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Research Article

INCORPORATION OF A SEQUENTIAL BMP-2/BMP-2 DELIVERY SYSTEM INTO CHITOSAN-BASED SCAFFOLDS FOR OSTEOGENESIS AND BONE HEALING

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ABSTRACT

It is advantageous to incorporate controlled growth factor delivery into tissue engineering strategies. The aim of this study was to develop a 3-D construct carrying an inherent sequential growth factor delivery system. BMP-2 was encapsulated in silk chitosan particles that were produced by a simple and very mild processing method. The dose–response of BMP-2-loaded chitosan particles was examined in C2C12 cells after 5 days of culture. The BMP-2 retained most of its activity as observed by the increase in alkaline phosphatase activity, which was much higher when BMP-2 was encapsulated into the particles rather than just surface adsorbed. After 2 weeks of culture, increased mineralization was observed with BMP-2-loaded particles in comparison to soluble added growth factor. No significant cytotoxicity was detected. When implanted in a rat ectopic model, bone formation was observed by in vivo micro-computed tomography after 2 and 4 weeks post-implantation with particles loaded with 5 mg BMP-2. An increase in bone density was observed over time. Our findings show that chitosan microparticles may present an interesting option for future clinical applications in the bone tissue engineering field.

INTRODUCTION

The limited ability of bone tissue to regenerate in the case of large defects creates the need for substitutes which are mostly of autogenic and allogenic origins [1]. Tissue engineered constructs are the promising alternatives to these grafts to form the viable and functional 3-D constructs. Polymeric foams [2], micro and/or nanospherical-based scaffolds [3–5], and rapid prototyped constructs [6,7] are among the structures that have been successfully employed as scaffolds for bone tissue engineering; however, control of cell activity, especially differentiation, within the scaffold has not been fully achieved in these systems.

Growth factors regulate cellular activities in vivo, and their application as external bioactive agents has been reported to enhance bone healing [8,9], control growth and differentiation of cells [10], and stimulate angiogenesis [11]. In nature, multiple growth factors such as bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) function in unison during the bone formation and fracture healing processes [12].

Among these, BMPs were shown to induce bone formation by inducing mesenchymal stem cells (MSCs) toward chondroblastic and osteoblastic differentiations [13]. BMP-2 and BMP-2 were shown to be the most effective ones that induce complete bone morphogenesis [14] and were approved by FDA for clinical applications [15,16]. Considering the mechanism of their action, BMP-2 was reported to be an early appearing factor, peaking at day 1 after the fracture, while BMP-2 was expressed approximately after 2 weeks [17]. Therefore, the fact that the delivery of combinations of these growth factors is a viable biomimetic approach towards bone healing was taken into consideration.The conventional strategy in growth factor therapy for orthopedic applications is to administer the agent in a large dose by either single or repeated injections, but in such applications, a considerable proportion of the agent was reported to be lost through leakage and/or loss of bioactivity [12]. Encapsulation of growth factors in carrier structures, therefore, could be of utmost importance in protecting the bioactivity of the agent and in prolonging its presence at the defect site. Various recent attempts to incorporate growth factors into scaffold structures in order to provide the necessary protection and prolongation of activity as well as to achieve proper control of cellular activities in the implant side, are found in the literature [18–20].

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The most recent developments observed in the preparation of scaffold/controlled delivery systems involve the combined delivery of several growth factors from the same scaffold. The few studies in the literature include BMP-2 and transforming growth factor-b3 (TGF-b3) delivery from alginate hydrogels transplanted in mice which revealed significant healing [21]. Dual delivery of VEGF and BMP-2 from gelatin microparticles embedded in porous degradable scaffolds had a positive effect on the repair of a rat cranial defect [22]. Similarly, sequential delivery of BMP-2 and then IGF-1 from two-layered gelatin coatings led to the elevated alkaline phosphatase (ALP) activity and mineralized the matrix formation in vitro [23].

The most recent study in the literature on the combined delivery of BMP-2 and BMP-2was reported by our research group where the growth factors were released from the complexed microspheres embedded in the porous poly(lactic acid-co-glycolic acid) (PLGA) scaffolds [24]. The positive effect of co-administration of BMP-2 and BMP- 2 on osteogenic differentiationwas shown in vitro. In the only other study made also by our group [25], a nano-scale controlled release system was developed to enable the sequential delivery of first BMP-2 and then BMP-2 from PLGA and chitosan (CHS) nanocapsules, respectively. The effect of various nanoparticle production parameters was investigated in order to achieve the proper release rates to constitute the parts of the sequential delivery system. Nanocapsules of PLGA and CHS were found to have adequately high encapsulation efficiencies, appropriate release rates, and smooth surfaces. This sequential BMP-2 delivery system enhanced the differentiation of MSCs into osteoblasts in vitro while decreasing the proliferation rate.

In the present study, the above mentioned nanoparticulate sequential delivery system was incorporated into and onto the sphericals of wet spun chitosan-based scaffolds to create bi-functional constructs serving both as a scaffold and also as the growth factor delivery system. In the previous study [25], the unentrapped (free) nanocapsules were used to release both the bovine serum albumin (BSA) and the BMPs, and were found to have similar kinetics, so in the present study, the spherical-incorporated nanocapsules were expected to behave as before, presenting a similar release kinetics for the BMPs and the BSA. Therefore, in the current study, only the release kinetics of BSA was studied.

Fibrous scaffolds have gained great attention over the last years as they have the appropriate porosity for cell penetration, nutrient exchange, and tissue ingrowth. These fibrous structures were produced by electrospinning [4], phase separation [5], and spherical bonding [26]. In this study, chitosan spherical particle scaffolds were used to house the nanoparticulate sequential growth factor delivery system. The effect of single, simultaneous, and sequential deliveries of BMP-2 and BMP-2 incorporated to the scaffolds by the two different techniques, set into and onto chitosan sphericals, were studied in vitro using the rat bone marrow MSCs.

MATERIALS AND METHODS

Materials

Low molecular weight chitosan (deacetylation degree 90.85%, i.v. 185 cps for 1% in 1% acetic acid) was purchased from Aldrich. Poly(ethyleneoxide) (PEO) (PolyoxTM WSR 301, MW 4 x 106) was obtained from Dow Chemical Company(USA). PLGA (50:50) (ResomerTM RG503H, i.v. 0.32–0.44 dL/g, for 0.1% in chloroform, 25 °C) was purchased from BoehringerIngelheim (Germany). CHS (PHV content 8% w/w), dexamethasone, b- glycerophosphate disodium salt, and L-ascorbic acid were bought from Sigma–Aldrich (Germany). BSA and polyvinyl alcohol (PVA) (MW 15,000) were from Fluka (USA). BMP-2 from InductOs kit (Medtronic, USA) and recombinant human BMP-2 from Ray Biotech (USA) were used as the growth factors. For cell experiments, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), trypsin/EDTA, penicillin/streptomycin (P/S), and phosphate buffered saline (PBS, pH 7.4) were purchased from Gibco BRL (Carlsbad, CA, USA). Cell Counting kit-8 (CCK-8) was purchased from Dojindo Co. Ltd (Kumamoto, Japan). Alkaline phosphatase (ALP) LabAssay kit was purchased from Wako Chemicals Inc. (USA). PKH26 red-fluorescent cell linker mini kit was obtained from Aldrich Sigma. Balb/c mice and Sprague Dawley (SD) rats were supplied by Nara Biotech (Seoul, Korea).

Preparation of BSA, BMP-2 and BMP-2 loaded nanocapsules

PLGA and CHS nanocapsules containing BMP-2 and BMP-2, respectively, or BSA in both types of nanocapsules, were prepared by the double emulsion-solvent evaporation technique as reported earlier [25]. Briefly, an aqueous solution of BSA or BMPwas emulsified in dichloromethane containing PLGA or CHS and this was then introduced to an aqueous solution containing PVA. Nanocapsules were collected by centrifugation and washed with Tris–HCl (pH 7.4). The nanocapsules were then resuspended in the buffer and lyophilized.

Micro-computed tomography

Mean porosity and porosity distribution of the 3-D scaffolds were assessed by using micro-computed tomography (m-CT 20, SCANCO Medicals, Switzerland). Scanner settings were 40 keV and 248 mA. Entire scaffolds were scanned in slices of 7 mm thickness. CT Analyser and CT Vol Realistic 3-D Visualization (SkyScan, Belgium) softwares were used for image processing in CT reconstructions, and in creation and visualization of the 3-D representations.

In situ release studies

Release from the nanoparticle incorporated constructs was simulated by using BSA as a model molecule to represent growth factors. Protein release was determined spectrophotometrically by using Coomassie Plus Assay (Pierce, USA).

Cell culture

Bone marrow MSCs were isolated from the 6 week old male Sprague–Dawley rats as reported earlier [27]. The rats were euthanized and their femurs and tibia were excised, washed with DMEM containing 1000 U/mL penicillin and 1000 mg/mL streptomycin under aseptic conditions. The marrow in the midshaft was flushed out with DMEM containing 20% FBS and 100 U/mL penicillin and 100 mg/mL streptomycin, the

cells were centrifuged at 500g for 5 min, and the resulting cell pellet was resuspended and plated in T-75 flasks. These primary cultures were incubated for 2 days. The hematopoietic and other unattached cells were removed from the flasks by repeated washes with phosphate buffered saline (PBS) (10 mM, pH 7.4) and the medium of the flasks was renewed every other day until confluency was reached. These primary cultures were then stored frozen in liquid nitrogen until use. Ethylene oxide (EtO) sterilized spherical particle scaffolds and nanoparticle incorporated constructs were then seeded with these cells at a seeding density of 50,000 cells/scaffold. The viable cell number during cell seeding was determined with the Nucleocounter (Chemometec, Denmark). Incubation was performed at 37 °C and 5% CO2 in DMEM supplemented with 10% FBS, 10 mM b-glycerophosphate, 50 mg/mL L-ascorbic acid, 10 nM dexamethasone, and penicillin/streptomycin/amphotericin B. Viable cell number was assessed with Alamar Blue assay (USBiological). ALP activity was determined by using Randox kit (USA) where the absorbance of p- nitrophenol formed from p-nitrophenyl phosphate was measured at 405 nm.

Scanning electron microscopy

The structure of the scaffolds, the nanocapsule incorporated constructs, and the cell attachment on the spherical surfaces after 21 days of incubation were studied by Scanning Electron Microscopy (SEM) after sputter coating with gold (Leica Cambridge S360, Germany). Cell seeded scaffolds were fixed after 21 days of incubation with glutaraldehyde (2.5% in cacodylate buffer, pH 7.4) for 2 h, and thenwashed with cacodylate buffer several times, and lyophilized prior to SEM examination.

Statistical analysis

The data from the MSC proliferation and differentiation assays ($n = 3$) were analyzed with the statistically significant values defined as $p < 0.05$ based on one-way analysis of variance (ANOVA) followed by Tukey's test for determination of the significance of the differences between different groups ($p < 0.05$).

RESULTS

Characterization of chitosan microspheres and CHS particle/CMs scaffolds

The chitosan microspheres loaded with BSA (a model protein) and BMP-2 were prepared by the emulsion method in the presence of TPP, carrying five negative charges which allows the electrostatic interaction with protonated chitosan in an aqueous acidic solution [32]. The microspheres were spherical in shape, and had a regular surface without any crack (Fig. 1). The particle size distribution was in the range of 10–60 μm based on SEM observation. Moreover, the average diameters of microspheres loaded with BSA and synthetic peptide were 33.9 and 39.0 μm, respectively. In this study, the chitosan concentration is crucial for the morphology of obtained microspheres. To prepare spherical microspheres with smooth surface, the chitosan concentration should be larger than 20 g/L; otherwise, only fragile microspheres with irregular surface were obtained. The crosslinking time is also important and it should be longer than 2 h because the diffusion of TPP molecules into microspheres is time-dependent and critical, in order to maintain the shape of the microspheres [20,33].

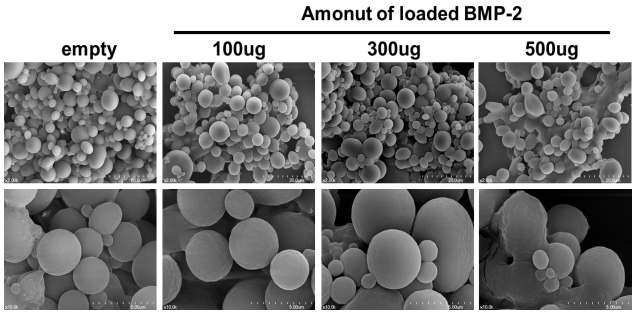

Fig 1 SEM micrographs of chitosan microspheres loaded with BMP-2 with different protein contents.

The morphology of CHS particle scaffolds before and after microspheres incorporation is shown in Fig. 2.

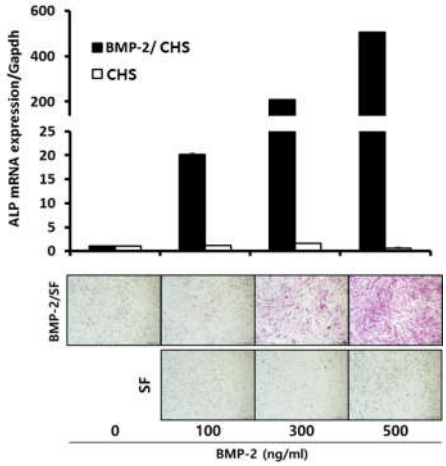

Fig 2 Alkaline phosphatase (ALP) activity of C2C12 cells after 5 days of culture. Particles were added in similar amounts to the cell cultures ontaining encapsulated BMP-2 (0.1, 0.3 or 0.5 mg, respectively). ALP activity is reported as nmol/min/mg of total protein.

The CHS particle scaffold (Fig. 2a) presented a typical microstructure of polymeric foam prepared by thermally induced phase separation [34]. The pore size ranged from 50 to 300 μm and the pores were interconnected. To investigate the influence of microspheres dosage on the microstructure of the composite, CHS particle/CMs scaffolds were prepared by varying the CMs contents while maintaining the nHAC and PLLA concentration constant in the dioxane solutions. At 10% (Fig. 2b) and 30% (Fig. 2c) CMs contents, the composites showed a continuous microstructure of interconnected pores with the diameter of 100–200 μm, which is very similar to the microporous structure of CHS particle scaffold. Moreover, the microspheres distributed uniformly throughout the whole scaffolds. These results suggested that, up to 30% contents, CMs did not influence the mechanism of the pore formation via crystallization and phase separation of dioxane. However, when the CMs dosage increased to 50%, the morphology of the composite appeared to be very different from the other two scaffolds (Fig. 2d). In this case, the pores were not interconnected and some microsphere aggregates could be seen on the pore walls. This mainly attributed to the decrease in the solvent volume relative to the total weight of nHAC, PLLA, and CMs during the process of composite preparation.

In vitro characterization of hMSCs

To evaluate the capacity of the CHS particle-coated particle scaffolds to support cell growth, we cultured hMSCs on CHS particle-coated particle scaffolds and monitored their growth using ESEM and CCK-8 assay, respectively. Representative morphological images of hMSCs cultured on the BMP-2 tethered CHS particle-coated particle scaffold exhibited that the hMSCshad spread quite well along the spherical structure (Fig. 3A). Furthermore, the cytoplasm of hMSCs appeared to integrate well with the ECM structure as assessed via a high magnification image. As the proliferation of hMSCs on each particle group was also examined for a period of 7 days, the cell number significantly increased on all ECM-coated particle groups compared to the control; no significant difference, however, was observed between the experimental groups (Fig. 3B).

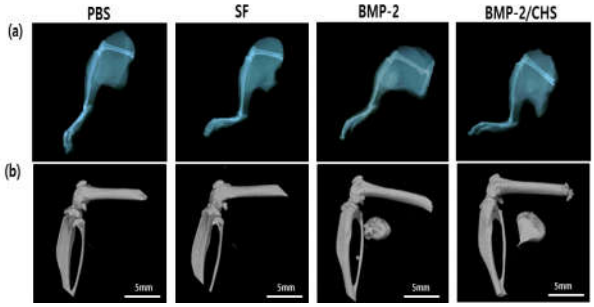

Figure 3 Ectopic bone formation induced by BMP-2/chitosan particle (CHS). A) soft radiograph images of ectopic bone formation. B) Detailed mCT images of ectopic bone formation induced by chitosan particles.

The effect of BMP-2-tethered CHS particle particled groups was examined by culturing hMSCs in the osteogenic media without supplementing the soluble BMP-2; cells in all other groups were cultured in osteogenic media added with the exogenous BMP-2 (50 ng/mL). Fig. 4A shows weak OC expression in both the control and FN groups at 2 weeks while BMP-2-tethered groups showed strong OC expression. As shown in Fig. 4B, a significantly greater level of ALP activity was observed at both 2 and 4 weeks from cells cultured on the two CHS particle-coated particle groups than from those cultured on FN or control groups: there was a statistically significant difference. Cells cultured on the BMP-2-tethered particle group showed a lower level of ALP activity at 4 weeks than those cultured on CHS particle particled groups supplemented with the soluble BMP-2 in the osteogenic medium.

In vivo osteogenesis of hMSCs via a mice ectopic model

After the 4 week implantation of PKH-26-labeled hMSCs (red color), particle scaffolds were removed from the implantation sites, frozen, sectioned, and stained for Col I (green color) (Fig. 4B). Red-labeled hMSCs could be seen around the implanted scaffolds for both CHS particle and CHS particle/BMP-2 groups. In particular, it is notable that the red-fluorescent hMSCs in the CHS particle/BMP-2 group appeared to be co-stained with green-fluorescent Col I, indicated by a yellow color, suggesting that the transplanted hMSCs themselves were able to contribute to the osteogenesis in the ectopic site. In addition, H&E staining was performed to assess for any inflammatory or adverse tissue reactions due to the polymeric particle scaffolds (Fig. 4A). The implanted particle scaffolds were clearly visible in the subcutaneous flap and some inflammatory signs or adverse tissue reactions were observed. Fibrocollagenous connective tissue, however, was noticed beneath the dermis. Interestingly, newly formed blood capillaries were detected around the particle scaffold, indicated by the presence of red blood cells in the round cross-sections. Mineralization was also assessed via von Kossa staining (Fig. 4A). While CHS particlecoated particle groups with or without BMP-2 showed abundant deposits of mineralized bone matrix (blue arrows) that penetrated deeper into the particle scaffolds, FN group displayed a marginal mineral deposition. In particular, significantly more mineralization was observed from the CHS particle/BMP-2 group (Fig. 4B), suggesting that the controlled release of BMP-2 in this system would take part in facilitatingthe osteogenic differentiation.

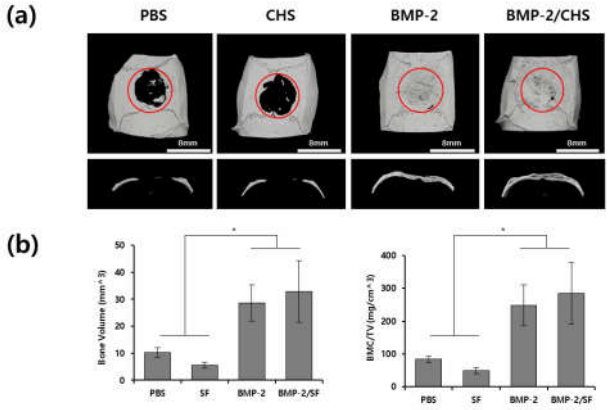

Figure 4 Effect of BMP-2/CHS microparticle on orthotopic bone formation in the rat calvarial defect model

In vivo bone regeneration in a calvarial defect model

A calvarial bone defect model was used to examine the effect of immobilized BMP-2 and ECM microenvironment on bone regeneration. 8 mm critical-sized defects were created on the rat skull, and were successfully filled with the particle scaffolds (Fig. 5A). A representative top view of the 3D reconstructed bone tissue was shown for each group (Fig. 5B). As expected, limited bone regeneration was observed in the defect-only groups. By 8 weeks, all experimental groups exhibited significantly greater qualitative bone density than the control group. Specifically, upon quantification, the defect area for the BMP1000 group was nearly regenerated after 8 weeks (Fig. 5C). Similarly, with regards to the volume of the newly-formed bone, that of the BMP1000 group was significantly higher than that of all other groups; no statistical difference, however, was observed between the CHS particle and BMP200 group (Fig. 5D).

Furthermore, H&E staining indicated a new bone formation across the defect areas. DAB- positive stains in the dark gray color showed the presence of Col I: the most abundant protein during the bone remodeling process appeared around the particle scaffolds (Fig. 5). Especially Col I was strongly expressed in the BMP-2-loaded (low and high) and CHS particle particled scaffolds (yellow (in the web version) arrows). Interestingly, compared to the control and the CHS particle groups, newly formed mature bone (M) was clearly observed in the defect site of the BMP- 2-loaded (low and

high) groups. Most notably, the high dose BMP-2 group (BMP1000) exhibited a nearcomplete closure of the defect by 8 weeks. Meanwhile it was difficult to distinguish the extent of integration at the interfacial region (I) between the transplanted scaffold (S) and the host bone (H) among the CHS particle-coated particle scaffolds (CHS particle, BMP200, and BMP1000), but the interfacial images may suggest a more advanced stage of bone healing for BMP1000 in terms of the maturity of the ingrowth tissues.

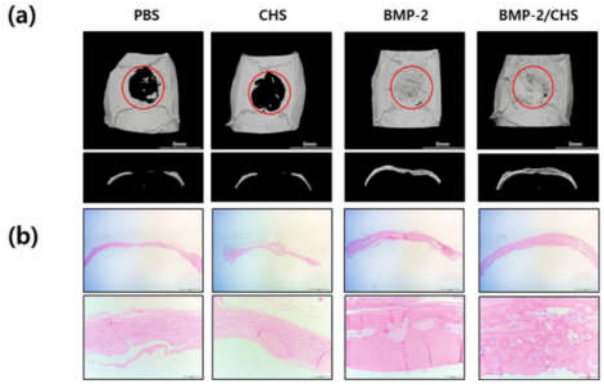

Figure 5 The effect of BMP-2/CHS micro-particle on the orthotopic bone formation in the rat calvarial defect model

DISCUSSION

The need for an efficient delivery system for BMPs as a way of providing effective and sustained stimulation of bone formation has been well recognized. The main role of a carrier is to retain these growth factors at the site of injury for a prolonged time frame, protecting the immobilized drugs from degradation and maintaining its bioactivity, while releasing the protein in a time- and site-controlled way to promote the formation of new bone at the treatment site. Chitosan biomaterials have been explored as novel alternatives for drug delivery and in tissue engineering as a result of their biocompatibility, biodegradability, mechanical strength, and versatility of formats into which the material could be processed. In our study, we have demonstrated that CHS particle not only provides physical cues in triggering the differentiation of osteoblasts but also serves as a natural reservoir of various cell differentiation. The natural chitosan polymer particle scaffolds demonstrate good porosity and biocompatibility, and are easy to handle. The basis for evaluating the activity of the BMP released by the chitosan particles was to test initially the particles loaded with growth factor in C2C12 cell line and then using a ectopic bone formation model. C2C12 murine premyoblast cells are well defined by their ability to rapidly differentiate into osteoblasts when cultured in the presence of BMPs. In these cells, chitosan particles loaded with BMP-2 were able to induce a significant increase in ALP activity (after 5 days of culture), thereby confirming that the growth factor was loaded into the particles, retaining its bioactive state. Similar cases were reported but using different methodologies. In one eport, chitosan particles retained the activity of loaded horseradish peroxidase, using lipid vesicles as particle templates, followed by methanol treatment. In our study, the activity recovery was estimated as 88 ± 6.1%, whereas no such results were presented in the former works using particles as carriers for BMP-2 delivery. To monitor the dose effect of BMP-2 in our system, we employed a calvarial bone defect model. Previous research by Palaez *et al*. using BMP-2-loaded collagen sponge carriers in a rat calvarial defect model, exhibited minimal difference of BMP-2 doses ranging from 1.25 to 20.0 mg. Therefore, a BMP-2 dose of less than 1 mg was chosen as the maximum loading concentration in our system. Microradiographic studies after 8 weeks posttreatment indicate that the most active bone regeneration in the calvarial defects was found in the BMP-2/CHS group. Furthermore, 3D reconstruction of the micro-CT scans display near-complete healing compared to that observed in the other experimental groups. Interestingly, there was no statistical difference in the newly regenerated bone volume between CHS particle and BMP2 group, but significance difference between the control and unload particle group, strongly indicating a positive role of CHS particle itself in the bone regeneration.

CONCLUSION

In the present study, a chitosan particle scaffold capable of immobilizing BMP-2 and releasing in a controlled manner was developed. We demonstrated that the BMP-2-loaded CHS particle allows the differentiation and growth of preosteoblasts in addition to the provision of a suitable microenvironment for osteogenic differentiation. We also demonstrated enhanced ectopic mineralization upon subcutaneous transplantation of the BMP-2-loaded particle system into mice. Finally, we prepared a rat calvarial defect model to show a significant increase of newly regenerated bone when using the CHS particle/BMP-2 particle scaffold. We envision that our CHS particle could be used to mimic the biophysical microenvironment, and when combined with BMP-2, act as a synergistic osteoconductive platform for bone tissue engineering applications.

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