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MANIPULATING THE INITIATION AND PROPAGATION OF MICROCRACKS IN COLLAGEN NETWORKS OF CARTILAGE

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INTRODUCTION

Macroscale and microscale injury regularly occur in the human knee joint resulting from complex and/or compound motions such as flexion, extension, and rotation, particularly under impact [1]. We previously demonstrated that low-energy mechanical impacts to articular cartilage, usually considered non-injurious, can in fact cause microscale cracks in the collagen network of visually pristine human cartilage [2]. Therein we defined collagen-network microcracks as fractures in the collagen network that are no wider than the diameter of chondrocyte lacunae (<30 μm). Such seemingly minute mechanical trauma may disrupt the microstructure of the extracellular matrix (ECM), altering the critical load-bearing capabilities and fluid retention properties of cartilage. We then quantified the extent to which microcracks initiate and propagate in collagen networks of cartilage during mechanical loading representative of normal activities [3].

Overall we aim to establish therapeutics to slow, stop, or even heal microcrack growth during cyclic loading, and thus minimize the possibility of subsequent cartilage and joint pathologies. Crosslinking of collagen can improve the mechanical stiffness of (especially monomeric) networks through several mechanisms, cf. [4]. Genipin is a well-established cross-linker for proteins and studies confirm that it can improve the mechanical strength of collagen networks [5]. While genipin cannot repair large (mm-scale) fissures [6], its ability to repair micron-scale fissures in collagen remains unknown. In this study we aimed to determine the effects of genipin as a preventative treatment: (1) to mitigate the initiation of microcracks under mechanical impacts; and (2) to mitigate the propagation of microcracks under cyclic compression (and specifically the effects of number and timing of genipin treatments). We hypothesized that treatments with genipin will interrupt initiation and progression of damage in the networks of collagen in cartilage, and thus potentially lead to new treatments.

METHODS

Mechanical Tests and Images via SHG. In total we tested 49 full-thickness, cylindrical osteochondral plugs (specimens) of 3 mm diameter. We pooled specimens from the lateral and medial femoral condyles, and assigned them to one of four different genipin treatment (dosing) groups, which included two time points: (A) before low-energy impact (2.5 mJ/mm^3) and (B) before unconfined cyclic compressions (10% of reference thickness at 1.44 Hz for 12,000 cycles), of either 0 mM (denoted –) or 11 mM (+) of genipin. Our control group (n=10) underwent the same mechanical treatments but no treatment with genipin (and subsequent crosslinking) [3]. We impacted all specimens with the same impact energy density and imaged them via Second Harmonic Generation (SHG) (Zeiss LSM 510, Oberkochen, DE) in Phosphate Buffered Saline (pH 7.4) at three time points, see **Fig. 1**.

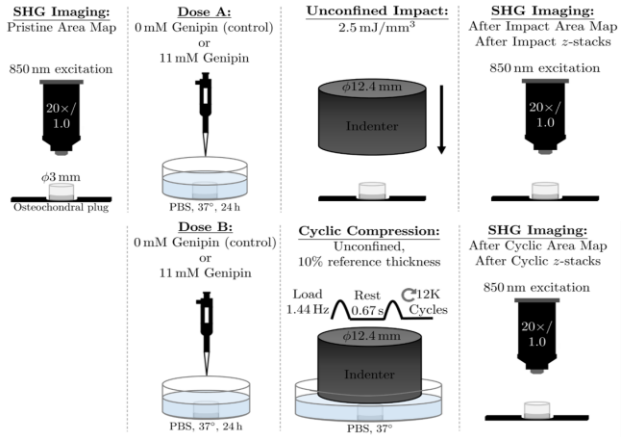

Figure 1: Schematic summary of our experimental protocol.

Analyses of Images. We used Fiji's Grid/Collection stitching plugin to generate images of the full circular cross section at a resolution of 1.2 μm/pixel. Using only the 3 × 3 tile grid centered on the main axis of the cylinder (to avoid edge effects), independent observers measured the length, width, depth, and principal angle (relative to the split-line direction) of each microcrack, cf. [3]. We calculated the length, width, depth, and orientation of all microcracks from both post-impact (PI) and post-cyclic (PC) compression phases of the mechanical experiment, and when possible, used the specific morphologies and orientations to track microcracks between the last two phases.

Statistical Analyses. We used separate mixed regression modeling to evaluate the effects of genipin treatment on microcrack density, and on the length, width, and depth of the microcracks over the course of the experiment. We included genipin treatment (dose) as a fixed effect and the thickness of each cartilage specimen as a covariate. We used post-hoc tests to evaluate differences among treatment combinations. To probe microcrack propagation, we analyzed our data from the tracked microcracks over the course of the experiment using the same mixed-model regressions, but with specimen included as an additional random factor. We completed all statistical analyses using SAS 9.4 (SAS Institute, Inc., Cary, NC) with a significance level $P < 0.05$.

RESULTS

We confirmed that our protocol successfully cross-linked the network of collagen. After 24 hours of incubation, the cartilage transformed from its normal white and glossy state to a dark color produced as genipin reacts with the amino groups. We imaged both treated and untreated specimens via Raman Spectroscopy and confirmed a characteristic fluorescence at 785 nm in only the treated specimens, thus confirming microstructural changes.

Genipin did not have a significant effect on the density and width of microcracks initiated under low-energy impacts, see **Figs. 2(a), (c)**. We did find statistically significant differences in the lengths and depths of microcracks initiated under low-energy impacts, see **Figs. 2(b), (d)**. Microcracks initiated in specimens treated with genipin tended to be longer and penetrate deeper into the cartilage specimens.

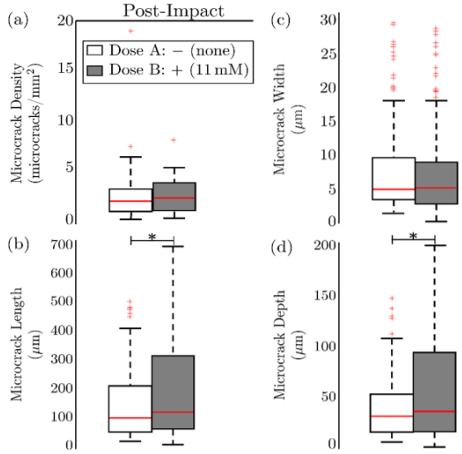

Figure 2: Microcrack Initiation under low-energy impact, untreated (white) vs. treated (gray) specimens. Box plots of the median (red lines) and interquartile ranges (black lines): (a) density, (b) length, (c) width, and (d) depth of microcracks.

Genipin had a significant effect on some aspects of microcrack propagation under cyclic, unconfined compression, see **Fig. 3**. Two treatments of genipin caused significantly greater propagation of microcracks (both longer and wider) than a single treatment of genipin, see **Figs. 3(a), (b)**. A single treatment of genipin did not have a statistically significant effect, but consistently resulted in marginally less propagation. Treatment with genipin had no effect on the depth of microcracks during cyclic, unconfined compression.

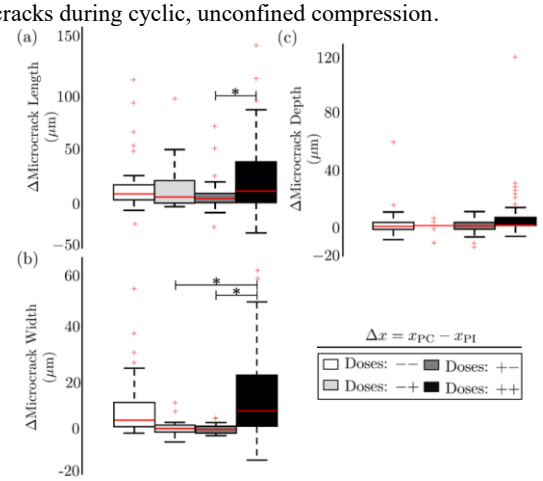

Figure 3: Microcrack Propagation post-impact to post-cyclic, unconfined compression, comparison of untreated (white), and treated (before impact = light gray, before cyclic compression = medium gray, before both = dark gray) specimens. Box plots of the median (red lines) and interquartile ranges (black lines): (a) length, (b) width, and (d) depth of microcracks.

DISCUSSION

In this study, we induced microscale damage to the network of collagen (i.e. collagen network microcracks) using low-energy impacts to cylindrical specimens of cartilage, and propagated these microcracks in unconfined, cyclic compression. The initiation and propagation of microcracks may deteriorate the mechanical function of cartilage and characterize pathogenesis of osteoarthritis, and may suggest therapeutic targets for future studies [3].

Our results do not support our hypothesis that treatments with genipin will improve the damage resistance of cartilage. In the dosing scheme that we tested genipin was not an effective treatment for preventing or repairing damage to the network of collagen in cartilage. Studies show that genipin can enhance the mechanical properties of collagen networks, particularly in engineering constructs. Specifically, genipin treatments to cartilage significantly increased stiffness in specimens treated with 10 mM for 24 hours [5]. In our study, the increased stiffness likely caused a reduction in ductility, which caused the cartilage to be less resistant to damage under impacts. Since crosslinking with genipin did not prevent microcrack initiation, the additional cross-links among collagen fibrils created by treatment with genipin were insufficient to prevent rupture of fibrils. Adding a second treatment of genipin further exacerbated the damage, suggesting that the stiffness may have further increased following the second treatment. While increased stiffness may be desirable in some engineering applications, it may be accompanied by reduced material toughness.

ACKNOWLEDGEMENTS

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