Injectable Composite Hydrogels for Bone Repair in a Sheep Bone Defect Model

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Statement of Purpose: Effective cell-based therapies for bone healing require the delivery of cells using instructive biomaterials that localize cells at the target site and provide instructional cues. Hydrogels are widely used in such applications, as they are chemically and physically tailorable, but they often lack the desired osteoconductivity of other common materials. To address this shortcoming, we entrapped mesenchymal stem cells (MSCs) in a novel composite hydrogel with enhanced osteoconductivity to promote bone regeneration in an ovine iliac crest bone defect. The composite hydrogel was composed of alginate, chosen for its tailorability, and hyaluronate, selected for its engagement of the CD44 cell receptor present on MSCs. Arginine-Glycine-Aspartic Acid (RGD) was conjugated to the backbone of both polymers to facilitate cell adhesion. Hydrogel osteoconductivity was increased by incorporating biomineralized polymeric microspheres into the network. We hypothesized that the transplantation of autologous ovine MSCs within this composite hydrogel containing mineralized polymeric microspheres would enhance hydrogel osteoconductivity, increase osteogenic potential, and promote bone healing in an ovine bone defect model.

Methods: Bone marrow was collected from 12 adult female Swiss Alpine sheep. Red blood cells and plasma were then removed from the aspirate, and the remaining cells were expanded and used for implantation. Sodium alginate was irradiated at 5 Mrad for faster degradation and covalently modified with G_4RGDSP using standard carbodiimide chemistry. Similarly, G_4RGDSP was covalently coupled to sodium hyaluronate using similar protocols. Poly(lactide-*co*-glycolide) (PLG) microspheres were formed using a double-emulsion process and underwent biomineralization by incubation in modified simulated body fluid. The gels were produced by mixing a 2% RGD-alginate and 1% RGD-hyaluronate at a 1:9 ratio with microspheres at a 3 mg/mL concentration. Control hydrogels were formed of RGD-alginate or RGD-alginate/hyaluronate (lacking RGD). Ovine MSCs were entrapped at 10 million cells/mL, and the gel was crosslinked *via* a supersaturated solution of $CaSO_4$. The hydrogels were characterized through SEM, NMR and mechanical testing. The osteogenic response of MSCs within the gel during *in vitro* culture was quantified by ALP expression and calcium deposition. Composite hydrogels were then injected into bilateral critical-sized iliac crest bone defects in adult female Swiss Alpine sheep. Repair of the bone defect at 12 weeks was evaluated through radiography, microCT and histology.

Results: NMR data confirmed that RGD was conjugated to both the hyaluronate and alginate. Alginate and alginate hyaluronate composite hydrogels exhibited similar swelling ratio and gross morphology. However, the compressive modulus and pore size decreased when hyaluronate was added. MSCs cultured in RGD-alginate/RGD-hyaluronate for 3 weeks had increased ALP activity and calcium accumulation compared to MSCs in RGD-alginate or RGD-alginate/hyaluronate. Therefore, RGD-alginate/RGD-hyaluronate gels were used for implantation. At 12 weeks, microCT revealed that bone defects treated with MSC-containing composite hydrogels had higher bone volume fraction and bone mineral density compared to sham and acellular groups (**Figure 1**). Histology indicated this group exhibited the highest vessel density. H&E, Masson's trichrome and Goldner's trichrome further confirmed more bone growth and collagen deposition within defects treated with MSC-containing gels.

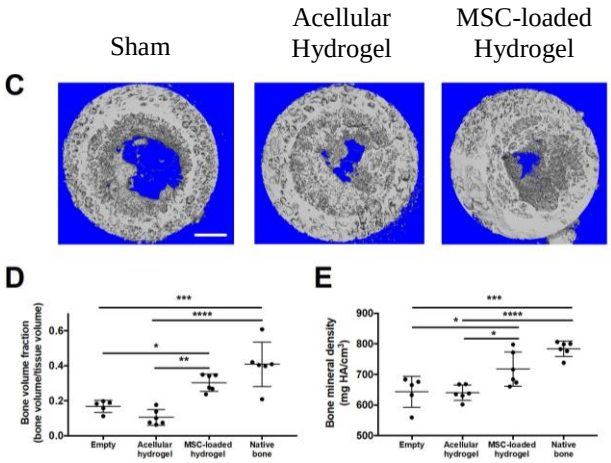

Figure 1. A) Representative microCT images at 12 weeks. Scale bar = 3 mm. (B) Bone volume fraction within the tissue defects. (C) Bone mineral density within the tissue defects. N=5-6; **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001

Conclusions: We have successfully developed and characterized a composite hydrogel formed of RGD-modified alginate and hyaluronate that contained apatite-coated polymeric microspheres. This material increased the osteogenic response of ovine MSCs *in vitro* while serving as an effective cell carrier for MSCs to promote bone regeneration and neovascularization in a sheep iliac crest defect. These data also reveal that RGD-hyaluronate, a material that engages both CD44 and RGD receptors, can enhance osteogenic differentiation to a greater degree compared to gels that engage each receptor alone. In light of these findings and its contribution to connective tissue homeostasis, the use of hyaluronate in instructive hydrogels merits consideration for use in other platforms designed to promote bone healing.