Genipin does not reduce the initiation or propagation of microcracks in collagen networks of cartilage

Stephany Santosa, Corey P. Neu, James J. Grady, David M. Pierce

ARTICLE INFO

Keywords:
Articular cartilage
Mechanical injury
Microcracks
Genipin
Cross-linking
Low-energy impact

ABSTRACT

Objective: We recently initiated microcracks, i.e. micron-scale cracks in the collagen networks of cartilage, using both single low-energy impacts and unconstrained, cyclic compressions. We also tracked the propagation of microcracks after cyclic compressions simulating 12,000 walking strides. In this study, we aimed to determine the effect of one or more genipin treatments on: (1) the initiation of microcracks under mechanical impacts and (2) the subsequent propagation of microcracks under cyclic, unconstrained compression. We hypothesized that treatments with genipin would improve the resistance of cartilage to microdamage, specifically reducing both the initiation of microcracks under impact loading and the propagation of microcracks under cyclic compression.

Design: We tested 49 full-thickness, cylindrical osteochondral specimens. We incorporated one or two doses of genipin in between mechanical treatments, i.e. single low-energy mechanical impacts to initiate microcracks and unconstrained, cyclic compressions to propagate microcracks. We also imaged specimens using second harmonic generation confocal microscopy, and analyzed the resulting images to quantify changes in morphologies (length, width, and depth) and orientations of microcracks. Finally, we used separate mixed-regression modeling to evaluate the effects of genipin treatments on mechanically induced microcracks.

Results: Specimens treated with genipin presented significantly longer and marginally deeper microcracks after mechanical impacts. Two doses of genipin caused significantly longer and wider microcracks under propagation versus one dose.

Conclusions: Our results do not support our hypothesis: unfortunately treatments with genipin, and the resulting mechanisms of cross-linking, do not provide resistance to microdamage, quantified as the initiation and propagation of microcracks.

1. Introduction

Injuries to articulating joints, e.g. the knee joint, occur relatively often due to complex and/or compound motions such as flexion, extension, and rotation [1]. Damage to the articular cartilage within such joints is a precursor to post-traumatic osteoarthritis (OA), a multifactorial cascade of degeneration. OA is a painful disease that affects more than 32.5 million adults in the United States with an overall economic burden estimated at $136.8 billion annually [2]. This economic burden has more than doubled over the last decade.

Severe joint injuries may present visible (arthroscopically or otherwise) millimeter-scale cracks or fissures on the surface of articular cartilage [3–6]. More subtle microdamage to cartilage may be difficult to identify (sub-millimeter, i.e. micrometer, scale) yet still compromise the load-bearing structure of cartilage, possibly invoking progressive degeneration. Recently we initiated microcracks, i.e. micron-scale cracks in the collagen networks of articular cartilage narrower than lacunae (30 μm), using both single low-energy impacts and cyclic unconstrained compressions [7,8]. To probe propagation we initiated microcracks in cartilage explants with low-energy mechanical impacts, and tracked the propagation of microcracks after cyclic compression simulating 12,000 walking strides [9]. Using second harmonic generation (SHG) microscopy, we measured microcrack area density before and after impact and after cyclic loading, and quantified changes in microcrack morphology (length, width, and depth) and orientation. The microcracks we initiated under low-energy impacts increased in length and width during...
subsequent cyclic compression that simulated walking, suggesting a poor outlook for joint health following injury even during routine physical activities [5,8,9].

Microdamage to the collagen matrix and loss of mechanical integrity in cartilage highlights the potential for repair strategies aimed at mitigating the initiation and progression of microcracks within cartilage. Extended deterioration of extracellular matrix including growth of microcracks, coupled with catabolic responses from cells [10], may define some of the key early stages of OA pathogenesis [11]. Consequently we sought to investigate possible therapeutics that would slow or arrest the progression of microcracks, or even heal them, and minimize the possibility of escalating pathologies within cartilage and joints.

Cross-linking of collagen can improve the mechanical stiffness and structural rigidity of (especially monomeric) networks, through chemical, mechanical, or combined radiative means [12]. The structural rigidity of collagen networks [16] and promote regeneration of cartilage [17]. Genipin stimulates intra- and inter-molecular cross-links of the amino residues on tropocollagen or proteoglycan molecules [18]. While genipin cannot repair large (millimeter-scale) microcracks remains unknown.

In this study, we aimed to determine the effect of one or more genipin treatments on: (1) the initiation of microcracks under mechanical impacts and (2) the subsequent propagation of microcracks under cyclic, unconfining compression. We hypothesized that treatments with genipin would improve the resistance of cartilage to microdamage, specifically reducing both the initiation of microcracks under impact loading and the propagation of microcracks within the network of collagen under cyclic, unconfining compression. To these ends, we incorporated one or two doses of genipin in between mechanical treatments, i.e. single low-energy mechanical impacts to initiate microcracks and unconfining, cyclic compressions to propagate microcracks, cf. Santos et al. [9]. Before and after mechanical treatments we imaged specimens using SHG confocal microscopy, and analyzed the resulting images to quantify changes in microcrack morphologies (length, width, and depth) and orientations. Finally, we used separate mixed-regression modeling to evaluate the effects of genipin treatments on mechanically induced microcracks. Understanding the effects of genipin treatments on the initiation and progression of microdamage in the network of collagen may suggest therapeutic targets for future studies and may lead to improved clinical outcomes.

2. Materials and methods

We tested 49 full-thickness, cylindrical osteochondral plugs (specimens). We pooled specimens from both the lateral and medial femoral condyles and assigned them to one of four different cross-linking (genipin) treatments, cf. Table 1. We treated specimens with Dose A prior to impact and Dose B after impact but prior to cyclic compression. The first row (–, –), i.e. specimens undergoing the same mechanical treatments but without cross-linking treatments, came from in a prior study and served as our control (n = 10) [9]. We applied the same impact (by energy density) and unconfining, cyclic compression treatments to all specimens. To quantify outcomes we performed imaging via SHG (LSM 510, Carl Zeiss, Oberkochen, DE) at three phases of the experiment (pristine, post-impact, and post-cyclic-compression). In Fig. 1 we show an overview of the experimental protocol.

2.1. Preparation of specimens

We received full bovine knees from three skeletally mature animals (18–30 months) packed on ice (Animal Technologies, Inc., Tyler, TX). We prepared specimens as described previously [9]. Briefly, we extracted cylindrical osteochondral plugs from visibly pristine load-bearing regions on both the lateral and medial condyles while recording the local split-line direction [20,21]. Using a scalpel, we removed a majority of the subchondral bone while ensuring that the remaining subchondral bone surface was visibly parallel to the articular surface. Using a digital camera (EOS 70D DSLR, Canon, Tokyo, JP), we imaged each cylindrical specimen and used standard image processing to determine the thickness of cartilage [22]. We immersed specimens not immediately tested in Phosphate Buffered Saline (PBS, pH 7.4) and stored them at ~80°C [20,23]. On the day of testing we thawed specimens and mounted them to custom, ultra-wear-resistant nylon plates using cyanoacrylate adhesive for subsequent imaging, and genipin and mechanical treatments.

2.2. Treatments with genipin

We received the chemical compound genipin (Adipogen Life Sciences, San Diego, CA) on ice, and stored it as a 200 mM stock solution in anhydrous dimethyl sulfoxide (DMSO) at ~20°C. We prepared working solutions of 11 mM genipin using PBS [24]. In + treatments we incubated specimens for 24 h in 300 μL of 11 mM genipin solution at 37°C [19,24,25]. In – treatments we incubated specimens for 24 h in 300 μL of PBS at 37°C. After the incubation periods, we rinsed specimens in PBS for at least 30 s prior to mechanical treatments.

2.3. Mechanical treatments

2.3.1. Low-energy impact

We applied low-energy impact treatments using our drop tower and protocol as described previously [9]. Briefly, we impacted the articular surface of unconfining, pristine specimens with an impact energy density of 2.5 mJ/mm² using a custom drop tower with a 12.4 mm diameter flat, stainless steel impactor at 0.5 m/s. Post-impact, we submerged specimens in PBS at 37°C for at least 1 h to equilibrate prior to subsequent imaging, and genipin and mechanical treatments [8].

2.3.2. Cyclic, unconfining compression

We applied unconfining, cyclic compression treatments using our uniaxial compression device and protocol as described previously [9]. Briefly, post-impact and post 24 h incubation with or without genipin, we conducted unconfining cyclic compression tests in PBS 37°C using a custom device based on a Bose L1M Electroforce linear motor with WinTest 7 software (Bose, Eden Prairie, MN). After force-controlled 0.2 N compression for 3000 s, we applied a pattern of cyclic compression including 0.69 s sinusoidal compression with an amplitude of 10% of the cartilage thickness, followed by 0.67 s recovery (total cycle time equals 1.36 s or 0.74 Hz), cf. Zhang et al. [26] and Santos et al. [9]. Post-cyclic compression, we submerged specimens in PBS at 37°C for at least 1 h prior to subsequent imaging [8].

| Dose | Dose | # Specimens | Total Measurements | Cluster Sizes |
|------|------|-------------|--------------------|--------------|
| A    | B    | 10          | 162                | 3, 13, 28, 23, 32, 43, 4, 15, 1 |
| –    | +    | 14          | 165                | 8, 10, 16, 3, 1, 2, 5, 87, 3, 11, 10, 5, 3 |
| +    | –    | 12          | 72                 | 5, 6, 5, 1, 9, 11, 2, 11, 12, 2, 7, 1 |
| +    | +    | 13          | 160                | 17, 2, 48, 5, 2, 13, 17, 23, 9, 3, 9, 6, 6 |
2.4. Images via second harmonic generation

We performed SHG imaging using our confocal microscope and protocol as described previously [9]. Briefly, we imaged specimens at three separate experimental phases (pristine–P, post-impact–PI, and post-cyclic compression–PC). We used tunable Ti: Sapphire lasers (Coherent Chameleon, Santa Clara, CA) at 850 nm for excitation and we post-cyclic compression. We also used a water-immersion objective (W Plan-Apochromat 20 × 13 nm) and a 600 × 600 μm (512 × 512 pixel) field of view. For each specimen, we acquired a 7 × 7 tile grid (100 μm tile overlap) of the entire articular surface at three separate experimental phases (P, PI, and PC). Additionally, both post impact and post cyclic compression we acquired through-thickness image stacks between 50 and 200 μm (slice increment of 2.5 μm) from a 3 × 3 tile grid centered on the articular surface (to avoid edge effects).

2.5. Analyses of images

We combined our SHG images using Fiji’s Grid/Collection Stitching Plugin [27] for ImageJ (National Institutes of Health, Bethesda, MD) 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 articular surface (3618.8 × 3618.8 μm, 3093 × 3093 pixels), independent observers measured the length, width, depth, and orientation (principal angle relative to the split-line direction) of each microcrack in each image (parallel to the articular surface), using the measurement tools in Fiji. We then calculated the length, width, depth, and orientation (angle from the split-line direction) of all microcracks from both post-impact and post-cyclic compression phases, and when possible, used specific morphology and orientation to track microcracks from post-impact to post-cyclic compression. We also calculated the microcrack area density for each specimen using the total number of microcracks within the centered 3 × 3 tile grid.

2.6. Statistical analyses

We used separate, mixed-regression modeling to evaluate the effects of genipin treatments on subsequent microcrack density, and on the length, width, depth, and orientation of microcracks. We included dose as a fixed effect and the thickness of each specimen as a covariate; and we considered measures made at each dose/thickness level as correlated clusters of data. We used post-hoc tests to evaluate differences among treatment combinations. To calculate means for our statistical analyses we considered crack morphologies (i.e. length, width, depth, and orientation) as individual data points associated with Dose A for each specimen. We report both the p-values and the estimates for our statistical tests.

To probe propagation of microcracks, we analyzed the subset of our data where we tracked individual microcracks over the course of the experiment using the same mixed-model regressions, but with specimen included as an additional random variable. To quantify the magnitudes of propagation of microcracks, we calculated the changes in length, width, and depth for each microcrack and used this change in the mixed model. In this case we report only the change in length, width, and depth of microcracks as ΔL = LP + Δr, where L represents a specific morphological feature. We also report the p-values for our statistical tests. We estimated all statistical models using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC).

3. Results

We completed combinations of genipin and mechanical treatments, and imaging, on a total of 49 specimens. We confirmed our protocol successfully caused cross-linking in the network of collagen since the cartilage specimens transformed from their normal glossy white color to a saturated black [28]. Additionally, we did not find any microcracks in the untreated controls. We successfully initiated microcracks in visibly pristine cartilage, and propagated the microcracks under cyclic compression.

3.1. Microcrack initiation

Comparing untreated cartilage specimens and those treated with genipin (Dose A) after low-energy impacts, we identified statistically significant differences in microcrack lengths (p-value = 0.001) such that the genipin treatments caused increases in the lengths of microcracks, see Fig. 2. We found no statistically significant differences in median area densities (0.905), widths (0.442), or orientations (0.831) of microcracks initiated in untreated cartilage specimens versus those treated with genipin. The genipin treatment caused a marginal increase in the depth of microcracks, but these changes were not statistically significant (0.062). Therefore, specimens treated with genipin tended to present longer and (marginally) deeper microcracks after mechanical impacts.

We summarize the statistical results in Table 2 and summarize the corresponding raw data in Appendix A, Table A4.

3.2. Microcrack propagation

Comparing the propagation of microcracks under our four different combinations of genipin treatments (Dose A was before impact, Dose B was before cyclic compression, cf. Fig. 1), we found that two doses of genipin caused significantly greater lengths and widths of propagated microcracks versus one dose, seen Fig. 3. Specifically, in comparing one dose of genipin before impact (Dose A) versus two doses (both Dose A and Dose B), microcracks propagated significantly by length (0.026) and width (0.030). Additionally, in comparing one dose of genipin before cyclic compression (Dose B) versus two doses (both Dose A and Dose B), microcracks propagated significantly only by width (0.034). Overall, one dose of genipin marginally decreased the change in the width of propagated microcracks compared to the untreated control, but these changes were not statistically significant. We saw no change in propagation by depth among any of the treatment combinations.
We summarize the statistical results in Table 3 and summarize the corresponding raw data in Appendix B, Table B5.

4. Discussion

In this study, we induced microscale damage (microcracks) to the network of collagen using low-energy impacts, and propagated the microcracks in unconfined, cyclic compression. Specimens treated with genipin presented significantly longer and (marginally) deeper microcracks after mechanical impacts. Specimens treated with two doses of genipin also presented significantly greater lengths and widths of propagated microcracks verses one dose. Microcracks compromise the load-bearing function of cartilage [7,19], likely by disrupting the ability of collagen to restrain densely-packed proteoglycan and retain fluid pressure. Continued propagation of microcracks under mechanical loads may create macroscale fissures that further deteriorate the mechanical function of cartilage. Cartilage degradation is associated with OA; however it is unclear whether microcracks are a precursor to OA. Nonetheless, mitigating, arresting, or even healing microcracks would provide insight towards understanding the mechanisms behind cartilage damage and repair.

Genipin, a cross-linker derived from plants, has received attention as a means to enhance mechanical properties of cartilage and tissue-engineered cartilage constructs [18,19], and as a potential repair for collagen damage [17]. Cartilage turns black in color when genipin reacts with the amino groups, and the color associates with oxygen radical-induced polymerization of genipin [28]. Its low cytotoxicity makes genipin an appealing option to stimulate structural changes that may improve outcomes after severe mechanical loading [17].

In this study, we aimed to evaluate the efficacy of genipin as a treatment against the initiation and propagation of microcracks. Our results do not support our hypothesis (that treatments with genipin improve the resistance of cartilage to microdamage, specifically reducing the initiation of microcracks under impact loading, and the subsequent propagation of microcracks within the network of collagen under cyclic, unconfined compression). Our statistical analyses produced some statistically significant results relating to both initiation and propagation of microcracks. Since there are significant results within our statistical analyses addressing both of these driving questions we are confident that where we didn’t find significance we did not commit a type 2 error. We determined the natural crosslinker genipin is not an effective treatment for preventing or repairing damage within the network of collagen, at least not in the treatments we tested.

| Measure      | p-value | Estimate |
|--------------|---------|----------|
| Density (#/mm²) | 0.905  | -0.170   |
| Length (μm)    | 0.001  | 108      |
| Width (μm)     | 0.442  | -1.37    |
| Depth (μm)     | 0.062  | 32.9     |
| Angle (°)      | 0.831  | 5.21     |
4.1. Microcrack initiation

We initiated microcracks using an impact-energy density of 2.5 mJ/mm², and a velocity of 0.5 m/s. At this impact-energy density, the probability of initiating microcracks in the network of collagen in human cartilage is approximately 40% [8]. The morphology of cracks initiated in this study matched those initiated at similar impact energy densities in prior work [9].

We aimed to improve the resistance of the collagen network to damage via increased cross-linking, however the resulting increase in stiffness reduced damage resistance. Genipin treatments to cartilage can significantly increase stiffness [18,19], e.g. in cartilage specimens treated with 10 mM for 24 h [19]. Considering genipin as a preventative treatment, adding genipin (11 mM) had no statistically significant effect on the total number of microcracks initiated under low-energy impacts. However, treatment with genipin did negatively change the morphology of the microcracks initiated, specifically in the length and depth of the resulting microcracks. Thus, the increased stiffness likely caused a reduction in ductility, which caused the cartilage to be less resistant to microdamage under impacts. Cross-linking may alter mechanisms of energy transfer and thus the ability to store energy (potentially to withstand greater loads) must be weighed against changes to the mechanisms of energy dissipation.

Genipin causes bonding between amino residues both between collagen fibrils (via intermolecular cross-linking), and within collagen fibrils (via intramolecular cross-linking) [18]. Damage mechanisms in collagen within cartilage include fibril breaking [29] and peeling [30]. The number of bonds induced by cross-linkers such as genipin may also depend on the collagen arrangement (fiber orientation) since increased cross-linking may restrain collagen from realigning to better withstand mechanical loads. The collagen microcracks that we measured resided principally within the superficial zone where fibers are relatively well-aligned and parallel to the articulating surface. For microcracks forming parallel to the split-line direction [9], breaks in the network of collagen likely occurred between fibrils. Since cross-linking with genipin did not mitigate the initiation of microcracks, the additional cross-links formed by genipin appear insufficient to prevent this microdamage. Perhaps cross-linking within the middle zone of cartilage would reinforce collagen to be more resistant to the initiation and propagation of microcracks.

4.2. Microcrack propagation

After initiating microcracks using impact loading, we propagated them using unconfined cyclic compression, a technique well-established in the literature [31–33], with an axial strain profile that simulated walking. Under these mechanical treatments and in the absence of genipin, microcracks typically propagated primarily by width and also by length, but not depth [9].

Bonitsky et al. [19] reported that impacted articular cartilage presented diminished wear resistance compared to undamaged cartilage; however, the decrease in wear resistance was completely reversed by cross-linking treatments with genipin [19]. Specifically, cartilage was worn at room temperature against 316L stainless steel within a pin-on-disk tribometer with a sliding velocity of 4 mm/s and an effective contact pressure of approximately 1.6 MPa (removed 45% of the total time to permit rehydration) [19]. We found that treatment with genipin did not mitigate propagation of microcracks via unconfined cyclic compression. These mechanical tests are fundamentally different, but do suggest that load rate and amplitude may affect the mechanical response of cartilage treated with genipin.

Changes in the propagation of microcracks due to treatments with genipin may result from changes in bulk stiffness or changes in the mechanisms of energy transfer within the network of cartilage. The significant increases in the propagation of microcracks reported here may relate to an inability both to store and to dissipate energy. It is also possible that increased stiffness from cross-linking restricts the realignment of networked collagen, preventing rearrangements that may better withstand loading.

4.2.1. Effects of number of treatments with genipin

We found no statistically significant difference in microcrack propagation between untreated specimens and specimens treated with one dose of genipin, either before impact (Dose A) or before cyclic compression (Dose B). Therefore, applying one preventative dose of genipin before mechanical treatments has no effect on the subsequent propagation of microcracks.

Specimens treated with two doses of genipin presented statistically significantly greater propagation of microcracks compared to one dose (either Dose A or Dose B alone). Cartilage specimens undergoing 12,000 cycles or unconfined cyclic compression at 1.44 Hz presented an increased effective stiffness due to compaction of the specimens [7]. Perhaps a combination of increased stiffness from compaction and alterations in structure and stiffness from multiple doses of genipin caused the relatively severe increases in propagation of microcracks we saw in the specimens treated with two doses of genipin. It is possible that the increased stiffness is coupled with a decrease in toughness since our data do not support our hypothesis that genipin (and additional doses) would improve the resistance of cartilage to microdamage, specifically reducing both the initiation and the propagation of microcracks within the network of collagen.

4.3. Limitations and outlook

We made every effort to balance our study across cows (three total) but this was difficult in practice. We found no statistically significant differences in mechanical responses among specimens from lateral versus medial condyles or from different cows (consistent with our prior results, cf. Santos et al. [34]) and thus we grouped them while controlling for cow as a random variable. Nonetheless the design of our study, which included a limited number of cows, likely created intra-class correlation among measures from the same cluster (see Table 1). Although our statistical models corrected for some of the correlation in our analyses, the study did have pseudo-replicates which may have induced correlations among data points that can lead to reduced variance and, in some cases, smaller than expected p-values [35]. Some specimens also failed during the course of the experiments which resulted in treatment groups with different sizes (n = 10–14).

Table 3
Summary of statistical results comparing microcracks propagated under unconfined, cyclic compression and our four different combinations of genipin treatments (Dose A was before impact, Dose B was before cyclic compression, cf. Fig. 1). To quantify the magnitudes of propagation of microcracks, we calculated the mean changes in length, width, and depth for each microcrack. We denote statistically significant differences (p < 0.05) with bold font.

| Measure | Length (μm) | Width (μm) | Depth (μm) |
|---------|-------------|------------|------------|
| Dose A  | ~ +         | +          | +          |
|         | 0.999       | 0.656      | 0.121      |
| Dose B  | +           | ~          | +          |
|         | 0.545       | 0.392      | 0.968      |
|         | +           | +          | ~ +        |
|         | 0.999       | 0.034      | 0.999      |
|         | +           | +          | + +        |
|         | 0.026       | 0.030      | 0.295      |

Bonitsky et al. [19] reported that impacted articular cartilage presented diminished wear resistance compared to undamaged cartilage; however, the decrease in wear resistance was completely reversed by cross-linking treatments with genipin [19]. Specifically, cartilage was worn at room temperature against 316L stainless steel within a pin-on-disk tribometer with a sliding velocity of 4 mm/s and an effective contact pressure of approximately 1.6 MPa (removed 45% of the total time to permit rehydration) [19]. We found that treatment with genipin did not mitigate propagation of microcracks via unconfined cyclic compression. These mechanical tests are fundamentally different, but do suggest that load rate and amplitude may affect the mechanical response of cartilage treated with genipin.

Changes in the propagation of microcracks due to treatments with genipin may result from changes in bulk stiffness or changes in the mechanisms of energy transfer within the network of cartilage. The significant increases in the propagation of microcracks reported here may relate to an inability both to store and to dissipate energy. It is also possible that increased stiffness from cross-linking restricts the realignment of networked collagen, preventing rearrangements that may better withstand loading.

4.2.1. Effects of number of treatments with genipin

We found no statistically significant difference in microcrack propagation between untreated specimens and specimens treated with one dose of genipin, either before impact (Dose A) or before cyclic compression (Dose B). Therefore, applying one preventative dose of genipin before mechanical treatments has no effect on the subsequent propagation of microcracks.

Specimens treated with two doses of genipin presented statistically significantly greater propagation of microcracks compared to one dose (either Dose A or Dose B alone). Cartilage specimens undergoing 12,000 cycles or unconfined cyclic compression at 1.44 Hz presented an increased effective stiffness due to compaction of the specimens [7]. Perhaps a combination of increased stiffness from compaction and alterations in structure and stiffness from multiple doses of genipin caused the relatively severe increases in propagation of microcracks we saw in the specimens treated with two doses of genipin. It is possible that the increased stiffness is coupled with a decrease in toughness since our data do not support our hypothesis that genipin (and additional doses) would improve the resistance of cartilage to microdamage, specifically reducing both the initiation and the propagation of microcracks within the network of collagen.

4.3. Limitations and outlook

We made every effort to balance our study across cows (three total) but this was difficult in practice. We found no statistically significant differences in mechanical responses among specimens from lateral versus medial condyles or from different cows (consistent with our prior results, cf. Santos et al. [34]) and thus we grouped them while controlling for cow as a random variable. Nonetheless the design of our study, which included a limited number of cows, likely created intra-class correlation among measures from the same cluster (see Table 1). Although our statistical models corrected for some of the correlation in our analyses, the study did have pseudo-replicates which may have induced correlations among data points that can lead to reduced variance and, in some cases, smaller than expected p-values [35]. Some specimens also failed during the course of the experiments which resulted in treatment groups with different sizes (n = 10–14).
We performed our experiments on cartilage explants and did not include cell culture nor any means to maintain cell viability. Our findings therefore only address the passive mechanical responses of the collagen network to external loads. Some cartilage specimens presented matrix damage at the free edges resulting from the extraction process. Thus we analyzed only a central region (centered on the main axis of the specimen) excluding the edges of the specimens to reduce edge effects [9].

Unfortunately treatments with genipin, and the resulting mechanisms of cross-linking, do not provide resistance to microdamage, quantified as the initiation and propagation of microcracks. Other available cross-linkers, such as carbodiimide or riboflavin/Ultraviolet-A may enhance the resistance of articular cartilage and collagen networks to microdamage.

Author contributions

SS contributed to conception and design; prepared specimens and conducted the experiments; analyzed and interpreted data; participated in drafting the article and revising it critically; and gave final approval of the version submitted. CPN contributed to conception and design; analyzed and interpreted data; participated in drafting the article and revising it critically; and gave final approval of the version submitted. JJG contributed to design; analyzed and interpreted data; participated in revising the article critically; and gave final approval of the version submitted. DMP oversaw the project; contributed to conception and design; analyzed and interpreted data; participated in drafting the article and revising it critically; and gave final approval of the version submitted.

Role of the funding source

The National Science Foundation and the Ford Fellowship Foundation had no involvement in the study design; in collection, analysis and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

Declaration of competing interest

We have no conflicts of interest to report.

Acknowledgments

This material is based upon work supported by the National Science Foundation under Grant Number (CAREER 1653 358) and the Ford Fellowship Foundation. We thank undergraduate research assistants Tiffany Addy, Avery Carroll, Margaret Daniel, Lauren Knapp, Annchi Li, Sophia Murphy, Millenia Polanco, Amanda Reid, Bryanna Samolyk, Guilmar Valle, Brianna Westenfeld, and Nayara Zainadine for assistance with imaging and analyses. We thank Chris O’Connell and Stephen Clow for assistance with the Zeiss LSM 510 microscope.

Appendix A

We summarize of our mechanical measurements on the initiation of microcracks in Table A4.

Table A4
Summary of mechanical measurements comparing microcracks initiated under low-energy impacts in untreated (Dose A: –) cartilage specimens and those treated with genipin (Dose A: +, 11 mM).

| Dose A | Measure     | Mean | SD   |
|--------|-------------|------|------|
| –      | Density (#/mm²) | 8.18 | 10.3 |
|        | Length (µm)   | 1.35 | 114  |
|        | Width (µm)    | 7.59 | 6.68 |
|        | Depth (µm)    | 37.5 | 30.1 |
|        | Angle (°)     | 38.3 | 24.7 |
| +      | Density (#/mm²) | 10.3 | 5.14 |
|        | Length (µm)   | 218  | 213  |
|        | Width (µm)    | 7.34 | 6.87 |
|        | Depth (µm)    | 54.6 | 49.7 |
|        | Angle (°)     | 45.7 | 15.4 |

Appendix B

We summarize our mechanical measurements on the propagation of microcracks in Table B5.

Table B5
Summary of mechanical measurements comparing microcracks propagated under unconfined cyclic compression and four different combinations of genipin treatments (Dose A was before impact, Dose B was before cyclic compression, cf. Fig. 1). To quantify the magnitudes of propagation of microcracks, we calculated the mean changes in length, width, and depth for each microcrack. SD = Standard Deviation, PI = Post Impact, PC = Post Cyclic Compression.

| Dose A | Dose B | Measure     | Phase | Mean | SD  |
|--------|--------|-------------|-------|------|-----|
| –      | –      | Length (µm) | PI    | 125  | 119 |
|        |        | Width (µm)  | PI    | 9.41 | 7.36|

(continued on next page)
### Table B5 (continued)

| Dose A | Dose B | Measure | Phase | Mean | SD |
|--------|--------|---------|-------|------|----|
|        |        | Depth (μm) | PC   | 13.1 | 22.4 |
|        |        |          | PI   | 36.2 | 28.5 |
|        |        |          | PC   | 32.5 | 35.1 |
|        |        | Length (μm) | PC   | 145  | 109 |
|        |        |          | PI   | 142  | 101 |
|        |        |          | PC   | 5.86 | 5.46 |
|        |        | Width (μm) | PC   | 7.41 | 16.9 |
|        |        |          | PI   | 38.7 | 31.8 |
|        |        |          | PC   | 29.8 | 20.6 |
|        |        | Depth (μm) | PC   | 108  | 143 |
|        |        |          | PI   | 141  | 171 |
|        |        |          | PC   | 3.85 | 2.01 |
|        |        |          | PI   | 4.32 | 2.56 |
|        |        |          | PC   | 28.3 | 20.3 |
|        |        |          | PC   | 35.7 | 26.0 |
|        |        | Length (μm) | PC   | 283  | 221 |
|        |        |          | PI   | 229  | 211 |
|        |        |          | PC   | 9.42 | 7.85 |
|        |        |          | PC   | 13.4 | 17.9 |
|        |        | Depth (μm) | PC   | 70.3 | 55.2 |
|        |        |          | PC   | 52.7 | 59.6 |

### References

1. R.A. Goes, L.R. Lopes, V.R.A. Cassich, V.A.R. de Miranda, O.N. Coelho, R.d.C. Bastosand, et al., Musculoskeletal injuries in athletes from five modalities: a cross-sectional study. BMC Musculoskel. Disord. 21 (2020) 122.
2. United States Bone and Joint Initiative, The Burden of Musculoskeletal Diseases in the united states (BMUS), fourth ed., 2020, 2021-05-12, https://www.boneandjointburden.org/fourth-edition.
3. U.R. Repo, J.B. Finlay, Survival of articular cartilage after controlled impact, J. Bone Joint Surg. Am. 59 (1977) 1–2.
4. A. Thambyah, V.W.P. Shim, L.M. Chong, V.S. Lee, Impact-induced Osteochondral Fracture in the Tibial Plateau, vol. 41, 2008, pp. 1236–1242.
5. F. Malekipour, C. Whitton, D. Oetomo, F.Y. Lee, Shock Absorbing Ability of Articular Cartilage and Subchondral Bone under Impact Compression, vol. 26, 2013, pp. 127–135.
6. J. Workman, A. Thambyah, N. Broom, The influence of early degenerative changes on the vulnerability of articular cartilage to impact-induced, Injury 43 (2010) 40–49.
7. J.T. Kaplan, C.P. Neu, H. Dristi, N.C. Emery, D.M. Pierce, Cyclic loading of human articular cartilage: the transition from compaction to, fatigue 65 (2017) 734–742.
8. B. Kalem, P. Maier, H. Dristi, D. Pierce, Low-energy impact of human cartilage: Predictors for microcracking the network of collagen, Osteoarthr. Cartil. 25 (2017) 544–553.
9. S. Santos, N. Emery, C.P. Neu, D.M. Pierce, Propagation of Microcracks in Collagen Networks of Cartilage under Mechanical Loads, vol. 27, 2019, pp. 1392–1402.
10. J. Workman, A. Thambyah, N. Broom, The influence of early degenerative changes on the vulnerability of articular cartilage to impact-induced, Injury 43 (2010) 40–49.
11. J.T. Kaplan, C.P. Neu, H. Dristi, N.C. Emery, D.M. Pierce, Cyclic loading of human articular cartilage: the transition from compaction to, fatigue 65 (2017) 734–742.
12. B. Kalem, P. Maier, H. Dristi, D. Pierce, Low-energy impact of human cartilage: Predictors for microcracking the network of collagen, Osteoarthr. Cartil. 25 (2017) 544–553.
13. J. Workman, A. Thambyah, N. Broom, The influence of early degenerative changes on the vulnerability of articular cartilage to impact-induced, Injury 43 (2010) 40–49.
14. J.T. Kaplan, C.P. Neu, H. Dristi, N.C. Emery, D.M. Pierce, Cyclic loading of human articular cartilage: the transition from compaction to, fatigue 65 (2017) 734–742.