In vitro and *in vivo* study to determine the influence of bone morphogenetic protein-7 incorporated chitosan microparticles on bone regeneration

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Statement of Purpose: Chitosan is a natural polymer available in abundance, but though it has shown to be osteoconductive, it lacks osteoinductivity. Therefore, chitosan scaffolds with growth factors is being considered as a potential alternative. During the bone healing process bone morphogenetic proteins (BMPs) are found to play an important role in recruiting mesenchymal stem cells which form soft tissue around the defect and also are involved in cell proliferation in intramembranous ossification at periosteal site. Along with vascular endothelial growth factor (VEGF), BMPs have also been shown to play a crucial role in blood vessel ingrowth by chondrocyte apoptosis and cartilaginous degradation [1]. Thus this study focuses on incorporating BMP-7 into chitosan microparticles and studying their effect on bone regeneration *in vitro* and *in vivo*.

Methods: ***In vitro***: Release of BMP-7 was from chitosan microparticles was quantified using enzyme linked immunosorbent assay ***In vivo***: 80 healthy skeletally mature male 8 weeks old inbred Lewis rats were used as experimental animals. A 4-5 mm hole was drilled through one of the posterior cortex in the mid shaft region of the femur, using an orthopedic microdrill. We had three different microparticles groups (n=10), only microparticles, BMP-7 encapsulated microparticles and BMP-7 coated microparticles. Defects without any filling were used as controls. After 6 and 12 weeks of implantation, rats were euthanized and analyzed by **i) µ-CT analysis-** To assess the amount and quality of bone grown at various radial distances into and around the scaffold, biopsy analysis was performed on each specimen Bone volume and the ratio of bone volume and tissue volume were determined for analysis. **ii) Histological procedures**: Samples were embedded in liquid paraffin and cut into 5 µm thick sections for hematoxylin and eosin (H&E) staining **iii) Confocal multiphoton second harmonic generation-** 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.

Results: i) ***In vitro* release kinetics:** The release study indicated that by day 18, nearly 98% of the BMP-7 was released from the coated microparticles, while only 36% of the BMP-7 encapsulated in the microparticles was released (Fig 1). SPSS two way ANOVA indicated that there is a significant difference ($p<0.001$) in the release of BMP-7 incorporated in the two different ways at all the time points from t=0 to t=18.

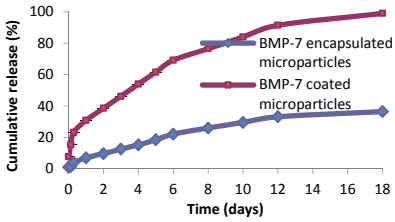

Fig.1: Cumulative release profile of BMP-7 encapsulated microparticles and BMP-7 coated microparticles over a period of 18 days.

ii) ***In vivo:*** None of the animals died indicating biocompatibility of the microparticles. Even after 12 weeks, the microparticles were found to be present intact at the defect site supporting the bone formation. **µ-CT** analysis indicated that there is a 6.99% increase in the bone formation in comparison with controls, in the presence of microparticles at 6 weeks. **Multiphoton SHG** results at 6 weeks indicated that collagen being formed in the bones defects containing microparticles was more in the bundled form in comparison to controls which had fibrous like collagen formation. **H&E** slides at week 6 indicated very minor inflammatory response, indicated by the presence of neutrophils around the microparticles. Active osteoblasts were found lying down bone in the regions adjacent to the microparticles. Active fibroblasts were observed in the collagen forming region. Many new capillaries were formed in the region of new bone formation indicating healthy bone formation. Hyaline cartilage formation sites were also observed in the region. Osteocytes were observed in between the newly formed bone. All this indicates the formation of the granulation tissue.

Conclusion: *In vivo* data indicates that chitosan microparticles positively influence bone formation, collagen was being formed in bundled fashion, indicating formation of stronger bone and there was no acute inflammatory response observed.

References: [1] Marsell R, et al., The biology of fracture healing. Injury 2011;42:551-555

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