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Fabrication, characterization and osseointegration of bonegraft incorporated with leaf extracts of *Ormocarpum Sennoides* and biocompatible polymers

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ABSTRACT

Allogenous grafts with excellent biocompatibility and immuno-compatibility play a major role in most of the biomedical applications. In this regard, the present study focuses on fabricating a novel bone graft material containing biopolymers and phytochemicals which can replace the use of autogenous graft with high biocompatibility and osteogenecity. Based on this, a bone graft material was synthesized using biphasic calcium phosphate (BCP), casein (CA), hen egg yolk (EY) and leaf extracts of *Ormocarpum sennoides* (Os). Two types of bone grafts namely group I (BCP-EY) and II (BCP-CA-Os-EY) were prepared and processed. The processed grafts were subjected to various characterizations like Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD), thermo gravimetric analysis (TGA), scanning electron microscopy (SEM), energy dispersive X-ray (EDX) analysis, and mechanical strength to show its chemical composition, stability and porosity. Further, the osteogenecity of the grafts were analyzed by performing invivo studies using wistar male albino rats. Samples were subjected to biochemical, radiological and histopathological analyses. Among the two grafts, graft II containing *Ormocarpum sennoides* extract showed excellent osteogenicity both invitro and invivo and hence it can be utilized in various biomedical applications like orthopedics, dental fillings, bone tissue engineering and in the treatment of rheumatoid arthritis.

1. Introduction

The widely used material for biomedical applications is ceramics (Christel et al., 1988). Ceramics can replace the use of autografts if they satisfy the conditions such as restoring the function of the surrounding tissue, filling this space, physiologically interact with body fluids, stimulate osteogenesis and provide skeletal repair which could bear a permanent stress. Calcium phosphate ceramics like hydroxyapatite (HA), β tricalcium phosphate (β-TCP) and Biphasic calcium phosphate (BCP) are used as composites as they can be bone bioactive thereby facilitating integration with bone tissue and osteoconductivity (Patlolla and Arinzeh, 2014). Bone grafts containing HA alone was unstable due to its fragility and hence β-TCP (Boden, 1999) or BCP (Jones et al., 1991; Ducheyne, 1992; Jacobson et al., 1992) are used in present studies. Biphasic calcium phosphate ceramics are made up of HA and β-TCP and this chemical composition are similar to natural HA of human bone and teeth. BCP showing a crystalline phase of 93 wt% of HA and 7 wt% of β-TCP in calcined bone when implanted in the body get dissolved in the surrounding fluid and releases its calcium and phosphate ions in the biological medium (Sasaki et al., 1989; Vogel et al., 2001). This dissolution property of BCP is determined by the β-TCP/HA ratio (Vogel et al., 2001; Schildhauer et al., 1999). During biodegradation dissolution of HA and β-TCP crystals happens individually (Vogel et al., 2001; Martin and Brown, 1997). BCP shows both the properties of HA and β-TCP by being stable and easily dissolve in the physiological environment. On the other hand, due to its high brittleness, ductility and toughness (Ramay and Zhang, 2004) the use of BCP as load bearing implants has been reduced. To strengthen the mechanical properties of the bone graft with superior biocompatibility, regenerative properties like osteoinduction, osteoconduction and osteointegration and better biodegradability (Kikuchi, 2013; Liu et al., 2013; Ohba and Tei Chung, 2013; Pastorino et al., 2014; Ma et al., 2014) biopolymers like collagen, chitosan, fibrin, gelatin, alginate, elastin casein etc. are added to BCP.

The present study shows interest in strengthening the BCP by including casein and egg yolk in the graft. Casein, a phospho protein contributes approximately 80% of protein in milk. Due to its hydrophobicity, casein has gained wide applications in the manufacture of plastics, food additives, adhesives, protective coatings, binders and

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fabrics (Somanathan et al., 2000; Diak et al., 2007). Recently casein is used in tissue engineering as it is inexpensive, readily available, biocompatible, non- toxic and highly stable. In bone tissue engineering casein is fabricated with other inorganic materials and used as a composite scaffold (Ritzoulis et al., 2005).

Egg yolk being a good emulsifying agent (Chung and Ferrier, 1991; Khan et al., 1998) contains Phosvitin, a phosphoprotein exhibits metal chelating property by binding with bivalent metals like calcium, magnesium and iron due to its high phosphate content (Taborsky, 1963; Grizzuti and Perlmann, 1973). Due to these properties, egg yolk was employed as an emulsifier, binder and pore creating agent (Fariza et al., 2011). The present study uses these properties of egg yolk and employs in preparing a porous bone graft for its application in fracture healing and tissue engineering.

Ancient Indians used herbal extracts for treating innumerable diseases. World Health Organization made an analysis on herbs and revealed that 80% of world's population uses herbal medicines as the prime treatment (Andy et al., 2008). These herbal extracts are enriched with bioactive compounds called phytochemicals which make them a potent medicinal plant. The important phytochemicals which imparts the medicinal value to the herbs are alkaloids, flavonoids, tannins and phenolic compounds. The prime purpose of using herbal extracts in medicine is that they are safe than allopathic medicines and has greater therapeutic benefits with less cost (Doughari and Obidah, 2008).

Researchers have done extensive study on the applications of herbs in fracture healing and bone tissue engineering. Many herbs are employed in bone tissue engineering such as *Acalypha indica, Cissus quadrangularis* (Muthusami et al., 2011), *Terminalia arjuna* (Krithiga et al., 2014), *Cassia occidentalis* (Santhosh Kumar et al., 2015a, 2015b), *Terminalia chebula* (Krithiga et al., 2011) and *Myristica fragrans* (Santhosh Kumar et al., 2015a, 2015b). The present study incorporates the leaf ethanolic extract of *Ormocarpum sennoides (Os)* in the bone graft. This plant belongs to the kingdom Plantae, phylum Magnoliophyta, class magnoliopsida and order fabales. The plant comes under the fabaceae family and is widely distributed in scrub jungles of India and Srilanka. It is found growing in the coromendal forest of Tamilnadu, in India (Thamacin Arulappan et al., 2014).

Ormocarpum sennoides is being known to the villagers as bone-knit or elumboti and used for fracture healing (Alves et al., 2004). Traditionally, the leaf powder of Os is consumed orally by mixing with honey or milk and also the leaf paste is applied topically on the fracture site and covered. Due to its fracture healing property, this study utilizes the phytochemicals of Os by incorporating the leaf extract in the bone graft and analyzing its properties thereby bringing the osteogenic property of this plant into light with scientific evidence.

2. Materials and methods

2.1. Chemicals

95% Ethanol, calcium hydroxide, ortho phosphoric acid, diammonium hydrogen phosphate, calcium nitrate tetra hydrate, ammonia and casein were purchased from Sigma Aldrich.

2.2. Preparation of the plant extract

Healthy leaves of *Ormocarpum sennoides* (Os) were collected from Pachakuppam hills, near Ambur, Tamilnadu, India. The leaves were washed with double distilled water, dried under shade and powdered in an electric blender. The dried powder was extracted using 95% ethanol in a Soxhlet apparatus. Using rotary evaporator the extract was concentrated, stored at 4 °C and the dried crude ethanolic extract was used for further study.

2.3. Synthesis of Hydroxyapatite (HA)

HA was synthesized by modifying the procedure of Bouyer et al. (2000). An aqueous solution of 0.5 M calcium hydroxide was prepared and to this 0.3 M ortho phosphoric acid was added 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 15 min. The precipitate was collected, rinsed with double distilled water and then dried at 100 °C for 7 h (Srividya et al., 2014).

2.4. Synthesis Of β-tricalcium Phosphate (β –TCP)

β-TCP was synthesized by modifying the procedure of Krithiga et al. (2011). An aqueous solution of diammonium hydrogen phosphate (25.76 g in 325 ml of double distilled water) was added to an aqueous solution of calcium nitrate tetra hydrate (69.675 g in 500 ml of double distilled water) under continuous stirring. To this, 16.5 ml of ammonia solution was added and stirred continuously for 2 h. The mixture was filtered, and the filtrate was rinsed with double distilled water (in order to remove the unreacted calcium and phosphate) 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 12 h followed by cooling to obtain a white fluffy precipitate of single phase β-TCP (Srividya et al., 2014).

2.5. Preparation of casein glue

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

2.6. Preparation of bone graft

BCP was prepared by mixing HA and β-TCP powders in the ratio of 60:40 (Feng-Huei et al., 1999). Two groups of grafts were prepared. In group I, 5 g of BCP and 3 ml of egg yolk was mixed and casted into cylindrical graft which was named as BCP-EY. Group II graft was prepared by mixing 5 g of BCP, casein glue, 500 mg of Os extract and 3 ml of egg yolk followed by casting into cylindrical grafts and named it as BCP-CA-Os-EY. The obtained cylindrical grafts were cut into required length and shape (disc or cylindrical shape) and allowed to cure at room temperature for 2–3 h. The cured grafts were dried at 55 °C overnight and sealed in polythene covers and sterilized by gamma irradiation at 2Mrads.

2.7. Characterization of the bone graft

The IR spectra of the prepared samples were read at 4000–400 cm^{-1} using Nicolet Impact 400 FTIR spectrophotometer using KBr pellet containing 1–2 mg of the sample. XRD analysis of the sample was done using an analytical X'Pert PRO alpha-1 with a RTMS X'Celerator detector. It utilizes Ni-filtered Cu Kα radiation over the 2θ range of 20–80° with a scan rate of 2.4°/min and at a sampling interval of 0.002° at 40 mA and 45 kV. The surface morphology was analyzed with a Zeiss Gemini Supra 55, SEM and EDX with Oxford instrument X-act. 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. Seiko SSC 5200 H is used for analyzing the bone grafts for thermo-gravimetric analysis in a nitrogen atmosphere (80 ml/min) at a heating rate of 10°c/min. Primary weight loss of the graft which was due to the function of temperature was recorded using this study.

2.8. In vivo animal studies

In vivo experiments were carried after getting the ethical committee approval (IAEC No. 03/008/2014). Eight week old male wistar rats weighing 150 – 200 g were divided into two groups each group containing 8 rats. The animals were classified based on the nature of the graft implanted. Group I was implanted with BCP-EY graft and Group II were implanted with BCP-CA-Os-EY graft. First the rats were anesthetized using ketamine 50 mg/kg of body weight intra-peritoneal injection. The skin on the either side of vertebra