Research in Veterinary Science 115 (2017) 1–9

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Effect of bone morphogenetic protein on Zn-HAp and Zn-HAp/collagen composite: A systematic *in vivo* study

Howa Begam [a], Samit Kumar Nandi [b,*], Abhijit Chanda [c], Biswanath Kundu [d]

[a] School of Bioscience and Engineering, Jadavpur University, Kolkata, India
[b] Department of Veterinary Surgery & Radiology, West Bengal University of Animal and Fishery Sciences, Kolkata 700037, India
[c] Department of Mechanical Engineering, Jadavpur University, Kolkata 700032, India
[d] Bioceramics and Coating Division, CSIR-Central Glass and Ceramic Research Institute, Kolkata, India

ARTICLE INFO

Article history:
Received 28 July 2016
Received in revised form 2 December 2016
Accepted 13 January 2017
Available online xxxx

Keywords:
Bone morphogenetic protein 2
Zinc doped hydroxyapatite
Collagen
In vivo
Bone healing

ABSTRACT

Due to good biocompatibility and osteoconductivity, hydroxyapatite (HAp) and its composite with different polymers have been widely investigated for the application in the field of bone tissue engineering. The present study reports the, *in vivo* performance of zinc doped HAp and HAp/collagen composite (HAC) using bone morphogenetic protein-2. It was done for a span of two months on New Zealand rabbit model. After two months post-operatively, there was no marked inflammatory reaction in experimental groups and control groups. The histological images showed well-formed bony matrix with well differentiated haversian system. From the fluorochrome labeling study, it was observed that higher amount of new bone formed in case of bone morphogenetic protein-2 (BMP-2) loaded Zn-HAp (50%) and HAC (27%) specimens than control. The percentage of new bone formation was significantly higher in case of BMP loaded Zn-HAp group than BMP loaded HAC group. From the SEM images similar trend was observed. As the HAC specimen consists of amorphous phase, it had a negative impact on new bone formation.

1. Introduction

Bone tissue engineering is an important tool for repair of large bone defects. Traditional autografts have some inherent limitations like availability of donor, donor site morbidity, *etc.* (Wu et al., 2014). The artificial tissue grafting is considered to be a better alternative however; it must ensure good biocompatibility, osteoconductivity, non-cytotoxicity properties along with adequate mechanical strength.

Human bone primarily consists of hydroxyapatite (HAp) crystals and collagen fibers. Synthetic calcium phosphates because of its structural and functional resemblance with natural HAp have drawn considerable attention as bone graft substitute. Its excellent biocompatibility and osteoconductivity has made it popular for last few decades but it does not show osteoinductivity. The approaches adopted so far to impart osteoinductivity include ion substitution, composite preparation using polymers (including biopolymers) and addition of bone morphogenetic proteins. The hexagonal structure of HAp can host variety of ionic substitutes. The insertion of foreign ions alters the crystallinity, stability, lattice parameters of HAp (Matsunaga et al., 2010; Miyaji et al., 2005; Ren et al., 2009; Gomes et al., 2010). Zinc is one such doping element and number of studies on zinc doped HAp have been reported so far (Esfahani et al., 2014; Galindo et al., 2016; Thian et al., 2013; Bhattacharjee et al., 2014). It is required for protein synthesis, DNA synthesis, mitosis, and cell proliferation (Wang et al., 2010). Zinc has stimulatory effects on bone formation as well as an inhibitory effect on osteoclastic bone resorption *in vitro*. Zinc doped HAp showed better bone bonding capacity and new bone formation when implanted in rabbit for two months (Bhattacharjee et al., 2014). Zn-HAp/Bisphosphonate increased bone formation and diminished bone resorption in rat model (Khajuria et al., 2016). Tao et al. reported that zinc, magnesium and strontium substituted HAp coating on titanium implant improve the implant osseointegration when implanted in rat models for twelve weeks (Tao et al., 2016).

Collagen is an osteoinductive, non-toxic and biodegradable material, acting as an excellent delivery system for bone morphogenetic proteins (BMPs) (Rodrigues et al., 2003). HAp/collagen composite prevents the dispersion of HAp in implants (Hsu et al., 1999). The use of HAp/ collagen composite is a promising approach for the generation of new bone substitutes and mimics the structure of natural bone. Collagen induces mineral deposition and also efficiently enhances osteoblast response of calcium phosphate (Hong et al., 2011). Calcium phosphate/collagen coating on titanium implant showed enhanced bone to implant contact (Alghamdi et al., 2013).

BMPs are members of the TGF-β super family. Recombinant human BMPs are already used for orthopedic applications and oral maxillofacial

* Corresponding author.
E-mail address: samitnandi1967@gmail.com (S.K. Nandi).

http://dx.doi.org/10.1016/j.rvsc.2017.01.012
0034-5288/

surgery. BMPs control the osteogenic activities of bone cells and can endorse new bone formation. Direct injection of BMPs into defect site is not generally efficient because of its rapid diffusion from the application sites. Therefore, for the delivery of BMPs suitable carrier is required. Various types of carrier materials *viz.* ceramics (hydroxyapatite, tricalcium phosphate), polymers (synthetic and natural) and biocomposite have been tested so far (Sachse et al., 2005; Ueki et al., 2003; Ohyama et al., 2004; Kaito et al., 2005; Lu et al., 2012; Cipitria et al., 2013; Zhang et al., 2012). Ceramics are generally used in the form of granules, particles, bulk and coating. HAp microspheres loaded with BMP-2 showed a significant amount of new bone formation in rabbit model (Baek et al., 2016). Notodihardjo et al. (Notodihardjo et al., 2012) investigated the effect of BMP on hydroxyapatite in rat model for four weeks and BMP/HAp group showed the highest level of bone induction than control. They showed that the relation between BMP and HAp has positive synchronization in the term of osteoinduction in the bone healing. BMP-2 has high attraction capacity for HAp and the release of BMP 2 from HAp is low. The adsorbed BMP-2 is strongly immobilized on the surface of HAp by electrostatic interaction and other bonding which makes it tough to move BMP-2 from HAp surface compared to other proteins (Xiao et al., 2013). Food and Drug Administration (FDA) approved BMP-2 and BMP-7 for spinal fusion and long-bone fractures treatment. BMP-2 have been demonstrated to be used for spinal fusion treatment in human (Sasmal and Begam, 2014; Bakhsheshi-Rad et al., 2014; Nandi et al., 2008). Haid et al. (Sasmal and Begam, 2014) studied posterior lumbar interbody fusion using rhBMP-2 with cylindrical interbody cages on 67 patients.

It is evident that there is no dearth of literature on the effect of doping, composite preparation with collagen and addition of BMP or growth factors but a continued approach is yet to be reported.

In this study, zinc doped HAp and Zn-HAp/collagen composite were prepared using wet chemical method. Collagen was extracted from fish skin in our laboratory. The specimens for implantation in animal were prepared using slip casting method. BMP-2 growth factor was loaded in implants. The effect of BMP-2 on Zn-HAp and Zn-HAp/Collagen composite were studied and compared after implantation in rabbit model for two months.

2. Materials and methods

2.1. Synthesis of materials

Hydroxyapatite doped with zinc oxide was synthesized using wet chemical method. The detail of synthesis steps is described elsewhere (Bhattacharjee et al., 2014). In brief, calcium hydroxide (Merck, India) was dissolved in boiled distilled water and then zinc oxide (Merck, India) in powder form was added in the solution. After proper mixing, 0.6 M orthophosphoric acid (Merck, India) aqueous solution was added drop by drop in stirring condition. The whole reaction was performed at 80 ± 2 °C and pH 11–12. After completion of synthesis reaction, it was aged overnight. Further, this was filtered and washed with distilled water and finally dried at 80 °C for 24 h. As prepared dried cake was crushed and powder obtained was further calcined at 800 °C for 2 h. The calcined powder was used for preparation of sample in animal study.

Type 1 collagen extracted from fish skin was used in this study. The extraction procedure is reported elsewhere (Sasmal and Begam, 2014). Briefly, the fish skin was treated with 0.1 M NaOH and 15% butyl alcohol to remove non collageneous tissue and fat. After that, the skin was immersed in 0.5 M acetic acid and the acid soluble type 1 collagen was extracted by centrifuging the solution. The extracted collagen was freeze dried at −40 °C.

Hydroxyapatite/collagen composite in ratio of 80:20 was prepared using wet chemical method. We prepared 10 g of HAp-collagen composite powder in one batch. In this method required amount of type 1 collagen was dissolved into 0.6 M H_3PO_4 aqueous solution. 1.008 M $Ca(OH)_2$ solution was prepared at room temperature by continuous stirring using a magnetic stirrer. The H_3PO_4 collagen aqueous solution was added drop by drop into the $Ca(OH)_2$ in stirring condition with pH 11–12 and the reaction temperature was maintained as 30 °C·The solution thus obtained was aged for 24 h followed by filtration. The filtrate was freeze dried for the removal of water content. The composite cakes were crushed using mortar and pestle followed by sieving. The composite powder was then used for characterization.

2.2. Characterization

The phase composition of calcined Zn-HAp powder and HAp/Collagen composite powder was analyzed by X-ray diffraction (XRD; Rigaku diffractometer, Model-Ultima III, Rigaku Co., Tokyo, Japan) using Cu Kα1 monochromatic X-rays (λ = 0.154059 nm) generated at 55 mA and 40 kV. Scans were recorded from diffraction angle (2θ) of 10°–80°, at a speed of 40/min with step size of 0.050.

The functional groups of HAp and collagen were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) which was obtained by using Perkin-Elmer, Model 1615 (USA) instrument. Powder was dispersed with pre-dried KBr in the proportion of 10 wt.% for measurements. Background noise was corrected with pure KBr data. The measuring resolution was 4 cm^{-1} and 256 readings were performed in the range from 400 to 4000 cm^{-1}.

2.3. Sample preparation

Samples were prepared for animal trial using slip casting technique. Slip was prepared in freshly prepared distilled water using 1% anionic dispersant, sodium polyacrylate (DN40). The mixing was performed using mechanical stirrer and then ball milled for 4 h. The slurry was poured into plaster of paris mould. The plaster of paris mould was prepared to obtain cylindrical specimens with 2–3 mm dia and 5 mm height (Fig. 1).

2.4. Loading of BMP

In this study, protein growth factor has been utilized in which Recombinant human bone morphogenetic protein-2 (BMP-2) was procured from Bio Vision Research Products, CA 94043, USA (both are >98% pure). The vial was centrifuged before opening and reconstitution.

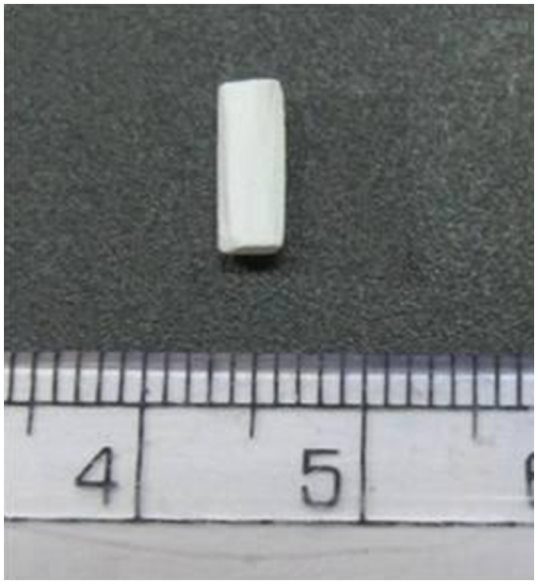

Fig. 1. Slip casted green specimen for implantation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)