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GENIPIN-CROSSLINKED FIBRIN HYDROGELS MODIFIED WITH COLLAGEN OR FIBRONECTIN AS AN ANNULUS FIBROSUS SEALANT

Clare Canal Guterl[1], Tyler Kim[1], Steven B. Nicoll[2], James C. Iatridis[1]

1. Leni and Peter W. May Department of Orthopaedics

Mount Sinai School of Medicine
New York, NY 10029
U.S.A.

2. Department of Biomedical Engineering

The City College of New York
New York, NY 10031
U.S.A.

INTRODUCTION

Low back pain (LBP) affects 80% of all adults at some point in their lives [1], and is often associated with intervertebral disc (IVD) degeneration, therefore, restorative solutions are of high importance. The IVD is composed of an outer ring of annulus fibrosus (AF) containing the pressurized nucleus pulposus (NP) center. NP replacement approaches and IVD procedures such as discectomy or discography involve rupture of the AF tissue for diagnosis or repair purposes. Even small defects to the outer AF can accelerate IVD degeneration [2]. Current closure techniques are limited to sutures and offer little in the way of tissue replacement or mechanical restoration of the AF [3]. A genipin crosslinked fibrin hydrogel offers some promise as an adhesive AF sealant [4]. Fibrin is FDA approved and genipin is a plant based chemical crosslinker with low cytotoxicity used with a variety of materials including fibrin for articular cartilage engineering [5]. Genipin crosslinked fibrin is a tunable material with comparable mechanical properties to native AF tissue and although cells remained viable on the gel, the relatively slow proliferation and presence of abnormal cells with rounded morphology motivated further optimization. To improve cytomorphology, key extracellular matrix proteins, collagen and fibronectin, were added to the formulation. It was hypothesized that the addition of these proteins would maintain the mechanical properties of this gel while improving cell morphology and viability. Several additional analyses were included to further characterize this gel including push-out strength, degradation and contraction tests.

The objective of this study was to: 1) develop a crosslinked fibrin gel with strong adhesiveness and high stiffness; 2) modify the formulations of this adhesive biomaterial with cell adhesion molecules to enhance cell growth and density; and 3) characterize biomechanical and biological performance of this biomaterial for its potential use as an AF sealant.

MATERIALS AND METHODS

Gel Formulation. Fibrinogen and thrombin, both isolated from bovine plasma (Sigma), were dissolved in PBS at 200 mg/ml and 100 U/ml respectively. Thrombin was added to the fibrinogen at a ratio of 1 U per 5 mg fibrinogen. In crosslinked groups, genipin (Wako) dissolved in DMSO (Fisher) was combined with the thrombin at a ratio of 0.25 mg genipin to 1 mg fibrinogen in the final mixture (0.25:1). In fibronectin-modified groups, fibronectin (Sigma) was added to the fibrinogen solution to create a final concentration of 50 μg/ml in the gel. In collagen-modified groups, collagen type I, isolated from rat tail tendon (BD Biosciences) was combined with 10x PBS, dH_2O, and NaOH and then added to previously mixed fibrinogen, thrombin and genipin to achieve a final concentration of 0.08 wt% or 0.8 mg/ml. Depending on the experiment, gels were prepared in a mold (rheological testing and degradation), sterilely on glass slides (cell viability), within cored out defects of AF tissue (push-out test) or as beads (contraction). All gels were allowed to set for 24 hours before testing or cell seeding. **Cell Culture**. 150 μl of gel was prepared under sterile conditions to produce approximately 1-mm thick by 14 -mm diameter gels, which were then placed into 12 well plates. Gels were seeded with 25,000 human AF cells (obtained from autopsy) in 2ml of media (DMEM with 10% FBS (Gibco), 0.5% fungizone, and 50 μl ascorbic acid (Sigma)). Cells used were P1-P2 with an average donor age of 53 ± 13. Cells were fed every 3-4 days until day of imaging when gels were rinsed with PBS and incubated with a solution of 4mM calcein in PBS (Invitrogen) for 15 minutes. 3 cell sources were used (N=3) and 5 images were taken per gel for a total of 15 images per gel formulation. ImageJ (NIH) was used to calculate the

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average cell count per mm^2. **Mechanical Testing**. 1.5-mm thick cylindrical gel specimens (∅5mm, n=8) were tested using a rheometer (TA instruments). The shear properties of the gels were characterized over a dynamic frequency and strain sweep as previously described [4]. **Adhesion testing**. Bovine caudal discs were dissected and 8-mm diameter specimens were punched radially from the disc. Samples were then sub-punched to create a 3-mm diameter defect which was then filled with genipin crosslinked fibrin gel or, as a control, the punched out AF tissue core was reinserted back into the defect without an adhesive. Samples were then placed in a confined chamber with 3.5 diameter top and bottom center holes and indented with a 2.8mm flat ended tip at a rate of 0.01 mm/sec until failure. **Degradation Tests**. 5 mm plugs of 0:1 and 0.25:1 genipin fibrin gels, were weighed (W_i) and then placed in PBS at 37°C for 1, 3, and 7 days to obtain both the wet (W_w) and dry weight (W_d) (n=4 for each time point and formulation). Swelling ratio (W_w/W_d) and weight loss ($(W_i-W_w)/W_i$) were calculated at each time point. **Contraction Tests**. 50 µl 0.25:1 genipin fibrin gel droplets (n=4) were placed between glass slides in a humidified environment and images were captured over a 60 minute time period to record contraction of the gel. **Statistics.** A two-way ANOVA with a Tukey's post-hoc test was used to detect significance between gel formulations and time points (GraphPad Prism).

RESULTS

Gel Stiffness. For brevity, only the dynamic modulus magnitude (|G*|) from the frequency sweep is reported (1 Hz at 10% strain). Results are compared to that of previously obtained values for human AF tissue (Figure 1). 0.25:1 ratio gels at 200mg/ml fibrinogen content are of similar stiffness to AF tissue. The addition of fibronectin or collagen to the gels did not significantly alter the stiffness. **Cell Culture.** Viability of cells increased from Day 7 to Day 21 on all gel formulations (Figure 2) and was significantly greater in the 0.25 + collagen and 0.5 groups ($p<0.05$). At day 21, fibrin gels were highly degraded with concentrated cell populations on remaining gel fragments. Representative images (Figure 2 bottom) illustrate cell morphology and typical density. Some variability was seen in cell concentrations throughout single gels. Cells seeded on collagen- and fibronectin-modified gels developed a spindle-like morphology that was maintained throughout culture. A rounded morphology was observed on non-modified crosslinked gels at day 7 (0.25:1 and 0.5:1), and was less apparent in those gels at day 21. **Push-Out Test.** An average failure strength of 60.7 ± 17 kPa and 8.6 ± 3 kPa was measured in bovine samples with defects filled with genipin crosslinked gel and control specimens respectively. Gel-filled defects failed when the indenter reached an average of 91 % of the specimen thickness; whereas controls failed at 35% through the specimen thickness. **Degradation and Contraction Tests**. After 7 days, uncrosslinked fibrin gels were completely degraded whereas those crosslinked with genipin lost an average of 23 ± 6% wet weight. The swelling ratio of genipin gels was consistent at 6.1 ± 0.4 for days 1, 3 and 7 whereas fibrin gels were significantly higher at 9.2 ± 0.7 and 10.1 ± 0.8 for days 1 and 3 ($p<0.05$). Over the 60-minute observation period, genipin crosslinked gels contracted a negligible amount (1.8 ± 4 % contraction).

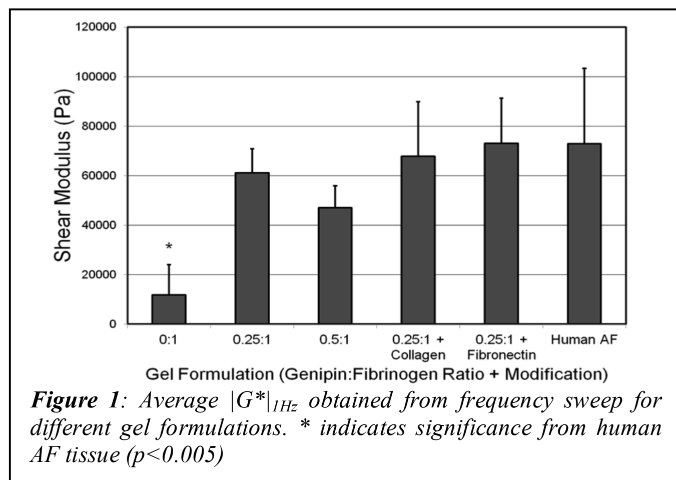

***Figure 1**: Average $|G^*|_{1Hz}$ obtained from frequency sweep for different gel formulations. * indicates significance from human AF tissue ($p<0.005$)*

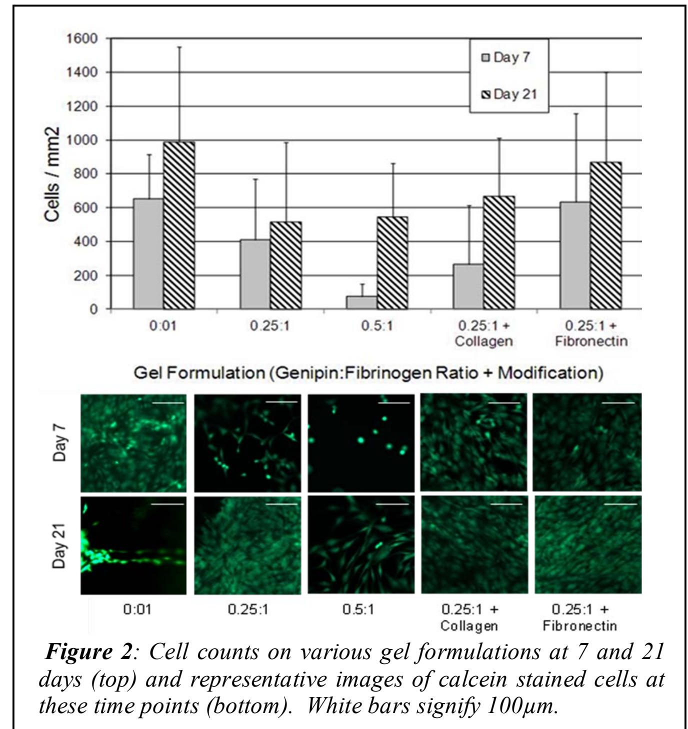

***Figure 2**: Cell counts on various gel formulations at 7 and 21 days (top) and representative images of calcein stained cells at these time points (bottom). White bars signify 100µm.*

DISCUSSION

This study developed and characterized a crosslinked fibrin gel modified to increase adhesive strength for potential use as an AF repair material. Although fibrin gels are utilized in a variety of clinical applications they degrade quickly, have low shear stiffness and do not show great potential as an AF sealant. With the addition of genipin, the stiffness of this crosslinked fibrin gel increased to a comparable level to native AF tissue and the degradation rate was significantly slowed. Furthermore, genipin crosslinked fibrin gels were dimensionally stable and did not contract upon setting. Our previous studies showed an altered AF cell morphology with genipin crosslinked fibrin gels, and in this study, the addition of either fibronectin or collagen improved cell morphology and density while maintaining gel stiffness.

Genipin crosslinked fibrin gels provide an adhesive biomaterial, with tunable material properties, that can be formulated with cell adhesion molecules for enhanced phenotype. This biomaterial offers promise for sealing small AF defects and may be useful as an adhesive in combination with other biomaterials for larger AF defects. Future optimization as well as in situ validation will be required to find and formulate the optimal adhesive to augment IVD annulus repair.

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