (figure 7a-c). There was a significant ($P < 0.05$) difference in viability between BCP-casein-CO and BCP at 150 and 200 µg concentrations, which could be attributed to the growth-promoting effect of CO extract. ALP levels were measured, as it is a phenotypic marker of osteoblasts and is characteristic of differentiation.⁵¹ Though there was not much difference in the levels of ALP between BCP-casein-CO and BCP (figure 8) on day 4, there was a significant difference ($P < 0.05$) in the levels on day 7 at 150 and 200 µg concentrations (figure 8), which is the characteristic of bone-like cells.⁴¹ Herbal extracts of *Terminalia arjuna*,⁵² Si-Wu-tang extract,⁵³ *Butea monosperma*,⁵⁴ *Cissus quadrangularis*.⁵⁵ etc. have been reported to induce proliferation and differentiation of osteoblast-like cells. From the *in vitro* study, it could be stated that BCP-casein-CO was able to induce healthy osteoblast functions viz., adhesion, proliferation and secretion of ALP, etc. in SaOS-2 cells.

4. Conclusion

BCP-casein-CO implants have shown improved mechanical properties and osteoinductive capacity. Moreover, BCP-casein-CO implant has good cytocompatibility with positive effects on cell growth and proliferation. The mechanical strength of the BCP-casein-CO implant can meet the necessary requirements for bone tissue engineering and regeneration. Further animal experiments will be carried out to

demonstrate the capacity of BCP–casein–CO implant for bone repair and regeneration.

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