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Converted marine coral hydroxyapatite implants with growth factors: In vivo bone regeneration

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ABSTRACT

Herein we report rabbit model in vivo bone regeneration of hydrothermally converted coralline hydroxyapatite (HCCHAp) scaffolds without (group I) and with growth factors namely insulin like growth factor-1 (IGF-1) (group II) and bone morphogenetic protein-2 (BMP-2) (group III). All HCCHAp scaffolds have been characterized for phase purity and morphology before implantation. Calcined marine coral was hydrothermally converted using a mineralizer/catalyst to phase pure HAp retaining original pore structure and geometry. After sintering at 1250 °C, the HCCHAp found to have ~87% crystallinity, 70–75% porosity and 2 ± 0.5 MPa compressive strength. In vitro growth factor release study at day 28 revealed 77 and 98% release for IGF-1 and BMP-2, respectively. The IGF-1 release was more sustained than BMP-2. In vivo bone healing of different groups was compared using chronological radiology, histological evaluations, scanning electron microscopy and fluorochrome labeling up to 90 days of implantation. In vivo studies showed substantial reduction in radiolucent zone and decreased radiodensity of implants in group II followed by group III and group I. These observations clearly suggest ingrowth of osseous tissue, initiation of bone healing and complete union between implants and natural bone in group II implants. A statistical score sheet based on histological observations showed an excellent osseous tissue formation in group II and group III scaffolds and moderate bone regeneration in group I scaffolds.

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1. Introduction

Concentrated efforts are being made across the globe to develop materials that can mimic both function and structure of natural bone tissue. In the past, surgeons mostly relied on autogenic bone grafts for orthopedic surgery as it is considered a gold standard due to its osteogenic, osteoconductive and osteoinductive potential [1,2]. Though autografts have proven their capabilities as an ideal bone grafts [3], their widespread clinical use has been restricted due to many important issues such as donor site morbidity, limited availability, risk of possible immune responses, and disease transmission [4,5]. The limitations of autografts and allografts had driven the development of alternative bone graft materials. For example, materials derived from natural materials can potentially exhibit superior mechanical properties and pore architecture than conventionally designed biomaterial. Further, the natural architecture of these materials make them ideal candidates for carrying and site specific delivering of growth factors (GFs) or drugs. One such material is coralline hydroxyapatite (HAp). Marine coral derived from exoskeleton of madreporic corals is an exciting biomaterial that exhibits porosity very close to the human cancellous/cortical bone and adequate mechanical and inherent osteoconductive and bioresorbable properties [6–11]. However, the presence of calcium carbonate in coral exoskeleton does not allow new bone formation due to rapid dissolution thus limiting their clinical use. Converted coralline HAp has been used as bone grafts and orbital implants because of its excellent pore interconnections allowing infiltration of blood vessels [12]. HAp powder has been derived from Indian coral [13], while Hu et al. converted Australian coral to single-phase HAp [14]. Relatively high extent of coral conversion has been reported in microwave processed coral HAP than conventional thermal processing [15].

It is known that bone derived growth factors such as bone morphogenetic protein (BMP), insulin like growth factor (IGF), vascular endothelial growth factor (VEGF), and transforming growth factor β (TGF-β) accelerate many biological activities leading to enhanced bone regeneration [16–18]. Among several growth factors, BMP-2 and IGF-1 are well-known cytokines to enhance bone and cartilage growth, and play vital role during embryonic patterning and early skeletal formation [19]. Therefore, delivery of such growth factors to local fracture/diseased site is an effective approach for early healing and recovery

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[20]. However, effective integration of growth factors and their carrier is a very challenging task and very limited reports are available on in vivo evaluation of naturally derived scaffolds loaded with growth factors [21, 22]. Therefore, in the present investigation we have made an attempt to evaluate the effectiveness of hydrothermally converted coral as a carrier and delivery vehicle of BMP-2 and IGF-1 growth factors to accelerate in vivo bone formation and osseointegration in rabbit bone defect model. Our hypothesis is that natural porous architecture of coral scaffolds enables effective growth factor loading and their release in enhancing in vivo bone regeneration.

2. Materials and methods

2.1. Fabrication and characterization of converted porous coral scaffold

Sea coral was converted to hydroxyapatite following hydrothermal exchange process [23]. The sliced corals (5 mm thick × 15 mm dia.) were washed thoroughly using double distilled water to remove soluble salts. After overnight drying at 60 °C the corals were ultrasonically cleaned in acetone bath for 15 min followed by overnight drying at 70 °C. The dried samples were fired at 900 °C for 2 h to convert them to carbonates while removing other organic compounds and impurities. A slow firing schedule was followed to reduce the chances of cracking (heating rate of 1 °C/min up to 500 °C followed by heating to 900 °C at a rate of 3 °C/min). Di-ammonium hydrogen orthophosphate (DAP) was used as source of PO_4^{3-} group and potassium di-hydrogen phosphate (KH_2PO_4) was used as a mineralizer. The fired scaffolds were soaked in 0.6 M solution (pH 10–11) of di-ammonium hydrogen orthophosphate (DAP) and potassium di-hydrogen phosphate (KH_2PO_4) and autoclaved at 150 °C for 8 days followed by sintering at 1250 °C to complete coral conversion process. The following is the proposed exchange reaction during above preparation:

$$10CaCO_3 + 6(NH_4)_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6(NH_4)_2CO_3 + 4H_2CO_3.$$

X ray diffraction (XRD) (Philips Analytical B.V., X'Pert Pro, Netherlands) and Fourier transformed infra-red spectroscopy (FTIR) (Perkin-Elmer, Spectrum 100, USA) were used to analyze phase constituents of as-received and hydrothermally converted coral samples. The microstructure and pore morphology of the samples were observed using scanning electron microscopy (SEM; Leo 430i Steroscan, U.K.). Compressive strength of hydrothermally converted coral scaffolds was determined using Universal Testing Machine (Instron 5500R, USA) with cross-head speed of 1 mm/min and the average of three measurements was reported.

Converted coral samples ($8 \times 3 \times 2.5$ mm^3) were vacuum infiltrated with a solution containing 30 μg of recombinant growth factors (IGF-1 and BMP-2) (G-Biosciences, USA) for 15 min (10 mm Hg pressure, 25 °C). High growth-factor loading was selected considering an initial burst release followed by a sustained release over planned 90 days of in vivo evaluation. To support this, in vitro release study was carried out using 1 mL of α-MEM (minimum essential medium) with 1% BSA (bovine serum albumin) in a sealed poly-propylene vial. The medium was replenished every 24 h during the release study up to 28 days. IGF-1 and BMP-2 release was quantified by using Human IGF-1/BMP-2 Quantikine ELISA kit (R&D Systems).

2.2. In vivo experiments

In vivo experiments were performed following recommendations of Guide for the Care and Use of Laboratory Animals of the Institutional Animal Ethics Committee, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, India. The protocol was approved by the Committee on the Ethics of Animal Experiments of the West Bengal University of Animal and Fishery Sciences. All surgeries were performed under standard anesthesia. This 90 day study evaluated early stage in vivo osteogenesis and bone remodeling using a bone defect model in the proximal tibia of New Zealand white rabbits.

Eighteen New Zealand white rabbits of either sex, weighing 1.5–2 kg were randomly distributed into three groups: control group I (6 animals with porous coral implants) and the test animals, group II and group III (6 animals each) with IGF-1 and BMP-2 loaded porous coral, respectively. Prior to surgery, the rabbits were housed in individual cages with alternating 12-hour cycles of light and dark in temperature and humidity controlled rooms, given water ad libitum and were without restriction of movement.

2.2.1. Surgical procedure

Surgeries were performed under aseptic conditions and sedation by intramuscular injection of xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride at 25 mg/kg body weight. A motorized drill was used to create $8 \times 3 \times 2.5$ mm^3 bone defect in the medial aspect of the proximal tibia. The implants were inserted in the defects and secured in position by suturing the muscle, subcutaneous tissue and skin in layers. Treated animals were daily administered with cefotaxime sodium (Mapra India, India) at 20 mg/kg body weight intramuscularly for 5 days at 12 hour interval and meloxicam at 0.2 mL/kg body weight. Surgical wounds were inspected daily and appropriate wound care was given. Fluorochrome (oxytetracycline dehydrate; Pfizer India, India), 25 mg/kg body weight, was intramuscularly injected 3 weeks before sacrifice. All animals were sacrificed after 90 days.

2.2.2. Post-operative evaluation

Bone regeneration in the defect was monitored using sequential radiographs taken immediately after implantation and once every month. The radiographs were examined to assess implant status, implant–bone interface and new bone formation. For histological analysis, bone specimens were collected from adjacent and bottom region of original bone defects, washed thoroughly with normal saline and were immediately fixed in 10% formalin for 7 days. Subsequently, the bone tissues were decalcified using Goodling and Stewart's fluid containing 15 mL formic acid, 5 mL formalin and 80 mL distilled water, followed by fixation with 4% paraformaldehyde. Finally, the samples were embedded in paraffin wax and 4 μm sections were extracted followed by standard preparation and staining with hematoxylin and eosin. Fluorochrome (oxytetracycline dehydrate; Pfizer India, India), 25 mg/kg body weight, was intramuscularly injected 3 weeks before sacrifice.

Undecalcified sections were prepared from implanted segments of the bone and the sections were ground to 20 μm thickness using different grades of sand paper. The ground sections were observed under ultraviolet incidental light using an Orthoplan microscope (Excitation filter, BP-400 range, Leitz, USA) for tetracycline labeling to determine the amount and source of newly formed bone.

After sacrificing the rabbits, bone samples were collected at day 90 to study the coral bone interface using SEM. Samples were fixed in 5% glutaraldehyde in PBS buffer for 48 h followed by gradual ethanol series drying. Dried samples were gold coated before imaging using a field emission scanning electron microscope (FESEM, FEI 200F, FEI, OR).

2.3. Statistical analysis

Histological images of all groups were taken with $n = 5$ per experiment and experimental group. All data were presented as means ± standard deviations. One-way ANOVA was performed on the data using SPSS® software for Mac (Version 16, SPSS Inc., Chicago, USA).

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