BMP-2 Induces de novo Bone Formation in Gelatin Hydrogel

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DISCLOSURE: Nothing to disclose.

INTRODUCTION: The repair of bone defects remains a severe clinical challenge. Various strategies such as stem cell and/or pharmaceutical therapies have exhibited positive effects in accelerating bone regeneration. However, such therapies are vulnerable to clearance and dilution in the systemic circulation; thus, their potency may be dampened and render them ineffective. Therefore, localizing and retaining therapeutics at the defect site is a priority to enhance their activities and minimize side effects. Gelatin hydrogels may serve as a scaffold to deliver therapeutic agents for bone growth: 1) to retain the therapeutics within the localized environment, 2) to serve as a scaffold for cell integration and osteo-differentiation, and 3) to reduce the extrusion of therapeutics to neighboring tissues and dilution into systemic circulation. Herein, we demonstrate for the first time a chemical-crosslinked gelatin hydrogel scaffold that supports bone induction and growth with the following specialized properties: 1) tunable stiffness, 2) encapsulation of recombinant human bone morphogenic protein 2 (rhBMP-2), 3) growth and osteo-differentiation of human mesenchymal stromal cells, and 4) formation of *de novo* bone *in vivo*. The unique properties of this tunable biomaterial will advance the use of BMP-2 in clinical application.

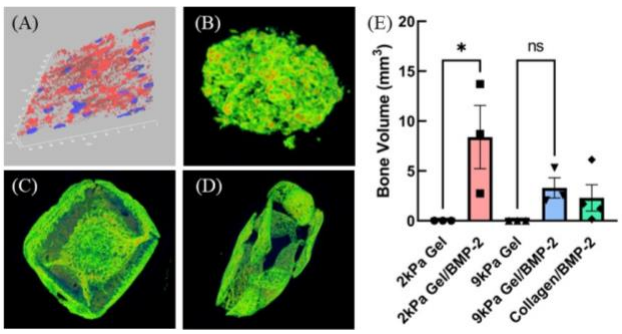
METHODS: The phenol moiety-functionalized collagen type I gelatin was reacted with horse radish peroxidase (HRP) and hydrogen peroxide (H_2O_2) to form crosslinked gelatin hydrogels. Differing doses of rhBMP-2 were incorporated into the gelatin solution before the hydrogel was crosslinked and solidified. The stiffness of gelatin hydrogels was determined by a rheometer (Anton Paar, MCR302e, Graz, Austria). To demonstrate the mineral deposition *in vitro*, human mesenchymal stem cells (hMSC, 10^6 cells/mL) were encapsulated with or without rhBMP-2 and cultured for 2 weeks. To detect mineral deposition, Alizarin Red S was used and was detected using confocal fluorescence microscopy (Model DMi8 automated, Leica Microsystems CMS GmbH, Mannheim, Germany). Five z-images were acquired for each sample, from the bottom to the top of each hydrogel. The quantification of mineral deposition was determined by calculating the ratio between positive Alizarin Red S and the number of nuclei within the field of view (n=3 per group). For *in vivo* bone formation, a rat subcutaneous, osteo-induction model was employed. Briefly, gelatin hydrogels (2 and 9 kPa stiffness), encapsulated with or without 15μg of rhBMP-2 (n=3 per group), were implanted on the ventral side of Sprague Dawley rats (5-6 weeks old). After 4 weeks, animals were euthanized, and biomaterials were isolated from the animal. Samples were immediately fixed in 10% neutral buffered formalin. Samples were analyzed for bone formation using microCT (SCANCO Medical AG, MicroCT 50, Switzerland) for 3-D mineralization analysis. One-way ANOVA statistical analysis with a multiple-comparison Tukey post hoc test was used to determine statistical significance ($p < 0.05$). Data were means $\pm$ SE.

RESULTS: Gelatin hydrogels were formed by covalent bond between the ortho-position of phenyl ring and hydroxyl group containing phenol moieties. The stiffness of gelatin hydrogels was controlled by altering H_2O_2 concentration. With H_2O_2 of 0.0045, 0.006, and 0.01%, gelatin hydrogels exhibited 1, 2, and 9 kPa, respectively. Gelatin hydrogels of 9 kPa did not support hMSC growth within the hydrogel matrices, even though the cells survived out to 14 days as determined by Live/Dead stain. The lower stiffness gelatin hydrogels (1 and 2 kPa) allowed hMSC proliferation and were used for subsequent assessment of *in vitro* mineral deposition. hMSC were encapsulated with varying concentrations of rhBMP-2 (0, 30, 90, and 120 ng/mL). After 14 days in culture, hMSC without rhBMP-2 did not exhibit any alizarin red stain while hMSC encapsulated with rhBMP-2 dose-dependently stained with alizarin red. Similar to *in vitro* findings, encapsulation of rhBMP-2 into gelatin hydrogel was sufficient to induce *de novo* bone formation in the rat subcutaneous osteo-induction model. The bone volume for 2kPa hydrogel/BMP-2 and 9kPa hydrogel/BMP-2 were 8.39 ± 5.48 mm^3 and 3.29 ± 1.76 mm^3, respectively, while no bone was detected in hydrogels (2kPa or 9kPa) without rhBMP-2. Further, gelatin hydrogels were not resorbed *in vivo* unlike the gold-standard collagen carrier typically used in this model.

DISCUSSION: Gelatin hydrogel stiffness influences hMSC growth *in vitro* and possibly cell penetration and differentiation *in vivo*. Our result suggests that higher stiffness is from a higher percent of crosslinking among gelatin and results in lower porosity within the hydrogel matrices. However, we cannot discount the possibility that diffusion of nutrients and waste (from cells) may be compromised with higher stiffness. This is the first evidence that rhBMP-2, which is currently used in the clinic for spine fusion, can induce *de novo* bone formation in a gelatin hydrogel carrier. rhBMP-2 encapsulation within the hydrogel was able to recruit resident cells to grow, differentiate, and form bone within the hydrogel scaffold. The preclinical data presented herein suggests that this osteo-inductive biomaterial may be used in the clinic to refine the dosage for BMP-2 and to reduce the known BMP-2 associated side effects.

SIGNIFICANCE/CLINICAL RELEVANCE: Gelatin hydrogels encapsulating rhBMP-2 can induce resident cells to make bone. This stable, cell-free scaffold shows promise to retain rhBMP-2 within a localized environment and thus may reduce the known side effects of rhBMP-2. Future studies with novel osteogenic small molecules integrated into this unique biomaterial will further advance treatments for musculoskeletal injuries and diseases.

ACKNOWLEDGEMENTS: This work was supported by NIH/NIAMS grant 1R01AR078892. We express our gratitude to Dr. Park at Ajou University (South Korea) for providing the biomaterials.



A, Maximum projection image of Alizarin Red S staining and DAPI of gelatin hydrogel encapsulated with rhBMP-2 and hMSC after 14 days in culture. Representative μCT heat-map images of collagen (B), 2kPa (C) and 9kPa (D) hydrogels, each containing 15μg of rhBMP-2.
E, Quantification of bone volume of implants isolated from rat subcutaneous, osteo-induction model.