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Original Article

SYNTHESIS AND CHARACTERIZATION OF A NOVEL BONE GRAFT MATERIAL USING BIPHASIC CALCIUM PHOSPHATE CASEIN CHITOSAN WITH THE EXTRACTS OF CORIANDRUM SATIVUM

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ABSTRACT

Objective: To synthesize a bone graft material using biphasic calcium phosphate (BCP) and chitosan (CH) with the phytochemicals of *Coriandrum sativam*(Cs).

Methods: Bone graft was prepared by mixing BCP, CH and casein glue with the phytochemicals of Cs, prepared in the form of cylindrical implants and dried.

Results: UV Spectrophotometric analysis of the implant revealed a gradual deposition of calcium and phosphorous from 12-21 days and FTIR pattern showed cross linking of the polymer with the ceramics and BCP without any disintegration. This indicates the stability of the implant performing osteogenesis. (XRD) pattern of BCP-Cs-CH-CA suggests the presence of nano HAP and increase in its crystalline and thermo gravimetric analysis showed a deposition of calcium phosphate crystals onto the BCP indicating the thermal stability of the graft. The SEM analysis showed the deposition of mineral phase (calcium phosphate) onto the crystals.

Conclusion: To summarize, in this study we have synthesized a bone graft with finer stability, ossification property, and good mechanical and biological properties and hereby recommend it as a novel bone graft for biomedical applications after its research on in vivo models.

Keywords: Coriandrum sativum, Biphasic calcium phosphate, Chitosan, Hydroxyapatite, β-tricalcium phosphate, Bone graft.

INTRODUCTION

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is an ideal bioactive material which is widely used as a bone graft material due to its similarities with the inorganic phase of bone [1], biocompatibility, osteoconductivity and stable chemical properties. In addition, HAP induces faster bone regeneration and exhibits direct bonding to the regenerated bone without intermediary connective tissue but has very low biodegradability. Beta tri calcium phosphate (*β*-TCP) has high resorbability, bioactivity and biocompatibility [2] and also has high extent of dissolution. Moreover, *β*-TCP has the ability to form a strong interface between the tissues and the graft [3, 4], but some researchers reported that *β*-TCP shows an increased rate of degradation for an ideal bonding to bone. [5]. A current study revealed that biphasic calcium phosphate (BCP) ceramics, consisting of a mixture of *β*-TCP and HAP, i.e. the weight ratio of 30/70 or 40/60 of *β*-TCP/HAP ceramics, have shown more efficiency in the repair of bone defects than pure HAP or pure *β*-TCP ceramic alone [5-7]. Also, BCP has a controlled resorbability and rapid bone formation around the implant site than pure *β*-TCP. Chitosan (CH), a natural cationic polysaccharide derived by deacetylation of chitin, is the second most abundant natural polysaccharide [8]. CH has a variety of promising pharmaceutical uses and is presently considered as a novel carrier material in drug delivery systems [9, 10]. In recent years, CH has been widely used in biomedical applications due to its biodegradability, non-toxicity, antibacterial activity, biocompatibility and has positive charge which acts as a binding site for other functional groups [11, 12] together with its extensive availability in nature and low cost. Casein (CA) being the major milk protein has excellent emulsification, gelation and water binding properties and is widely used in the food industry for various food products. Due to these properties it is utilized to prepare organic adhesive. In this study, CH is used as a biopolymer and a cross linking agent whereas CA imparts the adhesiveness to the graft and forms a tight bonding with BCP.

In the present study a fresh attempt has been made using the extracts of C*oriandrum sativum* (Cs) as a supporting factor for fracture healing. The Cs extract contains decanal, linalool, high amount of calcium, phosphorus, oxalic acid, vitamin A, B and C, iron, protein and fats. Cs is used for the treatment of chronic ulcers, rheumatism, swelling, neuralgia, bleeding piles etc. It has an astringent and aphrodisiac action, which can activate the release of sex hormones and thereby resulting in enhanced healing process [13]. In the current study a bone graft material was prepared using BCP, chitosan and the extracts of Cs, the resultant graft was characterized using conventional techniques.

MATERIALS AND METHODS

Preparation of plant material

Fresh coriander leaves were collected, washed with tap water and then rinsed with distilled water, air dried and ground to fine powder. 10g of this powder was mixed with 100 ml of ethanol in a conical flask and kept for shaking at 150 rpm for 4 days. It was then filtered and the extract is dried in the oven at 50ºC. The dried extract was stored in a sterile container.

Preparation of Nano HAP

An aqueous solution of 0.5 M calcium hydroxide was mixed with 0.3 M ortho phosphoric acid (Sigma Aldrich) drop by drop until the pH reaches 12.5. The mixture was kept for stirring for 24 h. The mixture was then centrifuged at 6000 rpm for 15min. The precipitate was rinsed with distilled water and then dried at 100ºC for 7 h.

Preparation of *β*-TCP

An aqueous solution of $(NH_4)_2HPO_4$ (Sigma Aldrich) (325 ml) was added to an aqueous solution of $Ca(NO_3)_2.4H_2O$ (Sigma Aldrich) (500 ml) under stirring. To this, 5 ml of ammonia solution was added and stirred for 2 h. The mixture was filtered and dried in the oven at 60˚C for 24 h. The flakes were then powdered and calcinated in the furnace at 850˚C for 12h followed by cooling to obtain single phase *β*-TCP.

Preparation of BCP

BCP was prepared by mixing HAP and *β*-TCP in the ratio of 60:40 [5-7].

Preparation of casein glue

2.0g of casein was soaked in 3ml of distilled water for half an hour and grounded to paste using a mortar and pestle. To this paste 1.0 g of calcium hydroxide in 4.0ml of distilled water was added drop by drop until the glue forms.

Preparation of BCP-Cs-CH-CA bone graft

0.5 g of Chitosan (CH) (Sigma Aldrich) was dissolved in 3.0 ml of distilled water. This CH solution was added to 5 g of BCP, 250 mg of plant extract and the casein glue and mixed well into a paste. With the help of a glass tube with a diameter of 1cm the paste was extruded as cylindrical implants. After 2 h of air drying the implants were cured at 60°C for 6 h.

Preparation of Simulated Body Fluid (SBF)

SBF was prepared by dissolving reagent grade NaCl 11.994 g, $NaHCO_3$ 0·525 g, KCl 0·336 g, $K_2HPO_4.3H_2O$ 0·342 g, $MgCl_2.6H_2O$ 0·4575 g, $CaCl_2$ 0·417 g and Na_2SO_4 0·1065 g in deionized water [11, 12]. The solution was buffered at pH 7.4 with tris (hydroxyl methyl) amino methane ($(CH_2OH)_3CNH_2$) and 1M hydrochloric acid at 36·5 ±1°C [14].

***In vitro* bioactivity test**

The prepared bone graft BCP-Cs-CH-CA was immersed in SBF solution for 21 days at 37°C. Later the implant was removed from the solution, washed and dried at room temperature.

Characterisation

UV spectrophotometric analysis was performed for the SBF supernatant at 210 nm at an interval of 3 days and it was plotted as graph. The IR spectra of the prepared samples were read at wave length range of 4000–400 cm^{-1} using Nicolet Impact 400 FTIR spectrophotometer using KBr pellet containing 1–2 mg of the sample. XRD analysis of prepared sample was conducted using an analytical X'Pert PRO alpha-1 with a RTMS X'Celerator detector. It used Ni-filtered Cu Kα radiation over the 2θ range of 20-80° at a scan rate of 2.4° /min and with a sampling interval of 0.002° at 40 mA and 45 kV. The surface morphology was analyzed with a Topcon, SM-300 SEM. The copper disc was pasted with carbon tape and the sample was dispersed over the tape. The disc was coated with gold in ionization chamber before microscopic analysis. The thermo-gravimetric analysis of the samples prepared was carried out using a Seiko SSC 5200 H in nitrogen atmosphere (80 ml/min) at a heating rate of 10°C/min. Primary weight loss of the implant as function of temperature was recorded using this study.

RESULTS AND DISCUSSION

***In vitro* bioactivity test**

The graft was stable for 21 days and hence subjected to further characterization. The SBF supernatant was collected and at regular intervals (3days) and subjected to UV analysis.

UV Spectrophotometric analysis

Fig. 1 shows the UV Spectrophotometric analysis of the implant in which a gradual deposition of calcium and phosphorous attains a stationary phase from 12-21 days. This indicates that the implant was stable and involve in osteogenesis by diffusing the calcium and phosphorous along with the phytochemicals to the surrounding tissues.

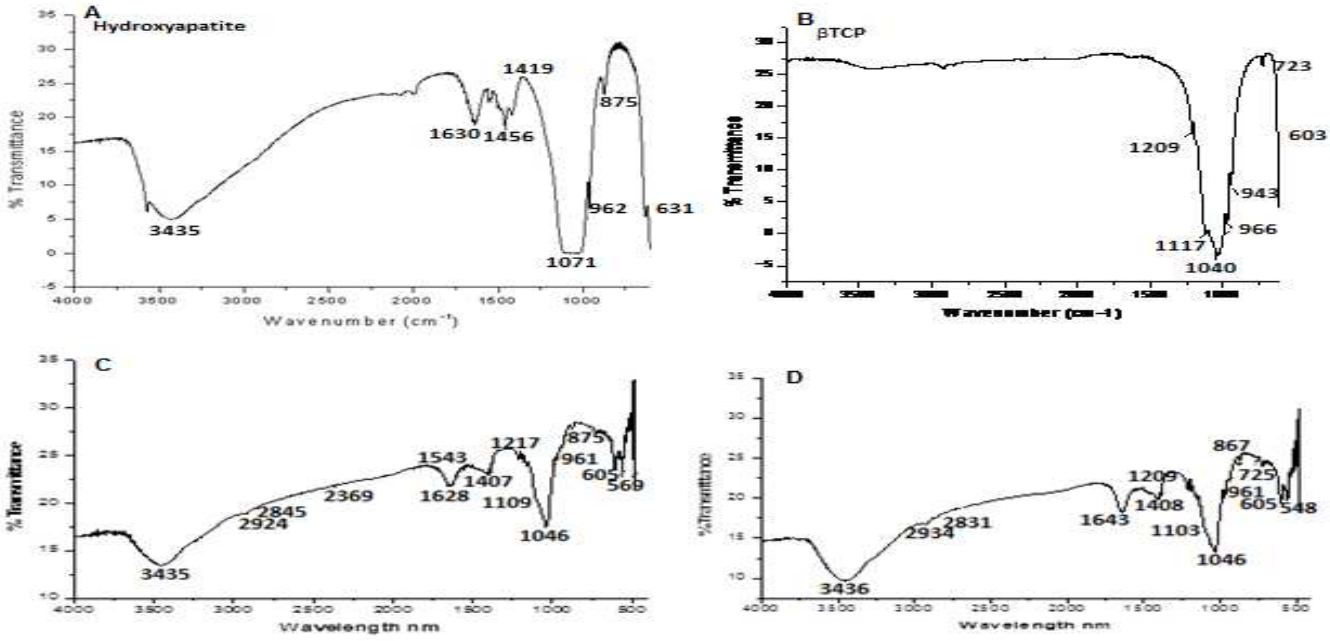

Fig. 2: FTIR pattern of (A) nano HAP (B) β-TCP (C) CH (D) BCP-Cs-CH-CA graft

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of nano HAP, *β*-TCP, CH, BCP-Cs-CH-CA implant are shown in fig. 2 (A) to fig.2 (D) respectively. The band at 605 cm^{-1} can be assigned to the anti-symmetric bending motion of phosphate groups in HAP [15]. The *v*1 phosphate bands representing *β*-TCP can be seen at 946 cm^{-1}. The –OH stretching bands of hydroxyl groups were seen at 3400 - 3700 cm^{-1}. The FTIR spectrum of implant exhibits amide I and II absorption bands of CA at 1649 cm^{-1} and 1543 cm^{-1}, respectively. The hydroxyl groups of CH were merged with those of HAP and were seen as broad band at 1046– 1110 cm^{-1}. FTIR spectra of CS/CA showed the absorption peaks at 3345 cm^{-1} (O-H), 2934 cm^{-1} (C-H), 1722 cm^{-1} (C=O), 1543 cm^{-1} (hydrogen bonded N-H) and 1030 cm^{-1}(C-O). The FTIR pattern for the bone graft before and after SBF showed the above wavelengths indicating the crosslinking of the polymer with the ceramics and also BCP is intact without any disintegration.

X-ray diffraction (XRD) Analysis

Fig.3 shows the X-ray diffraction (XRD) pattern of BCP-Cs-CH-CA suggests the presence of nano HAP, its crystallinity decreases with

increasing CH content. The peaks at 19.54º, 21.26º 26.10º,and 28.03º indicates the reflection from 111, 202, 002, and 210 crystal planes, respectively, thereby indicating the presence of phytonano HAP. The peaks at 31.31º, 33.199º, 48.33º and 52.39º indicates the reflection from 222,112,130 and 315 crystal planes, respectively thereby indicating the presence of β-TCP and CH [16]. All the samples show only characteristic peaks of β-TCP suggesting that its phase did not change into other phases during preparation. This is important for achieving good mechanical and biological properties of produced bone graft.

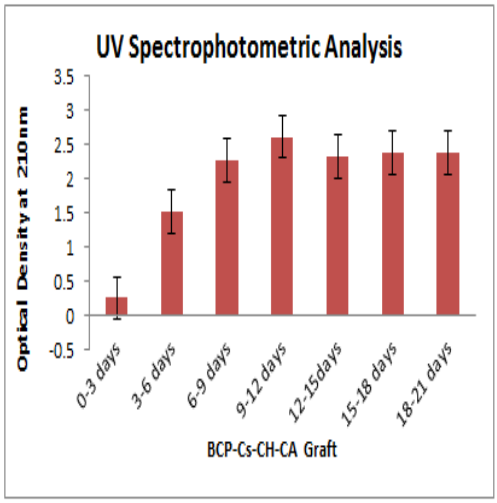

Fig. 1: UV Spectro-photometric analysis of the graft

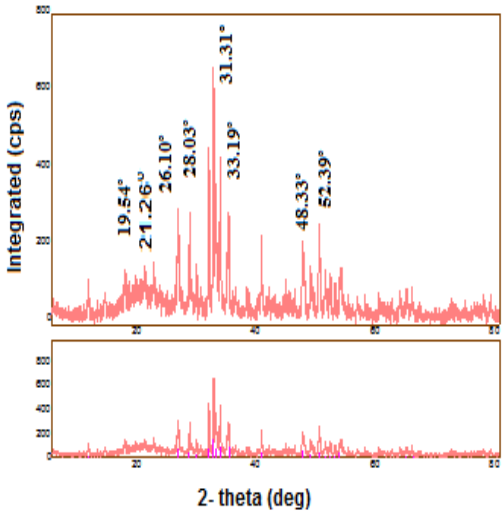

Fig. 3: XRD pattern of the implant

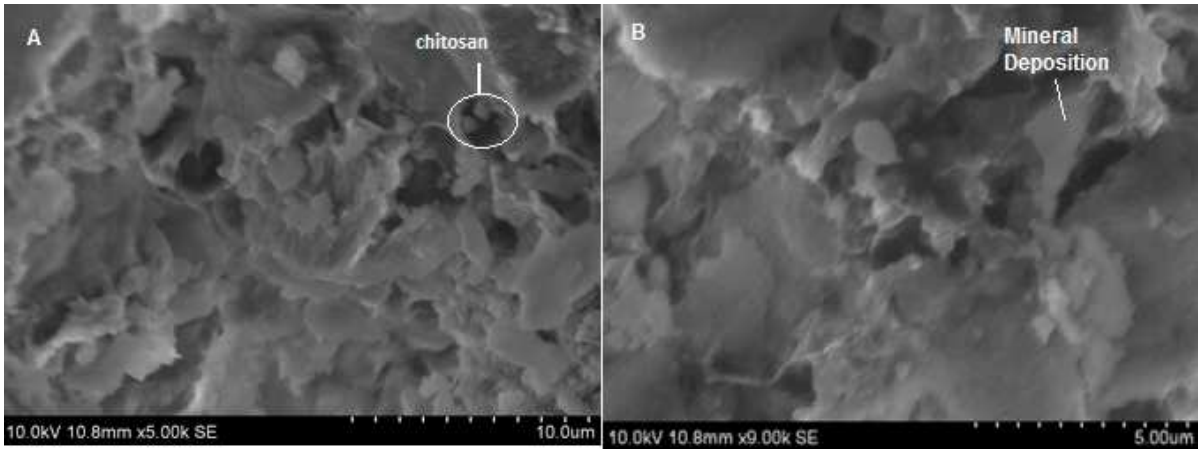

Fig. 4a: SEM image of BCP-Cs-CH-CA graft before SBF (B) SEM image of BCP-Cs-CH-CA graft after SBF.

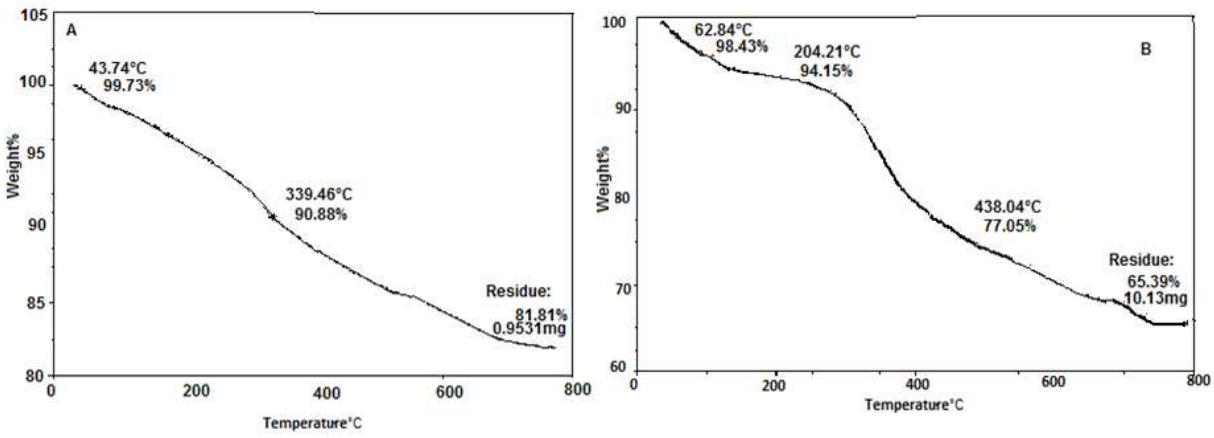

Fig. 5: Thermogram of (A) BCP-Cs-CH-CA graft before SBF (B) SEM image of BCP-Cs-CH-CA graft afterSBF

SEM analysis

Fig. 4(A & B)shows the SEM images of BCP-Cs-CH-CA graft before and after SBF respectively. The SEM images showed that spherical shaped surface indicating the presence of CH and the BCP imparts porosity to the material which is due to the presence of *β*-TCP in the graft. BCP and the phytochemicals were embedded in the porous matrix. The SEM picture of graft after SBF clearly showed the deposition of mineral phase (calcium phosphate) onto the crystals [17]. The aggregation was seen more due to the interconnection of the crystals via calcium phosphate deposition. In addition, it revealed the presence of porous matrix with mineral phase and also the integrity of the bone graft which will strengthen the ossification property of the graft.

Thermogravimetric analysis

BCP-Cs-CH-CA graft was subjected to thermal analysis using TGA and their results were shown in figure 5(A) & (B). A single-step weight loss was observed between 43.74℃ and 339.46℃ in the case of the graft not treated with SBF leaving a residual value of 0.9531 mg. This weight loss may be due to dehydration followed by decomposition of CA and CH. In the case of graft treated with SBF, a three-step weight loss was observed. The first loss was observed between 62.84℃, the second loss was at 204.21℃ and the third loss was at 438.04℃ yielding a residual weight of 10.13mg. There was an increase in the thermal stability of the graft subjected to SBF which may be due to the deposition of inorganic components from SBF. The increase in the residual content of the graft after treated with SBF revealed the fact that there was an increase in inorganic content due to which the thermal stability also increased. This increase in thermal stability reveals the deposition of calcium phosphate crystals onto the BCP while the graft was immersed in SBF.

CONCLUSION

In this study, a novel bone graft was prepared using BCP along with the phytochemicals of *Coriandrum sativum* conjugated with CH and CA. Here CA increases the stability of the graft by interacting with BCP and chitosan imparts an antimicrobial activity to the material. Moreover, the material was subjected to various physico-chemical techniques to reveal the composition and the integrity of the graft. Further, this graft can be used for fracture healing and other biomedical applications, due its better osteoconductivity, biocompatibility, and anti-bacterial activity. It can also be used in the treatment of rheumatism.

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