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OSTEOPOTENTIAL BONE IMPLANT CONTAINING POROUS BIPHASIC CALCIUM PHOSPHATE IMPREGNATED WITH CASEIN, EGG YOLK AND *ORMOCARPUM SENNOIDES* - AN *IN VITRO* STUDY

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ABSTRACT

A novel bone implant material containing biphasic calcium phosphate (BCP), casein (CA), hen egg yolk (EY) and the extracts of *Ormocarpum sennoides* (Os) plant was prepared. It has shown porosity, biocompatibility, cytocompatibility and better alkaline phosphatase activity that is required for osteoinduction. *In vitro* studies using SaOs-2 cell line have exhibited the osteoinductive nature of the implant. These results indicate the possible use of this implant bone tissue engineering.

KEY WORDS*:* Biphasic calcium phosphate, Bone implant, Casein, Egg yolk, O*rmocarpum sennoides*, Alkaline phosphatase, SaOs-2 cell line.

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INTRODUCTION

Calcium phosphate ceramics have gained wide clinical applications due to its biocompatibility and osteoinductivity. Hydroxyapatite (HAP) (Ca_{10} (PO_4) $_6$ (OH) $_{2)}$ is an ideal bone graft material due its similarities with the inorganic phase of bone, osteoconductivity and biocompatibility[1]. Though HAP has faster bone regeneration and good bioaffinity, it cannot be dissolved in the physiological environment. This was overcome by *β*-tricalcium phosphate (*β*-TCP) which has a greater degree of dissolution, degradation and high resorbable property[2-4]. These properties of *β*-TCP enhanced its utilization as bone cements and bone grafts. Since the use of pure *β*-TCP can cause the existence of a second phase which may result in weakening of its own properties. However, biphasic ceramics (a mixture of HAP and *β*-TCP) consisting of HAP/ *β*-TCP with ratios 60/40 or 70/30 have shown greater efficiency in tissue engineering [5-7]. BCP shows an efficient and rapid bone formation around the implant site and also exhibits controlled resorbability than *β*-TCP and HAP [2].Like other bone substitute materials, BCP also has osteoconductive property and acquires osteoinductivity through critical geometry of macroporosity [8, 9]. A bone implant should exhibit porosity in order to accommodate in the host tissue and to support cell growth. Many pore creating agents are available such as polymethylmethacrylate micro beads (PMMA), sodium chloride (NaCl), carbon beads, starch, ice and naphthalene. In this study egg yolk (EY) was used to impart the interconnecting porosity in the bone implant and to mimic human natural bones[10].EY is generally employed in food industry as an emulsifying agent and also a good binder. At 60-70°C, the proteins in egg yolk coagulate to form a semisolid or solid structure,this process enhances the binding nature of egg yolk and holds the elements together. In addition, it also preserves the moisture between the particles[11, 12]. Through these properties egg yolk imparts porosity to the bone implant. Despite of these advantages of EY, it gave less strength to the implant and hence we have used casein in the present study to give better strength to the implant. Casein (CA) being a phosphoprotein, has poor solubility in water and exhibits hydrophobic properties. It has many industrial applications like manufacture of adhesives, binders, protective coatings, plastics, fabrics and food additives [13, 14]. In recent studies, CA is used in tissue engineering as it is inexpensive, easily available, non-toxic and highly stable. Casein as microspheres is often used in drug delivery system [15 - 17]. In this study CA is used as binder, it also forms bonds between the surface phosphate groups of casein with divalent calcium ions thereby supporting the formation of hydroxyapatite. Herbs play a vital role in the prevention and treatment of diseases. The phytochemicals derived from certain plants induce bone formation and fracture healing. In the present study, Os extract is used as a promoter for fracture healing. *Ormocarpum sennoides (Os)* belongs to Fabaceae family and found growing in the scrub jungles of Coromandel, in India. This plant is known to the villagers as bone-knit plant and used for healingfractures[18]. In this study the extract of Os was used to find its capacity for inducing osteoblast differentiation. This paper also describes the *invitro* studies wherein SaOs-2 cell lines are used to find out osteoblast differentiation properties of the prepared bone implant.

MATERIALS AND METHODS

1. Synthesis of hydroxyapatite (HAP)

HAP was synthesized by modifying the procedure of Bouyer et al[19]. 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 in continuous stirring for 24 h. The resultant was then centrifuged at 6000 rpm for 15min. The precipitate was collected, rinsed with double distilled water and then dried at 100ºC for 7 h.

2. Synthesis of β–TCP

β-TCP was synthesized by modifying the procedure of Krithiga et al [20]. An aqueous solution of diammonium hydrogen phosphate (Sigma Aldrich) (325 ml) was added to an aqueous solution of calcium nitrate tetra hydrate (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 850º C for 12h followed by cooling to obtain single phase *β*-TCP.

3. Preparation of Ormocarpum sennoides (Os) extract

Healthy leaves of Os were collected and washed with double distilled water, dried under shade and powdered. The dried powder was extracted using 95% ethanol using a Soxhlet apparatus. Using rotary evaporator, the solvent was removed and the dried crude ethanolic extract was used for further study.

4. Preparation of BCP-CA-Os -EY and BCP – EY Implant

BCP was prepared by mixing HAP and β-TCP powders in the ratio of 60:40[21]. 5 g of BCP was taken in a mortar and finely powdered, to that, 2 g of casein 1 g of calcium hydroxide and finally 5mg of Os extract was added and mixed uniformly.3ml of egg yolk was added and mixed well to form dough. This dough was later extruded through a glass tube (1 cm diameter) to form cylindrical-shaped implants. Subsequently, bone implant was prepared without the addition of Os extract (BCP-CA-EY). The obtained implants were cut into required length and allowed to cure at room temperature for 2 to 3 h. The cured implants were dried at 55°C overnight and sealed in polythene covers and sterilized by gamma irradiation at 2 Mrads. The prepared implants were further subjected to cell culture studies.

CHARACTERIZATION

1. Cell culture studies

In vitro assay was performed on human osteosarcoma cell line (Saos-2) procured from National Centre for Cell Science, Pune, India. The cells were grown in McCoy's 5A medium(Hi Media) added with 15% fetal bovine serum (Gibco Laboratories), antibiotics(streptomycin, penicillin, and amphotericin B, (Sigma)), β-glycerophosphate (100 mM), ascorbicacid (100 nM) and dexamethasone (10 nM) and incubated under standard conditions (37°C, humidified, 5% CO2, 95% air).

2. Viability assay

Biocompatibility of the implant was evaluated using alamar blue viability assay[22]. SaOS-2 cells grown on 24 well plate was exposed to various concentrations (25, 50, 100, 150 and 200 µg/ml) of BCP- CA-Os-EY and BCP-CA-EY. The cell viability was measured after 24, 48 and 72 h. Alamar blue (100 µl) was added to the wells at the end of incubation period and the plates were incubated for 4 h at 37°C. Following incubation, media was aspirated and the absorbance was read at 570 and 600 nm using Tecan (infinite M200). The percentage reduction of each well was calculatedusing standard formula for alamar blue. The experiments were done in triplicate.

3. 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 200µg/ml) of BCP- CA-Os-EY and BCP-CA-EY and at different periods of incubation ALP activity was measured. After incubation cells were given a phosphate buffered saline (PBS) wash and then lysed using ice-cold lysis buffer (20 mM Tris-HCl – pH7.5, 150 mM NaCl, 1% v/v Triton X-100).The contents were then centrifuged at 3300g, at 4 °C for 5 min. The supernatant was carefully collected and stored at 4°C until further use. Working solution of alkaline phosphatase (ALP) kit was prepared as per the manufacturer's guidelines. Lysis buffer was used as blank for absorbance. The absorbance was measured at 405 nm after 10 min and the enzyme activity was calculated according to manufacturer's formula [23]. The experiments were done in triplicate.

4. SEM Analysis

The surface morphology of the implant was visualized by scanning electron microscopy (Zeiss Gemini Supra 55) attached with Oxford instrument X-act Energy dispersive X-ray spectroscopy (EDX), which 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, 20mA current and 70 s coating time, using a 15 KV accelerated voltage.

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5. Statistics

The results of the cell culture studies are given as mean±standard deviation of three Individual experiments (n=3). A one-way analysis of variance ($p<0.05$) was done by comparing the means followed by Duncan's multiple range analysis using statistical software package SPSS, version 13.0

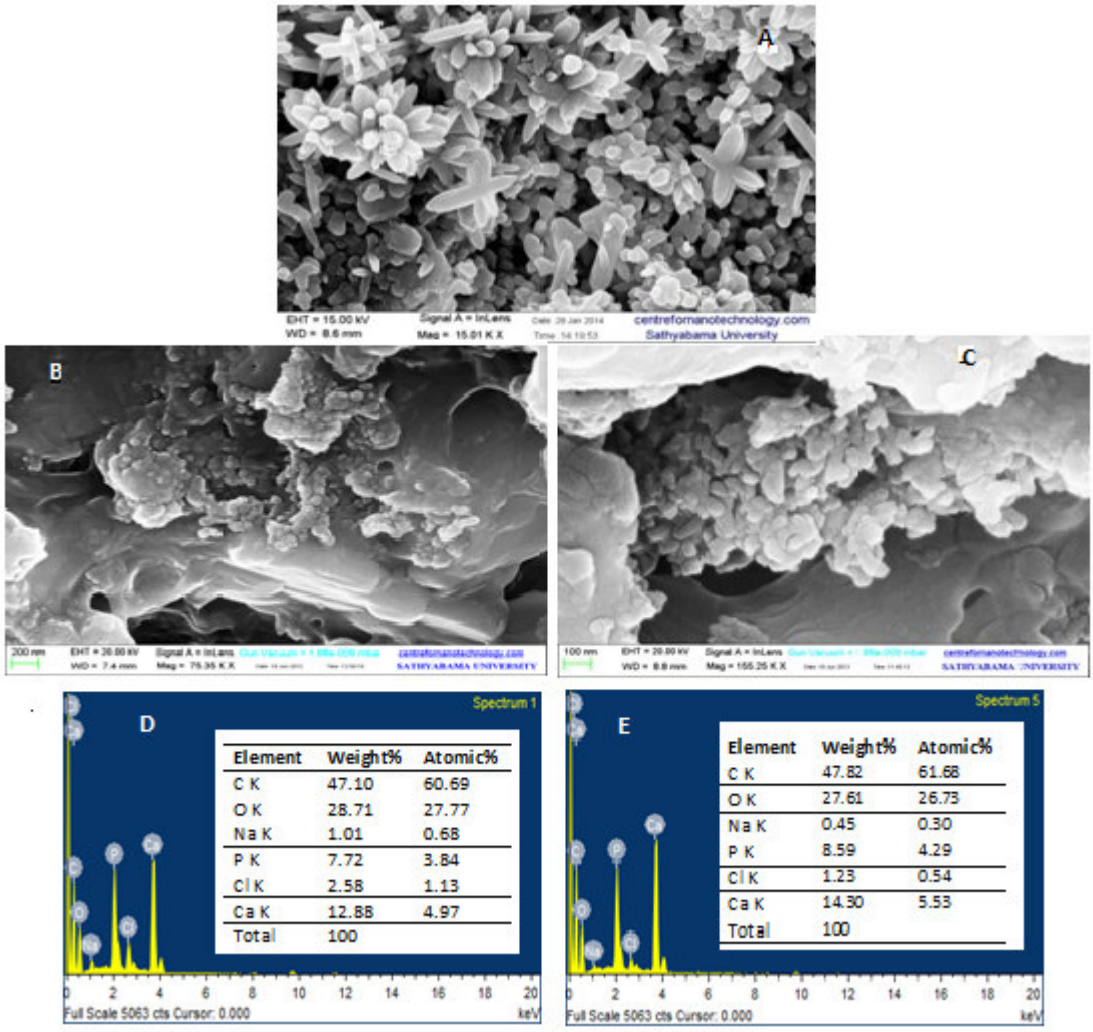

Figure 1

SEM image of (A) BCP (B) BCP-CA-EY (C) BCP-CA-Os-EY (D) EDX Spectrum of BCP-CA-EY and (E) EDX Spectrum of BCP-CA-Os-EY

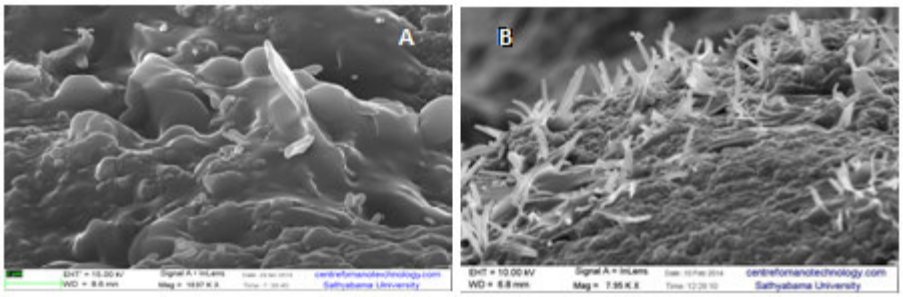

Figure 2
SEM image showing implant with SaOs 2 cells
(A) BCP-CA-EY and (B) BCP-CA-Os-EY

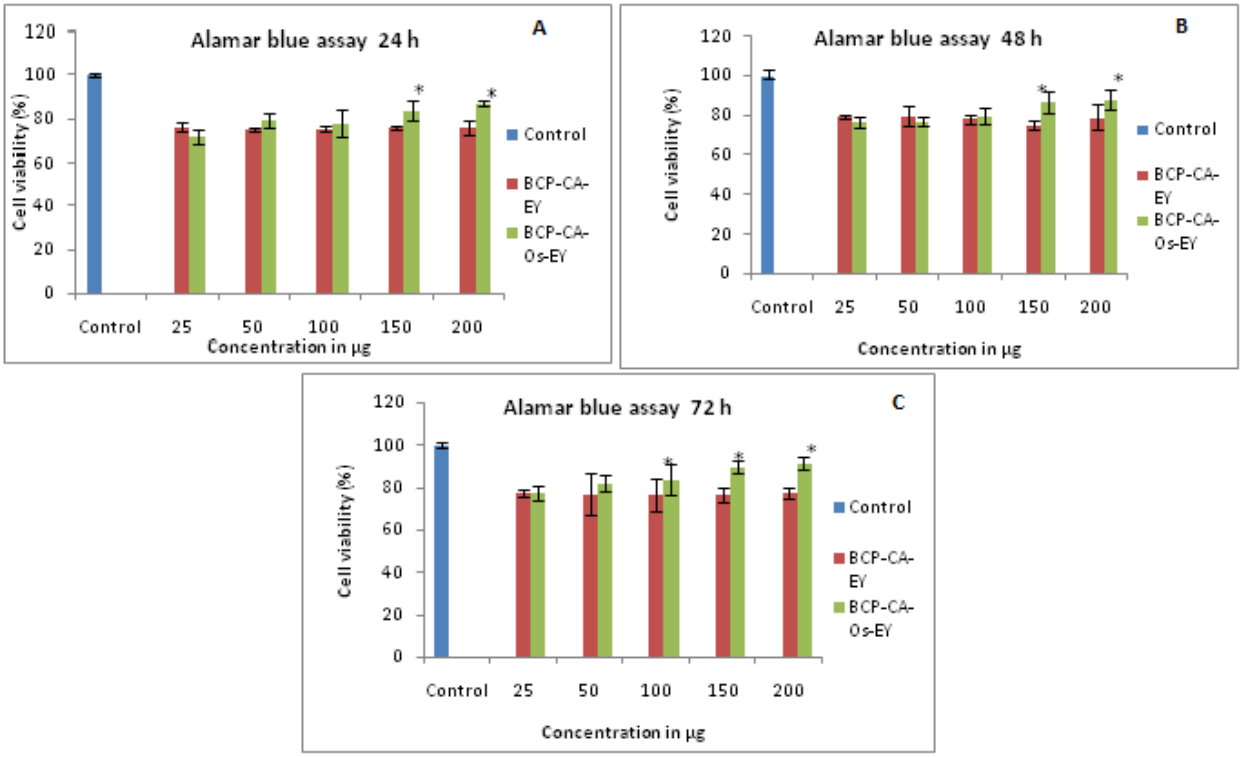

Figure 3
Alamar blue viability of BCP-CA-EY and BCP-CA-Os-EY(a) 24 h (b) 48 h(c) 72 h

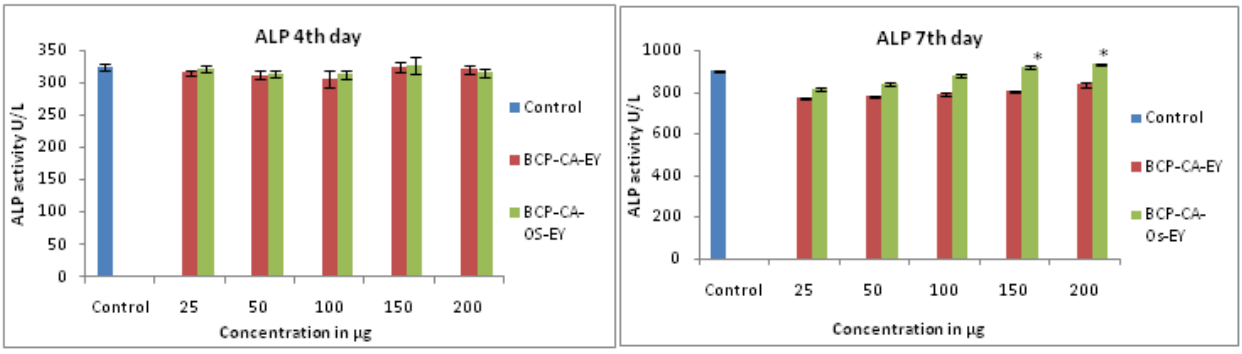

Figure 4
ALP activity of BCP-CA-EY and BCP-CA-Os-EY (A) 4th day (B) 7th day

RESULTS AND DISCUSSION

1. SEM analysis

Figure 1A shows the SEM micrographs of BCP in which HAP crystals appear as rod shaped crystals arranged in a flower like model with a particle size ranging from 40 – 100 nm and *β*-TCP crystals were found highly agglomerated. Figure 1B and 1C show the porous nature of the both the implants imparted by egg yolk. Figure 1D and 1E shows the EDX of both the implants with a Ca/P ratio of 1.66. Fig.2A and 2B show the SEM image of implant treated with SaOS2 cells after 24 h. The circumference of the implant was inclined at an angle of 45°and the image was taken so as to visualize the penetration of the osteoblast cells in the implant. In figure 2A, BCP-CA –EY implant treated with SaOS2 cells show the growth of the cell in a passive manner. Whereas in figure 2B, the cells in BCP-CA-Os-EY implant rapidly grows and extends its filopedia compared to BCP-CA –EY implant and hence reveals that the Os extract can enhance the osteoblast differentiation. The image clearly shows the SaOS2 cells extend their filopedia and penetrate through the porous implant thereby indicating viability and increased adherence to the implant [24].

2. Cell viability

Viability studies on the implants BCP-CA-Os-EY and BCP-CA-EY using SaOS2 cell-line, clearly indicate that the implants are highly biocompatible and the cells exhibit more than 75% viability at different time periods (Figure 3A, 3B and 3C). There is a significant ($p<0.05$) difference in viability between BCP-CA-Os-EY and BCP-CA-EY at 150 and 200 µg concentrations, which could be attributed to the growth promoting effect of Os extract.

3. Alkaline phosphatase (ALP) activity

Alkaline phosphatase levels were measured on day 4 and 7 respectively as ALP has a significant role as phenotypic marker of osteoblast and their differentiation [25]. On day 4, there was not much difference in the levels of ALP between BCP- CA-Os-EY and BCP-CA-EY (Figure 4A), but on day 7 there was a significant difference ($p<0.05$) in the levels at 150 and200 µg concentrations (Figure 4B), which resembles the feature of bone like cells [23]. From the *in vitro* study, it could be stated that BCP-CA-Os-EY was able to induce healthy osteoblast functions viz., adhesion, proliferation and secretion of ALP etc in SaOs-2 cells.

CONCLUSION

In this study, BCP-CA-Os-EY showed improved cytocompatibility and osteoinductivity properties. Moreover the SaOS2 cell treated BCP-CA-Os-EY revealed its porosity and further improves the osteoinductivity and also exerts positive effects on cell growth and proliferation. The implant shows that it can meet the requirements for bone tissue engineering and other biomedical applications. Further, *in vivo* studies will be carried out to find its efficacy as an osteoinductive material.

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