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Osteoinductive potential of biocomposite cylinders impregnated with *Glycyrrhiza glabra* for bone tissue engineering

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Abstract

Biocomposite cylinders containing biphasic calcium phosphate (BCP), collagen (CS), and an extract of *Glycyrrhiza glabra* (GG) were prepared and characterized for their physicochemical properties. Incorporation of BCP and GG enhanced the mechanical properties of the CS. The biomimetic mineralization capacity of CS–BCP–GG biocomposite cylinders was evaluated in a simulated body fluid solution. CS–BCP–GG biocomposite cylinders show over 80% viability in NIH-3T3 and SaOS-2 cell lines, compared to CS and CS–BCP. *In vivo* studies were performed using zebrafish embryos to find out the toxicity level of CS isolated from chrome shavings.

Keywords: Biphasic calcium phosphate; Collagen; Glycyrrhiza glabra; Zebrafish; Osteoinductive

1. Introduction

Bone is a mineralized connective tissue and serves vital structural and metabolic roles in the body [1,2]. Approximately 70% of the bone is a mineral phase, often described as naturally occurring hydroxyapatite (HA). The remaining 30% of the bone is organic phase which contains 90% type I collagen [3].

In recent years many efforts have been directed towards the repair and regeneration of bone using osteoinductive materials composed of various calcium phosphate compounds. An ideal bone graft material for hard tissue repair should be biocompatible, osteoinductive, resorbable and osteoconductive [4]. HA/tri-calcium phosphate (HA/TCP) mixture, which is being used as a bone graft substitute, is osteoconductive in nature [5,6]. The resorption of HA takes a very long time, whereas, β-TCP takes a very short time for resorption [7]. Hence, HA/TCP mixtures, which are believed to provide rapid fracture healing and resorption, are introduced [8,9]. Studies have proved that BCP is osteoinductive in nature [10]. It is porous ($Ca_3(PO4)_2$) and can replace bone tissue; further, β-TCP & HA are made into a scaffold and used for bone regeneration purposes [11,12].

In most developing countries, the use of medicinal plants has been observed as a normative basis for maintaining good health; however, industrialized societies are interested in the extraction of bioactive compounds from medicinal plants and in using them directly or indirectly as new drugs [13]. *Glycyrrhiza glabra* (GG), a medicinal plant commonly known as liquorice, has significant value in traditional and herbal medicine and has been used to treat fevers, gastric ulcers, sore throats, spasm, asthma, bronchitis, rheumatoid arthritis, bone healing, bone disease, cancer, and dysmenorrhea [14]. Liquorice is known to have a sweet principle glycyrrhizin, a biologically active triterpene glycoside [15]. The extract of GG in composition with various other plant extracts is being used as a drug to treat low bone mass, osteoporosis, bone fracture, bone defect, osteomalacia, bone loss, osteogenesis imperfecta, bone disease, and periodontal diseases [16].

Chrome shavings are the major solid wastes in the leather industry. From the environmental point of view, disposal of chrome shavings is identified as a serious problem due to their toxicity [17]. Chrome shavings contain CS and Cr(III) complexes; however, CS complexes can be isolated easily using

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chemical methods [18,19]. Protein products isolated from chrome shavings have gained importance in the field of cosmetics, adhesives, photography, and micro encapsulation and as additives in leather industries [20]. This connective tissue protein, CS, is extensively used in wound management [21–23].

To evaluate the toxicity of the isolated CS from chrome shavings, both *in vitro* and *in vivo* studies were carried out. In this study, zebrafish was used as a model to evaluate the toxicity of the CS because its tissue types are comparable to human tissue types and its genotypes are similar to those of human [24]. Moreover, rapid post fertilization development like organogenesis and vasculogenesis in less than a week has made it an ideal working model. This transparency has enabled us to visualize and study the cellular behavior and respective effects and disturbances, if any, that occurred after being treated with CS isolated from chrome shavings.

In the present study, a bone composite material in cylindrical form was prepared containing BCP, CS, and GG extract and was characterized for its physico-chemical properties. *In vitro* studies were performed using NIH3T3 and SaOS-2 cell lines and *in vivo* studies were performed using zebrafish embryos to find out the toxicity level of CS isolated from chrome shavings.

2. Experimental procedure

2.1. Materials

Chrome shavings were obtained from the Tannery Division of CLRI. All the other reagents used in this study were also of analytical grade.

2.2. Isolation of collagen(CS) from chrome shavings

CS was isolated from chrome shavings by following the procedure described earlier [25]. Briefly, chrome shavings were treated with 0.1 M Tris HCl (pH 8), 0.2 M β-mercaptoethanol, and 0.05 M EDTA for 3 days. The obtained CS fibrils were then suspended in 0.05 M acetic acid with sample/solution ratio 1:30 (W/V) containing pepsin/sample 1:10,000 (W/W) at 4 °C for 24 h with continuous stirring. The pepsin-stabilized CS was centrifuged at 10,000 rpm (6,6632 g) for an hour. The supernatant was further dissolved in 0.05 M acetic acid and subsequently dialysed with water, and the resultant CS solution was stored and used for further study, and it was denoted as CS. The solid content of the solution was found to be $15.21 \pm 0.96\%$.

2.3. Synthesis of hydroxyapatite (HA)

The HA powder used in the present study was prepared by employing earlier method [26]. In brief, to 0.5 M $Ca(OH)_2$ solution, 0.3 M H_3PO_4 solution was added drop by drop till the reaction mixture reached pH 12.3 to obtain calcium to phosphorus molar ratio of 1.6. The reaction was stopped after 24 h of vigorous stirring, and the precipitate formed was washed 2 to 3 times with distilled water and filtered to remove unreacted precursors. The precipitate was dried at 100 °C for 2 h, which resulted in synthetic HA.

2.4. Synthesis of β tri calcium phosphate (β-TCP)

β-TCP was synthesized according to the procedure followed in our laboratory [27]. In brief, H_3PO_4 was dissolved in methanol; subsequently $(CH_3COO)_2Ca \cdot H_2O$ was added slowly along with vigorous stirring and kept for 8 h for precipitation. Later, the precipitate was sintered at 800 ± 10 °C for 5 h using a muffle furnace. The resultant material is β-TCP.

2.5. Preparation of Glycyrrhiza glabra (GG) extract

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

2.6. Preparation of BCP loaded CS composite cylinders fortified with GG extract (CS–BCP–GG)

HA and β-TCP powders in the ratio of 60:40 were used for preparing films of CS–BCP (Fig. 1b) [28]. CS–BCP–GG films were prepared by mixing different stoichiometric ratios of the ingredients described in Table 2. Ethylene glycol was used as plasticizer and 0.25% glutaraldehyde (0.25 ml) solution was added as a cross linking agent [29]. The resultant solution was then poured in a polythene tray having measurements 10×10 cm^2 and dried at 30–35 °C. The dried films were then rolled into a tube form, then cut into 1 cm length tube. Dried CS–BCP–GG cylindrical composites were sealed in polythene covers and sterilized by gamma-irradiation at 2 Mrads (Fig. 1c). Subsequently, cylindrical composites were prepared without the addition of GG and dried at 30–35 °C. The dried samples were sealed in polythene covers and sterilized by gamma-irradiation at 2 Mrads. The sample no 3 in Table 2 exhibited better tensile strength, and this was selected for further studies.

2.7. In vitro bioactivity test

The biomineralization of the specimen was evaluated by their apatite forming abilities in simulated body fluid (SBF). It has the ionic concentration almost like human blood plasma. The SBF was prepared according to procedure established by Kokubo [30]. NaCl (8.035 g), NaHCO (3 0.355 g)$_3$, KCl (0.225 g), $K_2HPO_4 \cdot 3H_2O$ (0.231 g), $MgCl_2 \cdot 6H_2O$ (0.311 g), $CaCl_2$ (0.292 g), Na_2SO_4 (0.072 g), and Tris (6.118 g) were dissolved in 1 l of double distilled water, reagents were added, one after another, ensuring complete dissolution of the earlier