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Bone regeneration using injectable BMP-7 loaded chitosan microparticles in rat femoral defect

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ABSTRACT

Injectable chitosan microparticles were prepared using a simple coacervation method under physiologically friendly conditions by eliminating oil or toxic chemical, and employing low temperature and pressure for growth factor stability. Amount of 200 ng of bone morphogenetic protein-7 (BMP-7) was incorporated in the chitosan microparticles by two methods: encapsulating and coating techniques. These microparticles were tested *in vivo* to determine the biological response in a rat femoral bone defect at 6 and 12 weeks. Four groups ($n = 10$) were tested which include two groups for BMP-7 incorporated microparticles (by two techniques), microparticles without BMP-7, and defect itself (negative control). Healthy bone formation was observed around the microparticles, which were only confined to the defect site and did not disperse. Histology indicated minor inflammatory response around the microparticles at 6 weeks, which reduced by 12 weeks. Micro-CT analysis of bone surface density and porosity was found to be significantly more ($p < 0.05$) for microparticles containing groups, in comparison with controls, which suggests that the new bone formed in the presence of microparticles is more interconnected and porous. Collagen fibrils analysis conducted using multiphoton microscopy showed significant improvement in the formation of bundled collagen area (%) in microparticles containing groups in comparison with controls, indicating higher cross-linking between the fibrils. Microparticles were biocompatible and did not degrade in the 12 week implant period.

1. Introduction

Bone is a dynamic organ in the body that is constantly being resorbed and formed, through very meticulous processes. However, despite the excellent regenerative capacity of skeletal tissue, the biological process fails healing of large bone defects and fractures. Since several years, the gold standard treatment is the use of autografts, but its use is severely hampered by its short supply and the considerable donor site morbidity associated with the harvest. Risk of viral contamination and immunological response restrict the use of allografts [1–3].

Therefore, research is still focused on developing ideal bone graft substitute material that promotes healing through osteogenesis, osteoconduction and osteoinduction, in combination or alone. The selection of an ideal bone graft depends on several factors such as defect size, graft size, shape and volume, tissue viability, biomechanical characteristics, graft handling, cost, ethical issues, biological characteristics and associated complications [4,5]. Thus, optimizing scaffold design to satisfy all the requirements still remains a challenge. Apart from these characteristics, the biomaterial should finally degrade over time and be completely replaced by natural bone.

A number of biocompatible, biodegradable polymers – both natural and synthetic have been explored for repair and regeneration in the past decade [6–8]. Chitosan is a natural polymer available in abundance. Chemically, it comprises of β(1–4) linked D-glucosamine residues with *N*-acetyl-glucosamine side chains that makes it structurally similar to glucosaminoglycans (GAGs), which are usually the interacting molecules with osteoblasts [9–11], thus making it particularly attractive as a biomaterial for bone defects. Chitosan can also be degraded *in vivo* by lysozymes through hydrolysis of acetylated residues. It has also been found to provoke least foreign body response, leading to just normal granulation and neutrophils accumulation, which ultimately result in triggering local cell proliferation and integration of the scaffold with the host [12].

Although chitosan has shown to be osteoconductive, it lacks osteoinductivity. Therefore, chitosan scaffolds with growth factors is being considered as a potential alternative. Our lab has been involved in developing chitosan scaffolds, with and without growth factors through various techniques for application in bone tissue engineering [13–21]. Therefore, the aim of the present investigation is to study the

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effects of chitosan microparticles with and without bone morphogenetic protein-7 (BMP-7) on bone healing in rats. Three groups of microparticles were used: chitosan microparticles without growth factors; chitosan microparticles with BMP-7 coated onto it; and BMP-7 encapsulated into it. Bone formation was analyzed at 6 and 12 weeks. The morphology of the newly formed bone was quantitatively analyzed using micro-computed tomography (μ-CT). It is known that collagen is an important part of bi-phasic bone composite material, which plays an important role in regulating its mechanical properties. Though there are many studies, which focus on mineral phase of bone, very few studies focus on the collagen content and arrangement to determine its role. Therefore, this study also analyses the collagen arrangement in the newly formed bone using a novel technique of multiphoton second harmonic generation microscopy. The biological response to the microparticles and growth factors and their degradation was observed by histology.

2. Materials and methods

2.1. Materials

Chitosan (85% deacetylated, Molecular weight of 190–310 kDa), sodium tripolyphosphate (TPP), acetic acid, and phosphate buffered saline (PBS) were purchased from Sigma Chemicals, BMP-7 was purchased from Peprotech, 10% formalin and decalcification solution, Cal-Ex 11 was obtained from Fisher Scientific.

2.2. Methods

2.2.1. Fabrication of chitosan microparticles

Microparticles were prepared by a coacervation technique. In this technique, 2% chitosan was prepared by dissolving in acetic acid (1% *v*/v) at room temperature. The mixture was passed through a nylon mesh to remove insoluble substances. This mixture was then added drop wise to 50% sodium tri-polyphosphate (TPP) solution kept on ice-bath. The microparticles were allowed to cross-link overnight and then were air-dried. In order to prepare BMP-7 encapsulated microparticles, 200 ng of BMP-7 was dissolved in PBS (pH 7.4) and added to the chitosan solution before cross-linking. Each batch process resulted in a total of 7 mg of microparticles. BMP-7 coated microparticles were prepared by adding the same amount of the growth factor to 7 mg of microparticles after they were cross-linked and dried through the process of absorption.

2.2.2. Surgical procedure

Prior to the surgery, microparticles were packed in 1 ml syringes along with very small amount of saline. These microparticles were sterilized using gamma radiation for 5 min at 1358.5 rads. Inbred male Lewis rats, 8 weeks old, weighing between 240 and 290 g (Harlan Laboratories, IN), were used as experimental animals. The protocol was approved by the University of Toledo Institutional Animal Care and Use Committee (IACUC, Approval no: 105818) and national guidelines for care and use of laboratory animals were followed.

Surgery was performed under general inhalation anesthesia containing isofluorane and oxygen. A lateral approach was used to expose the right femoral diaphysis. After the exposure of the mid shaft region of the femur, a 5 mm × 1 mm × 1 mm (length x width x height) hole was drilled through one of the cortex until the bone marrow was exposed. After injecting the material, the soft tissues and skin were closed layer-by-layer using absorbable suture.

Each group constituted 10 animals, thus for 4 groups (including a control) and two time points, a total of 80 animals were used in this study. At 6 and 12 weeks post-operation, rats were anesthetized using over dose of ketamine (80 mg/kg) and xylazine (10 mg/kg), followed by the cardiac perfusion technique to fix the tissue.

2.2.3. Methods of evaluation of regenerated bone

2.2.3.1. Histological procedures. The fixed tissue was decalcified before sectioning. Samples were embedded in liquid paraffin and cut into 5 μm thick sections and fixed on microscopy slides for further analysis. The sections were stained with hematoxylin and eosin (H&E).

2.2.3.2. μ-CT analysis. Before decalcifying the bones, μ-CT was performed. Samples were firmly positioned in the sample holder using low-density foam. In order to obtain the highest contrast between the specimen and the surrounding medium, air was used as the scanning medium for all the samples. The fixed scaffolds were scanned with a high-resolution μ-CT scanner (μCT 35, Scanco Medical AG) at 70 kVp. Beam hardening of the X-ray was reduced by placing a beam-flattening filter in the X-ray path to narrow the energy spectrum. Signal-to-noise ratio (SNR) and scanning time had to be optimized to obtain good voxel information; therefore a tube current of 114 μA was used with an integration time of 100 ms per projection. The tradeoff between voxel size and scan time was also carefully considered in order to analyze the newly formed bone in rat and an isotropic voxel size of 12 μm was used for scanning all the bones. Region of interest (ROI) exceeding defect boundaries was used for scanning purposes (includes newly formed bone as well as old bone) and is approximately 20% of bone length. For each group ($n = 10$) samples were used.

The standard method of quantitatively describing bone architecture is the calculation of morphometric indices. Measurements such as bone volume fraction, specific bone surface, porosity of new bone, thickness of new bone fragments, spacing between newly formed bone fragments and number of new bone fragments were calculated using a preprogrammed algorithm to determine trabecular bone morphometry. But prior to computing the values of each of these outcome measures, a Gaussian filter (sigma = 0.8, support = 1.0) was applied for noise reduction. In order to determine all the above-mentioned parameters, contours were manually drawn carefully around the newly formed bone based on the visible threshold difference between the old and the new bone for all the groups. This threshold was carefully determined based on visual inspection of tomograms of regular cortical bone. Voxels above the threshold value of 289 and below 1000 were collected and the segmented bone was reconstructed for measurements.

2.2.3.3. Multiphoton second harmonic generation. Multiphoton second harmonic generation (SHG) imaging is based on the process of frequency doubling by which two near-infrared photons are converted into a single photon with exactly twice as much energy as the input photons. SHG signals arise due to interaction with anisotropic molecules, which in biological imaging, is most often present in collagen. We used Leica TCS SP5 laser scanning confocal microscopy (Leica Microsystems, Bannockburn, IL) equipped with a Ti-sapphire tunable multi-photon laser (Coherent, Santa Clara, CA). SHG for collagen was optimally imaged using 860 nm excitation (MP laser) for maximum efficiency and emission collection was in the range of 425–435 nm with a peak emission generated at 430 nm. H&E stained slides were used for imaging. The images obtained were analyzed using ImageJ 1.48j version software (NIH). During this analysis, collagen images were used to precisely match the collagen outline and converted to binary image for measurement. Three collagen attributes were acquired: total collagen area, collagen bundled area and collagen bundled area/total collagen area. Following an initial blind coded evaluation of 35 images, it was determined that an area of 3500 μm^2 was the minimum criterion for a collagen collection to be considered a collagen bundle. Accordingly, in each image, collagen bundle area with an area of at least 3500 μm^2 was summed to determine collagen bundled area of the entire image. The collagen bundle area was divided by the total collagen area to determine the relative amount of collagen bundling. For each group, $n = 15$ images were processed.