Microcracks and Metabolism within Articular Cartilage During Mechanical Load

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Contrary to popular thought, the most detrimental issue plaguing the human body is not cancer or heart disease, but rather the symptom that connects them: pain. Physical pain limits the quality of life of more than 20% of adults around the world. Joint injury and acute trauma account for a large portion pain. The most common joint injuries include ankle sprains, ACL tears in the knee, Patellofemoral syndrome (injury resulting from the repetitive movement of the patella against the femur), and Tennis Elbow (Epicondylitis). There is strong evidence that suggest these injuries trigger a cascade of events that lead to the development of the disease post-traumatic osteoarthritis (PTOA). PTOA affects a multitude of join components, including bone, meniscus, and most prominently, the articular cartilage. Articular cartilage is a complex tissue, comprised of solid and liquid constituent that continuously interact with each other.

In this work, we combined \textit{ex vivo} mechanical experiments with imaging techniques to determine how mechanical impacts affect the structure and function of articular cartilage. In particular, we used low-energy single impact loads, often followed by cyclic loading that mimics walking, as mechanical loads to the articular surface. For the studies presented herein, we used three driving questions: (1) How do microcracks propagate through the network of collagen? (2) Can impact loading produce positive (anabolic) responses? (3) Can we prevent microcrack formation and/or propagation?

Broadly, we found:
1. The microcracks we initiated under low-energy impact loading increased in length and width during subsequent cyclic compression that simulated walking. The extent of this propagation depended on the combination of impact and cyclic compression.

2. Low-energy mechanical impacts generally stimulate time-dependent anabolic responses in the superficial zone of articular cartilage and catabolic responses in the middle and deep zones.
Microcracks and Metabolism within Articular Cartilage During Mechanical Load

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Approval Page

Doctor of Philosophy Dissertation

Microcracks and Metabolism within Articular Cartilage During Mechanical Load

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University of Connecticut
2020
Foreword and Acknowledgments

*Do your best, don’t settle for less.*

- Belky Santos (my mother)

In July 2020, a few weeks before my dissertation defense, a young woman who just finished her first year as an undergraduate student in engineering at UConn asked me while we were together in an elevator, “How do you stay sane with everything you do, and not get overwhelmed?” I laughed, while I let the gravity of her question sink in. Her question alluded to my reputation at the UConn School of Engineering, where I am known as: the “Queen of BRIDGE,” a summer program for underrepresented or marginalized incoming first year students to UConn Engineering; the “Founding Mother of Engineering Ambassadors,” an outreach program striving to close educational inequities and false perceptions about engineering and engineers in K-12 students and the greater community; a teacher, a mentor, a role model, a sister, and a friend to thousands of students, but particularly ones that associate with the Engineering Diversity and Outreach Center. All while pursuing my Ph.D. in Biomedical Engineering, mentoring 10-15 undergraduate researchers in the lab, and volunteering on numerous university and national committees. After mulling her question, I responded with something along the lines of:

*How you experience life depends on your perspective. Time can be a powerful motivator, manager, and oppressor. You can’t look at time as a whole, rather*
take it day by day. There may be days that are incredibly busy, seemingly impossible, and borderline overwhelming, but you have to realize that these times are only temporary. Instead, look at short term goals that you can look forward to. Not everything in life is permanent, but you are in control of you perceive and handle every moment and every situation.

Dear Hannah - thank you for making me think about something I never have before.

When I started this journey, I didn’t know what it meant to be a graduate student, or a Ph.D. student at that. Over the years I realized everyone’s paths are different, including the baggage, the hurdles, the opportunities, and the wind beneath their wings. I’d like to acknowledge mentors I’ve accumulated over the years, to whom I am eternally grateful for:

- **Dr. David M. Pierce**, my advisor and guiding light through all things professional and h-indexy. Thank you for your patience and for pushing my persistence.

- **Aida Ghiaei**, my champion, therapist, and luminary through all things personal development.

- **Christine Haas**, my maven merging the worlds of communication, science, and justice.

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- **Dr. Kelly Cross**, my luminary who brought me into the world of mixed methods research.

- **Dr. Jenny Amos**, my role model and advocate who lifts me into the world of Engineering Education.
• **Dr. Daniel Burkey**, my icon, who created a world for me that merged my passions with my pursuits.

• **Kevin McLaughlin**, my sage, who gave me new beginnings with every degree I pursued at UConn, and big shoes to fill ahead.

I would be nothing without you.

They say it takes a village to raise a child; well it takes a tribe to construct a doctor/professor. I am deeply indebted to the following humans and groups that I view as nothing less than royalty:

• **Danica Chin**, my right hand. Thank you so much for being there every single day since July 5, 2008.

• **Esther Kim Chang**, my rhyme and reason. I am so grateful for all you are and all you do.

• **Velda Alfred Abney**, my keeper of answers to almost any question.

• **Dr. Deborah Dorcemus**, the reason I am a biomedical engineer.

• **Pierre Fils**, who never fails to ask me about my headspace. Thank you.

• **Randi Mendes**, my emotional ace, who teaches me what it means to lead every day.

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• **Donyeil Hoy**, we were the only for so long. Now we pave paths. You inspire me.

• **Cherish Vance**, an unrelenting motivator to finish.

• **Phoebe Szarek**, who went from student, to mentee, to colleague to friend. It’s an honor to inspire kids alongside you.

• **All my labmates past and present, particularly Franz Maier**, there aren’t enough words to express my gratitude for your help and perspective.
• The Ford Fellowship Foundation and Family, for absolutely everything - the financing, the culture, and the inspiration.

• My BRIDGE and EDOC Family, including EA, NSBE (undergraduate and graduate), SHPE, and SWE, who day after day give me reason to wake up in the morning.

To my unbelievable, inquisitive and independent undergraduate assistants and leaders. WOW. None of this work would be possible without you. Thank you to: Tiffany Addy, Maria Antony, Victoria Blair, Avery Carroll, Lauren Contenta, Jenna Clum-Russell, Margaret Daniel, Gina Digiacomo, Jacob Grosso, Zinnia Hall, Hannah Kackley, Lauren Knapp, Emilio Loret de Mola, Annchi Li, Kathryn Morozov, Sophia Murphy, Jennifer Pires, Millenia Polanco, Amanda Reid, Kelsey Richard, Bryanna Samolyk, Bryan Tassavor, Guilmar Valle, Brianna Westenfield, Paige Woods, and Nayara Zainadine.

Thank you to my wonderful collaborators, for providing wisdom and merging worlds in new and exciting ways. Thank you Dr. Caroline Dealy, Dr. Bin Feng, Dr. Melanie Fisher, and Dr. Corey Neu.

Sincerest thanks to my dissertation committee, Dr. George Lykotrafitis, Dr. Kristin Morgan, and Dr. Anna Tarakanova.

To my family, thank you for standing by me and for your unconditional love.

Sinceramente, gracias.
To the love of my life, **Darnell McLeod**.

Thank you for saving me. Thank you for lifting me out of the depths of mental despair.
Thank you for listening, and teaching me to listen.
Thank you for speaking, and teaching me to be open.
Thank you for being my motivation to push hard.
Thank you for creating time and space that became my reward after the push.
Thank you for staying up with me at all hours of the day and night, to make sure I stayed safe as I left the lab.

Thank you for your daily reminder and insistence that I am a human first, and employee/\-student second.
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1 Introduction

1.1 Motivation

The human race is plagued with mobility issues, all of which are due to one or a combination of environmental factors, body chemistry and genetics, acute injury, disparities in access to health care professionals, and systemic injustices that affect daily and generational life.

One in five adults experience some form of painful physical limitation. The prevalence of disability tends to double in successive age groups (Brault et al. 2009, Ramos-Pichardo et al. 2014). With respect to older populations, the World Health Organization reports up to 76% of adults over the age of 65 report difficulty in walking one kilometer (Capistrant et al. 2014). The knee, the most injury prone joint in the human body, often induces this gait discomfort and pain.

The issue with pain is that it often presents as a symptom too late. Eighty-nine percent of people with asymptomatic knees present evidence of at least one structural abnormality, and 50% present significant cartilage deterioration (Walczak et al. 2012). Asymptomatic abnormalities are concerning because they not only can cause subconscious changes in movement mechanics (i.e. antalgic gait: walking pattern that develops as a way to avoid pain while walking (Vezzetti and Bordoni 2018)), but if left untreated, can cascade into detrimental diseases such as osteoarthritis (Brunt et al. 2016).

Osteoarthritis (OA) is a complex disease that affects multiple facets in a joint, but the
hallmark and root cause of a majority of the pain is cartilage degeneration (Atkinson et al. 1998a, Peat et al. 2001, Logerstedt et al. 2010). Since Dr. John Kent Spender identified and coined the term osteoarthritis in 1886, doctors, scientists, and engineers synergized approaches to attack, cure, and prevent the disease. Unfortunately, OA is a complicated disease, with solutions remaining elusive on the horizon in 2020, in part due to the complexity of cartilage’s response to external stimuli.

Cartilage responds to both chemical and physical changes in the body. Stimuli such as limb motion or injury induce a cascade of changes that ultimately lead to degradation of the extracellular matrix and its solid constituents (Buckwalter 1997). Additionally, biochemical changes triggered by mechanical stimuli often influence signaling pathways, which may lead to changes in presence or activity of the proteins necessary for normal joint function. These chemical and physical responses are not separate, rather they continuously influence and inform each other, leading to a vicious cycle ending in severe OA (Ewers et al. 2001, Julkunen et al. 2013). To better understand the vicious cycle of destructive biochemical and physical changes, it is essential to understand the tissue’s function in the body, considering both the ultrastructure and microstructure, and how the structure and function relate to each other and contribute to joint behavior. This will contribute to improved diagnostic tools and methods, as well as prognostic insight for potential disease prevention and care.

1.2 Structure and Function of Cartilage

Cartilage is heterogeneous on several accords. Approximately 70-85% of the weight of the tissue is water and electrolytes (fluid component), with the remainder comprising of the matrix proteins proteoglycans and collagen (solid components) (Mow et al. 2005, Buckwalter and Martin 2006, Julkunen et al. 2013). These constituents reside in a poroelastic medium with a dynamic composition. Due to its composition and its biomechanical behavior, some regard articular cartilage as “an inert hyperhydrated hydrogel” in nature
Collagen, a fibrous protein, exists in over 16 forms. In articular cartilage, it is primarily types I, II, and III (Gelse et al. 2003). Collagen fiber arrangement varies through the thickness of the tissue. This contributes to the anisotropic behavior. Cartilage can be divided into four major zones between the articular surface and the subchondral bone: Superficial/Tangential, Intermediate/Middle, Deep/Radiate, and Calcified Zones, as seen in Fig. 1.1 B. In the superficial zone, cartilage fibers tend to orient themselves parallel to the articular surface.

A simple way to elucidate the surface fiber direction is by determining the split-line direction, as depicted in Fig. 1.2, and Fig. 1.3. Upon pricking the surface with a circular tool such as a needle, an ellipsoidal hole transpires, confirming the general direction of the cartilage fibers. Split-lines can vary across the articular surface and among the joint and condyle locations.

In the intermediate zone, collagen fibers adjust radially, and begin to randomly interweave and disperse isotropically. In the deep zone, the fibers align themselves in a near-perpendicular fashion with respect to the subchondral bone surface. In addition to orientation shifts, the individual fiber thickness also varies with depth, such that the thinnest fibers are in the superficial zone, and exhibit increased thickness in the deeper zones (Mansour 2003, Julkunen et al. 2013).

Considering the cellular level, chondrocytes are cells unique to articular cartilage. The differentiation process of mesenchymal cells into chondrocytes, is vividly apparent in the tissue structure, with newly differentiated chondrocytes near the superficial zone surface, and fully mature chondrocytes lining the calcified region. In addition to chondrocytes, another cell type, chondroprogenitors, also respond and influence the overall tissue response. These are resident mesenchymal stem or progenitor cells that are capable of differentiating into chondrocytes when needed (Jayasuriya and Chen 2015). Previously, the terms chon-
4 1 Introduction

Figure 1.1: Cartilage structural morphology. (A) Chondrocytes organization in articular cartilage, and the three major uncalcified zones: superficial tangential zone (STZ), middle zone, and deep zone. (B) Collagen fiber orientation and alignment (Reproduced from Buckwalter et al. (1994)).

drocyte and chondroprogenitors were used interchangeably; however, recent work shows these are distinct cell populations that can be identified with surface cell markers (Jayasuriya and Chen 2015).

Nonetheless, cells are usually densely packed and relatively flat at the articular surface. They then shift towards a columnar orientation perpendicular to the calcified surface, as in Fig. 1.1 A. These ovoid chondrocytes form and maintain the fluid extracellular matrix of cartilage. The solid, fibrous pericellular matrix surrounding the chondrocytes help form the lacunae that is several microns long and is clearly visible in histological staining.

Cartilage is a type of connective tissue that is found in various areas of the body. Broadly, there are three types of cartilage: Elastic, Hyaline and Fibrous. Elastic cartilage, the only type to contain elastin, provides flexible support and shape for various bodily structures, most notably the ear pinnae. Hyaline cartilage serves as a flexible yet firm protective agent on many interfaces, such as the rib cage, trachea, nose, and at joint articulations. Fibrocartilage is comprised of both flexible and rigid constituents, and provides stronger,

In the knee, AC covers the surfaces of the femoral condyles, tibial plateau, and the posterior face of the patella, as in Fig. 1.4, averaging a thickness of approximately $2 - 2.5\text{ mm}$ on average in humans, though the thickness varies throughout the surface up the tissue, as seen in Fig. 1.5 (Frisbie et al. 2006). Additionally, fibrocartilage -namely the menisci- serves as a “deep-dish socket” support on the tibial plateaus to provide a seat for the interfacing femur. The menisci are secured by a direct connection to capsular ligaments (Blackburn and Craig 1980). Therefore, to define function, the articular cartilage protects and lubricates, and the menisci provide further stability by holding the condyle in place.
1.3 Mechanical Testing of Cartilage

Mechanical testing of cartilage is not new, yet we still do not know the mechanism behind damage initiation, propagation, and repair. Early experiments investigating damage initiation showed large mechanical insults in the form of impact loading causing compactions between 5-15% of the overall thickness produced large macroscale fissures in the articular surface, as shown by SEM in Fig. 1.6. Cell death also accompanied fissure formation.

Cyclic loading also initiates damage on the articular surface. In one early experiment, SEM shows large scale crack formation after 1 Hz cyclic loading (Fig. 1.7. The average human walks at 1.44 Hz. This experiment also shows crack formation not only occurs in vitro in the laboratory setting, but also in situ.

Kerin et al. (2003) mechanically initiated macroscale fissures ex vivo on the articular surface, and propagated cracks at a slow rate (0.5 Hz). These fissures spanned lengths of 3-4 mm, which are easily viewed with the human eye. With improved technology and imag-
Figure 1.4: Human knee anatomy. (A) Anterior view. (B) Posterior view (Adapted from Stannard and Luck (2015))

...resolution, researchers can view smaller fissures in the articular surface. Malekipour et al. (2013) viewed small fissures with lengths spanning 1-2 mm and resolutions of 9 $\mu$m induced by cyclic loading (Fig. 1.8). Recently, Kaleem et al. (2017) was the first induce microcracks (fissures less than the width of a chondrocyte lacunae ($< 30 \mu m$)) in the network of collagen using low-energy impacts (Fig. 1.9). Much of the work in this thesis are based on low-energy impacts.
Figure 1.5: Human femoral head cartilage thickness map. (A) Normal (B) OA (Adapted from Carballido-Gamio et al. (2008))

Figure 1.6: Fissures in explants after in vitro loading via SEM (Reproduced from Repo and Finlay (1977))
1.3 Mechanical Testing of Cartilage

Figure 1.7: Fissures in explants after in vivo loading via SEM (Reproduced from Dekel and Weissman (1978))

Figure 1.8: Small fissures with lengths spanning 1-2 mm (Reproduced from Malekipour et al. (2013))
Figure 1.9: Microcracks induced by low-energy impacts (Reproduced from Kaleem et al. (2017))
1.4 Overview of Research Questions and Thesis Structure

We deduced the following observations from the literature:

1. Impact loading affects the collagen network and chondrocytes.

2. Impact loading may induce macro and microscale fissures that are detectable via imaging.

3. Chondrocytes’ mechanotransductive response is primarily cell death and changes in gene expression.

This lead to the following driving questions:

1. How do microcracks propagate through the network of collagen?

2. Can impact loading produce positive (anabolic) responses?

3. Can we prevent microcrack formation and/or propagation?

This thesis is structured such that each driving question is published or pending manuscript. The text from the manuscript is included in this dissertation as its own chapter. The final chapter of this dissertation includes a research outlook and future experiments.
2 How do microcracks propagate through the network of collagen?

This chapter is published as Santos et al. (2017).

2.1 Abstract

Objective We recently demonstrated that low-energy mechanical impact to articular cartilage, usually considered non-injurious, can in fact cause microscale cracks (widths $< 30 \mu m$) in the collagen network of visually pristine human cartilage. While research on macro-scale cracks in cartilage and microcracks in bone abounds, how microcracks within cartilage initiate and propagate remains unknown. We quantified the extent to which microcracks initiate and propagate in the collagen network during mechanical loading representative of normal activities.

Design We tested 76 full-thickness, cylindrical osteochondral plugs. We imaged untreated specimens (pristine phase) via second harmonic generation and assigned specimens to three low-energy impact groups (none, low, high), and thereafter to three cyclic compression groups (none, low, high) which simulate walking. We re-imaged specimens in the post-impact and post-cyclic compression phases to identify and track microcracks.

Results Microcracks in the network of collagen did not present in untreated controls
but did initiate and propagate under mechanical treatments. We found that the length and width of microcracks increased from post-impact to post-cyclic compression in tracked microcracks, but neither depth nor angle presented statistically significant differences.

**Conclusions** The microcracks we initiated under low-energy impact loading increased in length and width during subsequent cyclic compression that simulated walking. The extent of this propagation depended on the combination of impact and cyclic compression. More broadly, the initiation and propagation of microcracks may characterize pathogenesis of osteoarthritis, and may suggest therapeutic targets for future studies.

### 2.2 Introduction

Low-energy impacts to articular cartilage may initiate microcracks in the network of collagen (Kaleem et al. 2017), which may be one of the earliest (i.e. pre-clinical) detrimental changes to cartilage. Such changes may contribute to osteoarthritis (OA), a leading cause of disability in adults. Clinical OA, characterized by the weakening and loss of cartilage, afflicts over 26.9 million people in the US alone (Lawrence et al. 2008), leading to pain, disability, and total joint replacement. Following initiation of microcracks, repetitive mechanical loads to cartilage during normal daily activities may propagate these microcracks (within the extracellular matrix) and initiate the cascade of degeneration leading to OA. The extent of microcrack propagation during repetitive mechanical loads to cartilage during normal daily activities, however, is unknown.

The human knee is the largest joint in the body, and one of the most susceptible to injury (Peña et al. 2007). Within the knee, acute or chronic damage may occur in the ligaments and tendons, as well as in the cartilage and bone, possibly resulting in post-traumatic osteoarthritis (OA) (Buckwalter et al. 2005, Workman et al. 2017). While microcracks in bone have been characterized extensively (Burr et al. 1985, Zioupos et al. 1996, Martin 2002, Landrigan et al. 2011, Agcaoglu and Akkus 2013), and sub-millimeter-scale surface
fissures in cartilage are well known for early to advanced osteoarthritis (OA) (Finlay and Repo 1978, Atkinson et al. 1998b, Duda et al. 2001), we recently demonstrated that low-energy impact usually considered non-injurious can in fact cause micrometer-scale cracks (microcracks) in the collagen network of human cartilage (Kaleem et al. 2017). In previous work we defined collagen-network microcracks as fractures in the collagen network that are no wider than the diameter of chondrocyte lacunae (< 30 µm) (Clarke 1971, 1972). Furthermore, we probed the diminished functional response of cartilage under progressive cyclic loading (Kaplan et al. 2017b) and found statistically significant microcracking under some loading conditions.

Using second harmonic generation imaging (SHG) via confocal microscopy, we previously visualized micro-scale mechanical damage to the network of collagen that we initiated from low-energy impact. Other researchers applied compression (Novakofski et al. 2014), tension (Mansfield et al. 2015), or surgically induced injury (Kiyomatsu et al. 2015) and used 2-D or 3-D SHG visualize (but never quantify) microcracks in the network of collagen (referred to as microcracks, micro-splits, and micro-wrinkles). Microcracks also present in vivo, as seen by us and others, e.g. in mice with surgically induced OA (Kiyomatsu et al. 2015) and in ICRS Grade-I human femoral cartilage (Brittberg et al. 2000, Kumar et al. 2015).

In this study, we aimed to determine: (1) what combinations of impact and cyclic compression initiate microcracks in the network of collagen; and (2) whether and how cartilage microcracks propagate during cyclic, mechanical loading which simulates walking. Understanding these aims contribute to understanding the initial mechanisms of microscale damage in the network of collagen that may be a precursor to degradation characteristic of OA.

To these ends, we initiated microcracks in the network of collagen in cartilage explants using low-energy mechanical impacts, and tracked the propagation of microcracks after cyclic compression simulating 12,000 walking strides, approximately equivalent to com-
pleting a half marathon paced at 13-15 minutes per mile (Hoeger et al. 2008). Using 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.

2.3 Materials and Methods

In total we tested 7 full-thickness, cylindrical osteochondral plugs (specimens). We separated specimens from the lateral and medial femoral condyles, and assigned them to one of three different impact groups (none, low, high), with impact energy density as the independent variable, and thereafter one of three different cyclic compression groups (none, low, high) which simulate 12,000 walking strides. We also performed SHG imaging (Carl Zeiss LSM 510 or Nikon FN1) at three experimental phases (pristine, post-impact, and post-cyclic compression). In Fig. 2.1 we show a summary of the treatment groups. In total, we had seven different treatment groups, where one control group received neither impact nor cyclic compression, two control groups received only cyclic compression, and the remaining four groups received both impact and cyclic compression.

<table>
<thead>
<tr>
<th>Low-Energy Impact</th>
<th>Cyclic Compression</th>
</tr>
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<tbody>
<tr>
<td><strong>NC</strong></td>
<td><strong>LC</strong></td>
</tr>
<tr>
<td>n = 4</td>
<td>n = 12</td>
</tr>
</tbody>
</table>

Figure 2.1: Summary of 76 specimens separated into treatment groups, with specimens from the medial and lateral condyles pooled together. Low-Energy Impact: no impact--NI, low impact--LI (1.5-2.5 mJ/mm$^3$), high impact--HI (2.6-4.0 mJ/mm$^3$). Cyclic Compression: no compression--NC, low compression--LC (10%), high compression--HC (15%).
2.3.1 Preparation of Specimens

We received full bovine knees from five skeletally mature animals (18-30 months) packed on ice (Animal Technologies, Inc., Tyler, TX). We carefully exposed and identified load-bearing and visibly pristine regions on both the lateral and medial femoral condyles, and determined the local split-line direction along these surfaces (Athanasiou et al. 1991, Neu et al. 2007). We then extracted cylindrical specimens (3 mm diameter, full thickness) using a circular punch while keeping track of the local split-line direction. 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 (Schindelin et al. 2012). To store specimens not immediately tested, we immersed them in Phosphate Buffered Saline (PBS, pH 7.4) and stored them at -80°C (Athanasiou et al. 1991, Szarko et al. 2010). On the day of testing we thawed the specimens and mounted them to custom, ultra-wear-resistant nylon platens using cyanoacrylate adhesive for subsequent imaging and mechanical testing.

2.3.2 Mechanical Tests

Low-Energy Impact

We impacted the articular surface of unconfined pristine specimens using a custom drop tower with a polished, flat metal impactor (diameter much greater than 3 mm). We separated specimens from the lateral and medial femoral condyles and randomly assigned those specimens to three different impact groups (no impact-NI, low impact-LI, high impact-HI). Based on preliminary studies, we selected $LI = 1.5-2.5$ mJ/mm$^3$ and $HI = 2.5-4.0$ mJ/mm$^3$. Based on our previous work (Kaleem et al. 2017), we selected the intended impact velocity $v_{imp}^* = 0.5$ m/s. We calculated the required drop height $h = (v_{imp}^*)^2/2g$, with $g$ as gravitational acceleration. We then calculated the required total drop mass $m$ to achieve the
intended impact energy density $E_{imp}^{*}$ using

$$E_{imp}^{*} = \frac{mgh}{V},$$

(2.1)

where $V$ is the volume of the specimen.

During the test we measured the acceleration ($\pm 49000 \text{ m/s}^2$; 350A14, PCB Piezotronics, Depew, NY) and the force (22.24 kN; 200B05, PCB Piezotronics) at 100,000 Hz sampling rate. After the test, we integrated the acceleration once to determine the actual velocity at the moment of impact $v_{imp}$, and used this to calculate the actual the impact energy density ($E_{imp}$) applied to each specimen using

$$E_{imp} = \frac{mv_{imp}^2}{2V}.$$  

(2.2)

For the control group ($NI$ in Fig. 2.1), specimens rested in PBS for the duration of the test. Post-impact, we submerged the specimens in PBS at 37°C for at least one hour to equilibrate prior to subsequent imaging and mechanical testing (Kaleem et al. 2017).

**Cyclic, Unconfined Compression**

Post-impact, we conducted unconfined cyclic compression tests, a technique well-established in the literature (Mow et al. 1999, Korhonen et al. 2002, Park et al. 2004), using a Bose LM1 Electroforce linear motor with WinTest 7 software (Bose, Eden Prairie, MN). First, we submerged the tissue in PBS solution at 37°C and maintained force-controlled 0.2 N compression for 3000 seconds. We then applied a pattern of cyclic compression including 0.69 sec sinusoidal compression followed by 0.67 sec recovery (total cycle time equals 1.36 sec or 0.74 Hz), cf. Zhang et al. (2015). The amplitude of cyclic compression was either low compression – $LC = 10\%$ or high compression – $HC = 15\%$ of the cartilage thickness measured prior to impact testing, cf. Liu et al. (2010), Harkey et al. (2017). For the control group (high compression – $NC$ in Fig. 2.1), specimens rested in PBS for the
duration of the test. Post-cyclic compression, we submerged the specimens in PBS at 37°C for at least one hour prior to subsequent imaging (Kaleem et al. 2017).

2.3.3 Images via Second Harmonic Generation (SHG)

We performed SHG imaging (Zeiss LSM 510, Oberkochen, DE or Nikon FN1, Tokyo, JP) at three separate experimental phases (pristine–\(P\), post-impact–\(PI\), and post-cyclic compression–\(PC\)). We used tunable Ti: Sapphire lasers (Zeiss: Coherent Chameleon, Santa Clara, CA or Nikon: Spectra Physics, Santa Clara, CA) at 850 nm for excitation of the nonlinear signal. We acquired the signals in non-descanned detection using a specialized filter (Zeiss: 425 ± 13 nm band-pass filter; FF01-425/26-25, Semrock, Rochester, NY or Nikon: 492 blocking edge short pass filter; FF01-492/SP-25, Semrock). For all images from the Zeiss, we used a water-immersion objective (W Plan-Apochromat 20x/1.0) and a 600 × 600 \(\mu\)m (512 × 512 pixel) field of view. For all images from the Nikon, we used a water-immersion objective (CFI75 Apochromat LWD 25x MP) and a 516 × 516 \(\mu\)m (1024 × 1024 pixel) field of view. For each specimen, we acquired a 7 × 7 tile grid (100 \(\mu\)m 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 (slice increment of 2.5 \(\mu\)m) from a 3 × 3 tile grid centered on the articular surface (to avoid edge effects). We acquired image stacks 50 \(\mu\)m into the specimen measured from the articular surface and scanned for microcracks. We stopped imaging if we found no microcracks. If we identified microcracks, we continued imaging up to 200 \(\mu\)m into the specimen (the approximate focal length of the microscopes). We also mounted each specimen to keep the placement, and thus orientation of the images with respect to the split-line direction, consistent.
2.3.4 Analyses of Images

We stitched together our SHG images using Fiji’s Grid/Collection Stitching Plugin (Preibisch et al. 2009) for ImageJ (National Institutes of Health, Bethesda, MD) to generate images of the full circular cross section at a resolution of 1.2 \( \mu m/pixel \) (Zeiss) or 0.50 \( \mu m/pixel \) (Nikon). In SHG, collagen fibers/fibrils create the strongest SHG signals (He et al. 2014). The collagen signal was white (Zeiss) or blue (Nikon), and we identified microcracks as absence of SHG signal (black) (He et al. 2014, Kaleem et al. 2017). Using only the \( 3 \times 3 \) tile grid centered on the articular surface (Zeiss: \( 3618.8 \times 3618.8 \mu m, 3093 \times 3093 \) pixels; Nikon: \( 3092.5 \times 3092.5 \mu m, 6185 \times 6185 \) pixels), independent observers measured the length, width, and principal angle (relative to the split-line direction) of each microcrack in each image (parallel to the articular surface) manually, using the measurement tools in Fiji. To determine the microcrack depth, each observer first determined the height of the articular surface (defined as the axial position when 50% of surface was visible/non-black in each individual image, and then followed each microcrack axially through the image stacks (slice interval of 2.5 \( \mu m \)) until each disappeared. We recorded these data overall in the pristine, post-impact, and post-cyclic compression phases of the experiment. Additionally, where we could positively track individual microcracks by feature matching from the post-impact to the post-cyclic compression phases of the experiments, we recorded the specific morphology and orientation of each tracked microcrack as a subset of the overall data.

To validate inter-observer agreement in our measured data, we calculated the Cohen’s kappa coefficient \( \kappa \), a 95% confidence interval, and the inter-rater reliability using SAS 9.4 (SAS Institute, Inc., Cary, NC). We also verified inter-observer reliability by comparing the means using a \( t \)-test with \( p < 0.05 \) to determine significant differences among observers. If we found agreements greater than 75% from two independent observers, we averaged these results (McHugh 2012, Tavakoli et al. 2012). If we found practically significant differences between observers, we included a third independent observer and averaged the two sets of
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We 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. For each specimen, we also calculated the overall microcrack area density using the total number of microcracks in the $3 \times 3$ tile grid centered on the articular surface.

2.3.5 Statistical Analyses

We used separate mixed-model ANOVAs to evaluate the effects of impact and cyclic compression on microcrack density, and on the length, width, depth, and angle of microcracks. We included condyle (medial or lateral), impact level (low or high), cyclic compression level (10% or 15%), and phase (post impact or post cyclic compression) as fixed effects, specimen as a random effect, and the thickness of each cartilage specimen as a covariate. We also included all two-way interactions between impact, cyclic compression, and phase, as well as the interaction between sample thickness and impact level.

Prior to analysis, we $\log(x+1)$ transformed mean microcrack density, width, and depth, and $\sqrt(x+1)$ transformed mean microcrack length, to meet the assumptions of ANOVA. Even after transformation, the data for mean microcrack length failed to meet the assumption of normality of residuals due to two outliers. We tested if the outliers were driving our results for mean microcrack length by running the model with and without the outliers included. Removing the outliers did not alter the significance of any variables in the model; for brevity, we present only the results of the analysis using the full data set (including the outliers). We used post-hoc tests to evaluate differences among treatment combinations for any statistically significant interactions between fixed effects. Finally, we used separate simple regressions to investigate interactions that we identified as statistically significant between specimen thickness and each impact level for mean microcrack width. This involved generating the residuals from a reduced, mixed-model ANOVA that did not include thickness or the thickness $\times$ impact level interaction, and then testing for
2.4 Results

We analyzed the subset of our data where we tracked individual cartilage microcracks over the course of the experiment using the same mixed-model ANOVA, but with specimen and microcrack included as additional random factors (to account for non-independence at those scales), and the three-way interaction among impact, cyclic compression, and phase. Prior to analysis, we log($x + 1$) transformed mean length, width, and depth of the tracked microcracks to meet the assumptions of ANOVA. Data for the orientation of the tracked microcracks met the assumptions of ANOVA without transformation.

2.4 Results

We completed mechanical treatments and imaging on a total of 76 specimens including controls ($NI$, $NC$ in Fig. 2.1). We did not find any microcracks in the untreated controls. We found no statistically significant differences between lateral and medial condyles, so we pooled data from these two groups to increase our statistical power. We successfully initiated microcracks in verified, visibly pristine cartilage, and propagated the microcracks under cyclic compression (Fig. 2.2).

We found statistically significant differences in the density, length, width, depth, and angle of microcracks depending on the combinations of mechanical treatments (Table 2.1, Appendix A and Table 2.2, Appendix B). We found no microcracks in any of our control specimens (column $NI$ in Table 2.1), nor any microcracks in our pristine specimens prior to impact treatments (row $P$).

Under our loading combinations, we found that when microcracks form, they most likely present with the following morphologies: lengths < 40 $\mu$m, widths < 20 $\mu$m, depths < 30 $\mu$m, and angles either $0^\circ$ or $45^\circ$ to the split-line direction (Fig. 2.6, Appendix C).
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Figure 2.2: Representative SHG images of a control specimen (a-c) resting in PBS for the duration of the mechanical tests, and of two mechanically treated specimens (d-i) presenting microcracks: (d, g) pristine; (e, h) post impact at 2.5-4.0 mJ/mm²; and (f, i) post-cyclic compression at 10% and 15% respectively. We co-registered images for each specimen using relative coordinate mapping and feature matching.
2.4 Results

2.4.1 Microcrack Initiation

We initiated microcracks during low-energy impacts, and both initiated and propagated microcracks during unconfined cyclic compressions. We found no statistically significant differences in median microcrack densities, lengths, or depths in our controls, and under our combinations of impact and cyclic-compression treatments (Fig. 2.3). We did find statistically significant larger median angles (from split-line direction) of microcracks in specimens from the high-impact compared to the low-impact treatment group at 15% cyclic compression ($p = 0.0252$).

Median microcrack widths were: (1) larger for those initiated at high impacts than for those initiated at low impacts overall ($p = 0.0213$), (2) larger for those initiated at high impacts than for those initiated at low impacts and subsequently undergoing 10% cyclic compression ($p = 0.0252$), and (3) smaller for those undergoing 10% as compared to 15% cyclic compression after high impacts ($p = 0.0398$) (Fig. 2.4). We found that log microcrack width correlated with cartilage thickness under high ($\log(W + 1) = 0.983T + 1.23, p = 0.0025$) but not low impacts.

2.4.2 Microcrack Propagation

In tracked microcracks, the main effect of phase (post-impact versus post-cyclic compression) showed microcrack lengths and widths measured post cyclic compression were statistically significantly greater than those measured post impact ($p < 0.0001$). Regarding length specifically, the interaction between level of cyclic-compression and level of impact was also statistically significant ($p = 0.018$), but we did not pursue this with post-hoc tests. Regarding the width of tracked microcracks, two interactions with impact level were significantly different in our post-hoc tests: impact $\times$ cyclic compression ($p = 0.025$) and impact $\times$ phase ($p = 0.043$). To further probe these interactions, we ran separate models for each impact (thus dropping terms or interactions containing impact). These
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Figure 2.3: There are no statistically significant differences in densities, lengths, or depths of cartilage microcracks under different combinations of impact and cyclic compression treatments. Plot shows distributions in (a) density, (b) length, (c) depth, and (d) angle (from the split-line direction) of cartilage microcracks observed after no impact (left column), low impact (middle column), or high impact (right column) and one of two cyclic compression treatments. Control specimens (no impact/no cyclic compression) presented no microcracks in any specimens (not shown). Here we show raw data. We determined statistical significance using mixed-model ANOVAs for each response variable. We transformed these data prior to analyses as described in Section 4.3.6.
Figure 2.4: We found statistically significant, large differences in widths of cartilage microcracks under different combinations of impact and cyclic compression treatments (see Table 2.2 for statistical results). Plot (a) shows distributions in width of cartilage microcracks observed after no impact (left column), low impact (middle column), or high impact (right column) and one of two cyclic compression treatments. Control specimens (no impact/no cyclic compression) presented no microcracks in any specimens (not shown). Plot (b) shows log microcrack width versus cartilage thickness for both low and high impact treatments. Here we show raw data unless indicated otherwise. We determined statistical significance using mixed-model ANOVAs with 47 degrees of freedom for each response variable. We transformed these data prior to analyses as described in Section 4.3.6.
analyses found that after high impact, widths grew from the post-impact phase to the post-cyclic compression phase. Additionally, we found statistically significantly larger widths after 10% cyclic compression than after 15%, but only in the low-impact treatment group (Fig. 2.5).

We found no statistically significant differences in the depth of tracked cartilage microcracks between post impact and post-cyclic compression, but did find different combinations of impact and cyclic compression influenced the microcrack depth ($p = 0.014$). Similarly, we did not see statistically significant changes in microcrack angle (with respect to the split-line direction) from post impact to post-cyclic compression, but did find different combinations of impact and cyclic compression influenced the angle of microcracks ($p = 0.020$).
2.5 Discussion

Regular mechanical loading is one of the most important environmental factors in maintaining cartilage and joint health, but severe loading can also have degenerative effects that influence the development of OA (Buckwalter et al. 2005). Because cartilage undergoes various combinations of mechanical loading \textit{in vivo}, mechanical analyses of cartilage should employ similarly complex loading treatments.

We were the first to successfully identify and track progression of individual microcracks in the collagen networks of cartilage \textit{in-vitro} mechanical loading while maintaining the full thickness of cartilage. In this study we determined combinations of impact and cyclic compression that affect the propagation of microcracks.

In articular cartilage, covalent cross-links among fibrils and fibers stabilize the network of collagen (Gelse et al. 2003). These cross-links predominantly connect telopeptides of adjacent molecules (von der Mark 1999). There are several damage mechanisms within articular cartilage that may contribute to OA, including breaking (Henzgen et al. 1996, Andriacchi et al. 2004) and peeling of collagen fibrils (Lewis and Johnson 2001). Bonitsky et al. (2017) found they could not repair (macro-scale) fissures with cross-linking treatments. Given the significance of damage to the collagen network within cartilage, understanding the initiation and propagation of microcracks could provide essential insight into the initiation of OA.

2.5.1 Microcrack Initiation

We initiated microcracks using relatively low impact energy densities of $1.5 - 4 \text{ mJ/mm}^3$ (approximately $0.042 - 0.11\text{ J}$) given the average dimensions of our specimens. Rapid impact loading leads to tensile stresses within the collagen fibers and renders them susceptible to rupture (Kafka 2002). Larger impact energies yielded microcracks with overall greater initial microcrack widths. Duda et al. (2001) impacted fully intact porcine patella
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with comparably low impact energies (0.06 – 0.2 J) and did not find fissures using electron microscopy, but did find damage to chondrocytes. Using SHG, we were able to see microcracks in the network of collagen at comparable (or lower) impact energies. These differences may be due to differences in experimental design, species and/or location of tissue, and/or imaging modality. In some cases, we also initiated microcracks during cyclic compression. Larger tensile stresses within the compromised collagen network may then cause the formation of new microcracks (Workman et al. 2017).

Based on preliminary studies, we initiated our microcracks at an impact velocity of 0.5 m/s. Kaleem et al. (2017) initiated cartilage microcracks with impact velocities ranging from 0.75 m/s to 1.0 m/s and impact energies in the range of 0.05 – 0.09 J, but did not include cyclic compression among their mechanical treatments. With these impact treatments, they determined that impact energy densities of approximately 1.5 – 3 mJ/mm³ initiate microcracks in human cartilage, and that increasing impact velocity did not correlate with increasing occurrence of microcracks.

We found that cartilage microcracks can form at various angles ranging from parallel to perpendicular to the split-line direction; however the majority of microcracks tended to form either parallel to the split-line direction or at 45° to it. Microcracks forming parallel to the split-line direction may indicate voids between aligned collagen fibers forming as cross-links rupture (Gelse et al. 2003). Cartilage microcracks forming at 45° to the split-line direction may indicate rupture of both cross-links and collagen fibers.

We found that cartilage thickness significantly correlated with median width of initiated microcracks for high (1.5 – 2.5 mJ/mm³) but not low (2.5 – 4.0 mJ/mm³) impacts (Fig. 2.4(b)), i.e. specimens with greater thicknesses generate microcracks with greater initial widths. In previous research we found that other mechanical responses of cartilage correlate with thickness, e.g. energy dissipation under shear deformations decreased with increased cartilage thickness (Santos et al. 2017).
2.5.2 Microcrack Propagation

In several instances while tracking microcracks, we identified microcracks in the post-impact phase but could no longer find them in the same locations in the post-cyclic compression phase. Post high impact, we also found that microcrack widths grew less after subsequent 15% cyclic compression than after 10% cyclic compression. Since we did not use living cartilage for these experiments, these microcracks likely closed or fused by readjustment of the extra-cellular matrix under cyclic compression. Many studies demonstrate that the collagen network within cartilage realigns during cyclic loading (Duda et al. 2001, Greene et al. 2010). Brown et al. (2012) suggest that collagen fibers bundle near the cartilage surface after damage to the network of collagen, and that such bundles are visible via SHG as brighter white lines. We did see bright white lines near the cartilage surface, perhaps resulting from fiber realignment to create fiber bundles. Such realignment within the extra-cellular matrix may account for our disappearing microcracks.

2.5.3 Limitations and Outlook

We used 3 mm diameter plugs, which may not fully represent in vivo conditions. Thus, this study is a first investigation of the mechanisms of microcracking. Correlations to in vivo conditions require further experiments. We found small cartilage microcracks near the edges of the cut cylindrical surfaces in several pristine specimens, these resulting from extraction of our specimens. Some of these microcracks propagated towards the center of the explants, likely influencing the integrity of the collagen network during our mechanical treatments. During imaging we considered only the center region of our explants to (largely) avoid including these microcracks in our analyses, but they may have influenced propagation of surrounding microcracks initiated during our mechanical treatments.

Post-traumatic osteoarthritis (PTOA) involves mechanical insult and an inflammatory cascade. In previous studies using SHG, we screened cartilage from total knee arthroplas-
ties (TKAs) and found microcracks occurring naturally in human cartilage with very early-stage OA. Similarly, Kumar et al. (2015) identified, via SHG imaging, cartilage microcracks (which they called microsplits and wrinkles) in very early-stage OA (ICRS Grade-I) femoral cartilage from TKAs. OA involves biomechanical, biochemical, metabolic, and genetic changes often triggered by injury and inflammation pathways (Moos et al. 1999, Tetlow et al. 2001). Future studies aimed at understanding the interplay of mechanical and cellular mechanisms in cartilage microcracks, e.g. connecting microcrack propagation in vivo and marathon running (Schueller-Weidekamm et al. 2006), may enable new treatment targets and detection of pre-clinical/early OA (Regatte et al. 2006).

In summary, our results show: (1) changes in overall microcrack width significantly depend on loading conditions where greater propagation occurs after low impacts; and (2) microcracks propagate by increasing in both length and width, but not depth. Of note, the microcracks we initiated during low-energy impact increased primarily in width during loading profiles that simulated walking. The extent of this propagation depended on the combination of impact and cyclic compression. More broadly, the initiation and propagation of microcracks may characterize pathogenesis of osteoarthritis, and may suggest therapeutic targets for future studies.

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**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. NE analyzed and interpreted data; participated in drafting the article and revising it critically; and gave final approval of the version submitted. CPN analyzed and interpreted data; participated in drafting the article and revising it 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.

**Appendix A**

In Table 2.1 we present the medians and interquartile ranges of the lengths, widths, depths, and angles of microcracks in the pristine, post impact, and post-cyclic compression phases of our experiment under one of three different impact treatments (none, low, high), and thereafter one of three different cyclic compression treatments (none, low, high).
Table 2.1: Plots show the medians and interquartile ranges \([Q_1, Q_3]\) of the lengths, widths, depths, and angles of microcracks in the pristine \((P)\), post impact \((PI)\), and post-cyclic compression \((PC)\) phases of our experiment under the three different impact treatments (no impact–NI, low impact–LI \((1.5-2.5 \text{ ml/mm}^3)\), and high impact–HI \((2.6-4.0 \text{ ml/mm}^3)\)) and thereafter the three different cyclic compression treatments (no compression–NC, low compression–LC \((10\%)\), and high compression–HC \((15\%)\)).
Appendix B

In Table 2.2 we summarize our statistical findings (p-values and F-values).

<table>
<thead>
<tr>
<th>Fixed Effect</th>
<th>Density (#/µm²)</th>
<th>Length (µm)</th>
<th>Width (µm)</th>
<th>Depth (µm)</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condyle</td>
<td>p</td>
<td>0.782</td>
<td>-</td>
<td>0.733</td>
<td>-</td>
</tr>
<tr>
<td>Impact</td>
<td>p</td>
<td>0.088</td>
<td>-</td>
<td>0.122</td>
<td>-</td>
</tr>
<tr>
<td>Cycl. Comp.</td>
<td>p</td>
<td>0.012</td>
<td>-</td>
<td>0.015</td>
<td>-</td>
</tr>
<tr>
<td>Impact×Cycl. Comp.</td>
<td>p</td>
<td>0.353</td>
<td>-</td>
<td>0.310</td>
<td>-</td>
</tr>
<tr>
<td>Phase</td>
<td>p</td>
<td>0.213</td>
<td>-</td>
<td>0.257</td>
<td>-</td>
</tr>
<tr>
<td>Phase×Impact</td>
<td>p</td>
<td>0.113</td>
<td>-</td>
<td>0.221</td>
<td>-</td>
</tr>
<tr>
<td>Phase×Cycl. Comp.</td>
<td>p</td>
<td>0.004</td>
<td>-</td>
<td>0.004</td>
<td>-</td>
</tr>
<tr>
<td>Thickness</td>
<td>p</td>
<td>0.194</td>
<td>-</td>
<td>0.363</td>
<td>-</td>
</tr>
<tr>
<td>Thickness×Impact</td>
<td>p</td>
<td>0.356</td>
<td>-</td>
<td>0.356</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.2: Summary of statistical p-values and F-values via ANOVA with significant differences (p < 0.05) in bold and highlighted. Init. = Initiation; Prop. = Propagation; Cycl. Comp. = Cyclic Compression. Degrees of freedom (df): initiation columns df = 83; propagation columns df = 234.

Appendix C

In Fig. 2.6 we show the normalized probability of finding microcracks at given lengths, widths, depths, and angles relative to the local split-line direction in the post impact and post-cyclic compression phases of our experiment under the three different impact treatments (none, low, high), and thereafter the three different cyclic compression treatments (none, low, high).
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Figure 2.6: Normalized probability of finding microcracks at given lengths (a-d), widths (e-h), depths (i-l), and angles with respect to the split-line direction (m-p). The solid red line in each figure represents data post impact, while the black dashed line represents data post-cyclic compression.
3 Can impact loading produce positive (anabolic) responses?

At the time of publication, this manuscript is in preparation.

3.1 Abstract

We aimed to determine the longitudinal effects of low-energy (generally considered non-injurious) impact loading on (1) chondrocyte proliferation, (2) chondroprogenitor cell activity, and (3) EGFR signaling. In an in vitro study, we assessed 127 full-thickness, cylindrical osteochondral plugs of bovine cartilage undergoing either single, uniaxial unconfined impact loads with energy densities in the range of $1.5 - 3.2 \text{ mJ/mm}^3$ or no impact (controls). We quantified cell responses at two, 24, 48, and 72 hours via immunohistochemical labeling of Ki67, Sox9, and pEGFR antibodies. We compared strain, stress, and impact energy density as predictors for mechanotransductive responses from cells. Our study demonstrates that low-energy mechanical impacts ($1.5 - 3.2 \text{ mJ/mm}^3$) generally stimulate time-dependent anabolic responses in the superficial zone of articular cartilage and catabolic responses in the middle and deep zones. We also found that impact energy density is a more consistent predictor of cell responses to low-energy impact loading. These spatial and temporal changes in chondrocyte behavior result directly from low-energy me-
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Mechanical impacts, revealing a new level of mechanotransductive sensitivity in chondrocytes not previously appreciated.

3.2 Introduction

Acute joint trauma, often incurred during accidents or sports injuries, may trigger a cascade of degenerative events that lead to post-traumatic osteoarthritis (OA). OA, the most common chronic joint disease in the world, afflicts over 30 million people in the US alone (Jia et al. 2016). In-vitro studies link post-traumatic OA to catabolic changes in articular cartilage following joint injury including increased degradation of collagen and proteoglycan (Setton et al. 1995), decreased synthesis of proteoglycan, switched synthesis from collagen type II to type I (Lahm et al. 2010), and apoptosis of chondrocytes (Chen et al. 2001), among other mechanotransductive responses. Such catabolic changes lead to articular cartilage degeneration and loss of joint function that greatly reduces quality of life, and is a significant cause of morbidity in aging populations (Ryan et al. 2009).

Mechanical loading can trigger catabolic behaviors from chondrocytes. Lee et al. (2005) and Chan et al. (2005) subjected cartilage explants to acute trauma and reported increased expression of genes that cause matrix degradation among other catabolic changes. However, in these experiments the mechanical treatments caused visible macroscale damage to the surface of articular cartilage such that catabolic responses from chondrocytes may manifest as secondary effects of load-induced macroscale damage. We recently found that even low-energy impacts usually considered non-injurious can cause micron-scale cracks in the network of collagen (microcracks less than the diameter of chondrocyte lacunae (< 30 μm)) (Kaleem et al. 2017). We do not know if mechanical impact that does not induce visible damage to cartilage triggers catabolic or anabolic responses from chondrocytes.

Anabolic responses from chondrocytes, including increased chondrocyte proliferation
and matrix synthesis (Shepard et al. 2013), lead to homeostasis and/or growth of articular cartilage. Anabolic (repair) responses may come from either chondrocytes, the mature cells in articular cartilage, or chondroprogenitor cells, multipotent cells that are capable of chondrogenic differentiation (Seol et al. 2012). Interest is growing in the development of mechanically-informed therapeutic approaches for post-traumatic OA prevention or treatment that would halt catabolic changes in articular cartilage, and/or stimulate anabolic ones (Anderson et al. 2011).

There are no studies investigating the mechanotransductive behavior of chondrocytes or chondroprogenitor cells in response to low-energy impacts generally considered non-injurious. Chondrocytes present mechanotransductive responses to mechanical treatments with strains as low as 6% (Perera et al. 2010). Chondrocytes also respond to fluid-induced stresses as low as 0.02 Pa (Saha and Kohles 2010). Microcracks in the network of collagen occur after impact treatments to the articular surface resulting in energy densities as low as 1.5 mJ/mm³ (Kaleem et al. 2017, Santos et al. 2019), but we do not know the corresponding cell responses. Furthermore, which mechanical measures of impact best predict the responses of chondrocytes or chondroprogenitor cells?

In this study, we aimed to determine the longitudinal effects of low-energy impacts generally considered non-injurious on (1) chondrocyte proliferation, (2) chondroprogenitor cell activity, and (3) EGFR signaling. To these ends, we assessed full-thickness, cylindrical osteochondral plugs of bovine cartilage undergoing either single, uniaxial unconfined impact loads with energy densities in the range of 1.5 – 3.2 mJ/mm³ (Santos et al. 2019) or no impact (controls). We quantified cell responses at two, 24, 48, and 72 hours via immunohistochemical labeling of Ki67, Sox9, and pEGFR. We also compared strain, stress, and impact energy density as predictors for mechanotransductive responses from cells.
3.3 Materials and Methods

3.3.1 Preparation of Specimens

We received full bovine knees from three skeletally mature animals (18-30 months) packed on ice and within 48 hours from slaughter (Animal Technologies, Inc., Tyler, TX). We then extracted cylindrical specimens (3 mm diameter, full thickness) from load-bearing and visibly pristine regions on the medial femoral condyles. We removed as much of the subchondral bone as possible while ensuring that the bottom surfaces were visibly parallel to the articular surface and measured the cartilage thicknesses ($h_0$) using calipers. We immediately immersed specimens in sterile Phosphate Buffered Saline (PBS, pH 7.4) until testing, which occurred less than two hours from extraction.

3.3.2 Mechanical Impact Test

We randomly assigned specimens to one of three impact groups (none, 1.5 mJ/mm$^3$, 3.2 mJ/mm$^3$), with impact energy density as the independent variable (Santos et al. 2019). We impacted the articular surface of specimens from the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups in unconfined compression using a custom drop tower with a 12.4 mm diameter flat metal platen Kaleem et al. (2017), Santos et al. (2019). We measured the acceleration ($\pm 49000$ m/s$^2$; 350A14, PCB Piezotronics, Depew, NY) and the force (22.24 kN; 200B05, PCB Piezotronics) at 100,000 Hz (sampling rate) for the full duration of the test. Post-impact, we submerged specimens in PBS at 37°C for at least 1 h prior to subsequent processing. Specimens from the control group rested in PBS for the duration of the test.

3.3.3 Data Analyses

We determined the actual velocity at the moment of impact $v_{imp}$ and the maximum compression of a specimen $\Delta t_{max}$ for each test by integrating the acceleration data once and
3.3 Materials and Methods

We also determined the maximum force applied to each specimen $f_{max}$ from the measured force data. We then calculated the maximum engineering strain $\epsilon(\cdot)$ as $\epsilon = \Delta t_{max}/t_0$ where $t_0$ is the corresponding reference thickness. Next, we calculated the maximum first Piola-Kirchhoff (nominal) stress $P$ (MPa) as $P = f_{max}/(\pi r_0^2)$ where $r_0$ is the initial radius of the specimen (1.5 mm). Finally, we calculated the impact energy density $E_{imp}$ or $E$ (mJ/mm$^3$) as $E = m v_{imp}^2/(2 \pi r_0^2 t_0)$ where $m$ is the total drop mass.

3.3.4 Cell Culture and Fixation

Immediately after rinsing the specimens in PBS, we fixed the two-hour post-impact specimens from the control ($n = 14$), the 1.5 mJ/mm$^3$ impact ($n = 9$), and the 3.2 mJ/mm$^3$ ($n = 9$)impact groups in 4% paraformaldehyde (Sigma, St. Louis, MO) for four days (see Fig. 3.1 for further detail). We placed the remaining 95 specimens in 1 mL of sterile media comprised of DMEM/F12 (Gibco, Gaithersburg, MD), 50 mg/ml ascorbic-acid-2-phosphate (Sigma), 0.1% bovine serum albumin (Sigma), 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 100 units/mL penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) for culture at 37°C and 5% CO$_2$ for 24, 48, or 72 hours post-impact. Once removed from culture, we also fixed these specimens in 4% paraformaldehyde for four days. We then decalcified all specimens using 14% EDTA with NH$_4$OH for four days at 4°C with rocking. After decalcification, we washed specimens in PBS and dehydrated through a series of solutions increasing in percent ethanol up to 70% ETOH. Finally we paraffin embedded and sectioned specimens (at 8 µm sections) for histology and immunohistochemistry.
3.3.5 Image-Based Assessments

We imaged slides with a Nikon Eclipse E800 microscope using 4×/0.20 NA and 20×/0.50 NA objectives, and 1248 × 936 pixel resolution with a pixel size of 1.75 µm. Using the 4× objective, we obtained single images of the full through-thickness cross-section (cartilage and bone) of each specimen for every histological and immunohistochemical stain and time point. Using the 20× objective, we obtained three to five images through the thickness of every specimen stained for immunohistochemistry (spanning from the SZ to the subchondral bone), with 0 – 600 pixels of image overlap.

Histology

We stained sections with 0.1% Safranin O (Sigma) and counterstained with Weigert’s Iron Hematoxylin (Poly Scientific, Bay Shore, NY) and 0.02% aqueous Fast Green (Fisher Scientific, Hampton, NH) (Shepard et al. 2013), and imaged the resulting slides. We used these images to qualitatively assess the articular surface integrity and the full-thickness health of the cartilage matrix (Kamekura et al. 2005).

Immunohistochemistry

In a preliminary study, we tested the immunolocalization of C3 protein over 72 hours and did not find appreciable cell death. Thus, we did not continue to test cell viability for this study. We performed immunohistochemical staining as previously described Shepard et al. (2013). Briefly, we de-paraffinized, rehydrated, and incubated slides with 3% hydrogen peroxide in water for 15 minutes. We then blocked rabbit anti-bodies using 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA), following standard protocol, and incubated the slides with primary antibodies in blocking buffer overnight at 4°C. In this study we diluted the following primary antibodies to 1:1000: rabbit anti-Ki67 (Abcam, Cambridge, MA, USA); rabbit anti-Sox9 (Abcam); and rabbit anti-pEGFR (Y1092; Abcam). We washed with Tris Buffered Saline (TBS) pH 7.6 containing 0.1% Tween 20
and incubated the slides with 1:200 biotinylated goat anti-rabbit IgG (Vector Laboratories). We then washed again and incubated the slides with Vectastain Elite ABC Reagent (Vector Laboratories) and developed them with DAB Reagent (Vector Laboratories). Finally, we counterstained with Harris’ Hematoxylin (Shandon, Cambridge, UK).

To recreate full-thickness cross-sections from the 20× images, we first selected regions with little or no matrix staining, and excluded specimen edges from the field of view. We then performed image stitching using Fiji’s Grid/Collection Stitching Plugin (Preibisch et al. 2009) for ImageJ (National Institutes of Health, Bethesda, MD). Using the resulting full-thickness images, we first determined boundaries for the superficial, middle, and deep zones by assessing lacunae morphology and cellular arrangement (Youn et al. 2006) and defined boundaries between zones as full-width horizontal lines at the average transition height (Pedersen et al. 2013). We also calculated the percent thickness of each zone with respect to total thickness of cartilage. We then quantified both the positive and negative cellular localization of each antibody within each zone, and calculated the percent positive cells within each zone for all images.

### 3.3.6 Statistical Analyses

To pre-process the data, we created subsets for each antibody and zone, for a total of 9 subsets. We used these subsets as the basis for our $t$-tests in our statistical analyses. Using the Shapiro-Wilk Test we confirmed that our measured percent positive cellular expressions of Ki67, Sox9, and pEGFR were normally distributed. First, we used separate two-sample $t$-tests to compare the means of percent positive cellular expressions of the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ treatment groups for each corresponding subgroup of equal antibody, time, and zone. We used the pooled $t$-statistic when groups had equal variances, and the Satterthwaite $t$-statistic when groups did not have equal variances. Additionally, we used separate two-sample $t$-tests to compare the means of percent positive cellular expression at every time point. Second, for significant differences detected by the $t$-tests, we used separate linear
regressions (including $R^2$ values) to investigate interactions between mechanical stimuli (maximum engineering strain $\epsilon$, maximum first Piola-Kirchhoff (nominal) stress $P$, and impact energy density $E_{imp}$) and percent positive cellular expressions. We completed all statistical analyses using SAS 9.4 (SAS Institute Inc., Cary, NC) and using $p < 0.05$ to determine significance.

In Fig. 3.1, we summarize the experimental protocol and the treatment groups.

### 3.4 Results

We confirmed the overall health and structural integrity of each specimen by analyses of histological images with staining by Safranin O and Fast Green, cf. Fig. 3.1. We found our low-energy impacts did not induce macroscale damage or visible fissuring at the articular surface. We also qualitatively compared the histological staining of non-impacted control specimens with impacted specimens at the same time points and noted that impacts caused a visible difference in immunohistochemical responses.

Our image analysis confirmed similarities in the ultrastructure of mature bovine and human tissue. Specifically, we found the SZ, MZ, and DZ in bovine represented $12\pm3.9\%$, $35\pm17.9\%$, and $54\pm17.7\%$ of the overall cartilage thickness, respectively. In human, these distributions range from $10–20\%$, $40–60\%$, and $20–50\%$ respectively (Buckwalter et al. 1994, Athanasiou et al. 2010).

We quantified immuno-labeling (Fig. 3.2; Appendix A, Fig. 3.6) and differences between impact-treatment groups (Table 3.1), and quantified the localization of each antibody by through-thickness zone and time post impact.

#### 3.4.1 Effects of Low-energy Impacts on Ki67 and Cell Proliferation

In the SZ we did not find significant differences in chondrocyte proliferation between impact groups for any time point, as determined by Ki67 labeling. In the MZ at 24 hours we
Figure 3.1: *Graphical Representation of the Experimental Protocol.* Summary of the experimental protocol and the treatment groups: (a) specimen extraction and unconfined impact test, (b) time course of specimens in culture by treatment group, (c) representative images from histology (Safranin O/Fast Green in red) and immunohistochemistry with antibody identification. Solid arrows indicate positive antibody expression while dashed arrows indicate negative antibody expression.
We found significant differences between the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups occurred in all zones, with longitudinal differences in zonal activity. Longitudinal distributions of percent positive cell labeling for Ki67, Sox9, and pEGFR after both 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impacts within the (a) superficial zone (SZ), (b) middle zone (MZ), and (c) deep zone (DZ). Here * indicates differences with statistical significance ($p < 0.05$) and $p$-values indicate marginally significant differences ($0.05 < p < 0.10$).

found significantly increased ($p = 0.0156$) Ki67 labeling in the 1.5 mJ/mm$^3$ impact group.

We also found a significantly increased ($p = 0.0312$) Ki67 labeling in the DZ at 72 hours in the 1.5 mJ/mm$^3$ impact group.
Table 3.1: We found statistically significant temporal and zonal differences between the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups for all antibodies. Summary of $p$-values from $t$-tests comparing 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups for the superficial zone (SZ), middle zone (MZ), and deep zone (DZ). We indicate statistical significance ($p < 0.05$) with * and dark gray shading, and marginal statistical significance ($0.05 \leq p < 0.10$) with light gray shading.

<table>
<thead>
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<th>24</th>
<th>48</th>
<th>72</th>
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<td>0.003*</td>
<td>0.022*</td>
</tr>
<tr>
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<td>pEGFR</td>
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<td>0.581</td>
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</table>

3.4.2 Effects of Low-energy Impacts on Sox9 and Progenitor Cell Populations

In the SZ at two hours we found significantly increased ($p = 0.0433$) labeling of Sox9 in the 3.2 mJ/mm$^3$ impact group compared to the 1.5 mJ/mm$^3$ impact group. We also found significant differences at 48 hours ($p = 0.0029$) and 72 hours ($p = 0.0223$) post impact.

At 24 hours labeling of Sox9 decreased in the 1.5 mJ/mm$^3$ impact group, while this increased in the 3.2 mJ/mm$^3$ impact group. At 48 hours we only found marginally significantly increased ($p = 0.0958$) labeling of Sox9 in the DZ of the 3.2 mJ/mm$^3$ impact group. At 72 hours we found that this trend flipped such that we found increased labeling of Sox9 in the 1.5 mJ/mm$^3$ impact group compared to the 3.2 mJ/mm$^3$ impact group.
3.4.3 Effects of Low-energy Impacts on pEGFR and Signaling Pathways

In the SZ we found a greater \((p = 0.0619)\) activation of pEGFR in the 3.2 mJ/mm\(^3\) impact group compared to the 1.5 mJ/mm\(^3\) impact group, but only at two hours post impact. At 24 hours in the MZ we found marginally greater \((p = 0.0822)\) activation of pEGFR. We found no difference in activation of pEGFR in the deep zone.

3.4.4 Linear Regressions as Predictors of Cell Labeling

We created linear regressions fitting the probability of percent positive labeling with respect to our mechanical impacts for all statistically significant differences from Table 3.1. In Figs. 3.3, 3.4, and 3.5 we summarize the linear regressions with 95% confidence intervals where we found statistically significant differences \((p < 0.05)\), and marginally statistically significant differences \((0.05 \leq p < 0.10)\) between the 1.5 and 3.2 mJ/mm\(^3\) impact treatment groups.

The key differences among Figs. 3.3–3.5 are the total number of statistically significant linear regressions quantifying the relationships between the independent variable (engineering strain, first P-K stress, and impact energy density) and the dependent variable (percent positive Ki67, Sox9, and pEGFR cells). We found that several of the marginally significant differences between the 1.5 mJ/mm\(^3\) and 3.2 mJ/mm\(^3\) impact groups did not produce strong/significant linear regressions, see Table 3.2.

We found that impact energy density is a more consistent predictor of percent positive cells, with nine strong correlations spanning both multiple through-thickness zones and multiple time points post impact. We found six and five strong correlations using engineering strain or first P-K stress as the independent variable, respectively.
3.5 Discussion

In this study, we investigated the cellular responses to two different levels of low-energy impact applied to articular cartilage. Previously, we found that low-energy impacts induce micron-scale cracks in the network of collagen in articular cartilage (Kaleem et al. 2017). Matrix repair and regulation after mechanical trauma depends on the chondrocytes
Can impact loading produce positive (anabolic) responses?

Figure 3.4: Linear Regressions for Stress. We found that first P-K stress was not a consistent predictor of percent positive cell labeling. Summary of linear regressions for first P-K stress $P$ (with 95% confidence intervals) where we found statistically significant differences ($p < 0.05$) and marginally statistically significant differences ($0.05 \leq p < 0.10$) between the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups. We summarize the corresponding $p$-values, equations, and $R^2$ values in Table 3.2.

(Aigner et al. 2007), however their ability to synthesize new matrix and produce more cells is extremely limited (Mankin 1982). At least three factors may drive the cascade of degeneration that leads to post-traumatic osteoarthritis: (1) a diminished ability of chondrocytes to repair cartilage matrix (Aigner et al. 2007), (2) an increase in joint fluid adumbrates
3.5 Discussion

Figure 3.5: Linear Regressions for Impact Energy Density. We found that impact energy density may be a better predictor of cell responses in proliferation, labeling, and pEGFR activation compared against both engineering strain and first P-K stress. Summary of linear regressions for impact energy density $E$ (with 95% confidence intervals) where we found statistically significant differences ($p < 0.05$) and marginally statistically significant differences ($0.05 \leq p < 0.10$) between the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups. We summarize the corresponding $p$-values, equations, and $R^2$ values in Table 3.2.

chondrocytes’ repair efforts (Buckwalter et al. 1994), and (3) an insufficient number of chondrogenic progenitor cells present (Candela et al. 2014).

To test chondrocyte ability and viability, we performed a preliminary study to examining cell death. In this study, we tested the immunolocalization of C3 protein, a sensitive
Table 3.2: Impact energy density ($E$) and engineering strain ($\epsilon$) are better predictors of percent positive cell labeling than first P-K stress ($P$). Summary of equations for linear regressions by antibody (Ki76, Sox9, and pEGFR), through-thickness zone (superficial zone (SZ), middle zone (MZ), and deep zone (DZ)), and time post impact (2, 24, 48, and 72 hours). We indicate statistical significance ($p < 0.05$) with * and dark gray shading, and marginal statistical significance ($0.05 \leq p < 0.10$) with light gray shading.

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>$p$</th>
<th>Equation</th>
<th>$R^2$</th>
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<td>Ki67</td>
<td>MZ</td>
<td>24 h</td>
<td>0.043*</td>
<td>$-0.604\epsilon + 0.716$</td>
<td>0.243</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 h</td>
<td>0.029*</td>
<td>$-0.121E + 0.710$</td>
<td>0.279</td>
</tr>
<tr>
<td></td>
<td>DZ</td>
<td>72 h</td>
<td>0.054</td>
<td>$-0.471\epsilon + 0.722$</td>
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<td></td>
<td>0.042*</td>
<td>$-0.065E + 0.626$</td>
<td>0.200</td>
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</tbody>
</table>

indicator of apoptotic cell death, in a few test sections. We found a minute number of positive cells, which were present only on the surface of both unloaded and loaded explants.
after 72 hours of *in vitro* culture. From this preliminary study, we determined that neither our extraction protocol nor our low-energy impacts caused substantial cell death. Our findings are supported by the literature, where appreciable cell death occurred at impact energies $3 - 10 \times$ greater (Duda et al. 2001, Martin et al. 2009, Szczodry et al. 2009) than ours ($10.6 - 35.3$ mJ), and at first P-K stresses $6 - 800 \times$ greater (Duda et al. 2001, Jeffrey et al. 1995, Ewers et al. 2001) than ours ($0.25 - 1.5$ MPa).

We also aimed to determine whether measures of strain, stress, or impact energy density could predict the cellular responses. Impact loads may stimulate mechanotransduction in chondrocytes, including changes in gene expression (Seol et al. 2012, Novakofski et al. 2015) and signaling patterns (Rosenzweig et al. 2012). This study is the first to demonstrate altered protein levels of intra-cellular Sox9 and activation of pEGFR in chondrocytes following low-energy impact loading. We found this non-injurious mechanical stimuli altered mechanotransductive responses and that the specific responses depended on the level of load. We used 1.5 and 2.5 mJ/mm$^3$ as non-injurious, low-energy impacts that have $\sim 25\%$ and $\sim 40\%$ probability of microcracking the network of collagen (Kaleem et al. 2017). These impact energy densities are also below the threshold that induces cell death, which can be as low as 2-5 mJ/mm$^3$ (Duda et al. 2001). We identified a range based on impact energy densities where mechanical thresholds may exist for up-regulation of Sox9, a chondroprogenitor cell marker, activating EGFR signaling (as indicated by pEGFR labeling), as well as proliferation, as identified by Ki67. We also determined that impact energy density was a better predictor of cell responses than measures of strain or stress.

### 3.5.1 Effects of Low-Energy Impacts on Ki67 and Cell Proliferation

We found that low-energy impacts stimulated proliferation by chondrocytes in articular cartilage at least 24 hours post-impact. We found no detectable difference in the amount of proliferation in the SZ. Rather, the significant differences in cell proliferation between impact groups occurred in the MZ, and marginally in the DZ. We found implications that
Can impact loading produce positive (anabolic) responses?

A threshold may exist, evidenced by a shift from anabolic to catabolic activity between 1.5 and 3.2 mJ/mm³. This may suggest targets for controlled, load-informed rehabilitation for post-traumatic OA.

However, Ki67 may not indicate cell division or an increase in cell number. Rather, Ki67 is a proliferation marker that is expressed during all active phases of the cell cycle (Scholzen and Gerdes 2000), and may not predict actual cell division. We did not find significant increases in the total number of cells present in the tissue, thus low-energy impact loading may not cause a significant increase in the total number of chondrocytes (at least for time courses up to 72 hours after impact). However, we did not investigate cell viability and thus changes in total cell count could be masked by flux in proliferating cells and necrotic cells. Static compression (Ryan et al. 2009) and cyclic loading (He et al. 2016) may have greater influences on cell proliferation and division than the single impact loads used in our experiments.

3.5.2 Effects of low-energy impacts on Sox9 and Progenitor Cell Populations

Sox9 is an important transcription factor that serves as a master regulator of cartilage formation and differentiation (Bi et al. 1999, Lefebvre and Dvir-Ginzberg 2017). It also maintains cartilage health by further recruiting transcriptional co-activators, histone-modifying enzymes, and other essential cellular subunits (Akiyama and Lefebvre 2011). Both chondroprogenitors (Shepard et al. 2013) and mature (adult) chondrocytes (Fitzgerald et al. 2004, Lee et al. 2005) express the “master chondrogenic regulatory factor” Sox9 (Shepard et al. 2013) until hypertrophy (Lefebvre and Dvir-Ginzberg 2017). In addition to these anabolic activities, Sox9 also helps regulate osteogenic differentiation and loss of the cartilage phenotype, which are both catabolic activities Liao et al. (2014). However, loss of Sox9 in articular cartilage leads to upregulation of catabolic and degradative pathways and shuts
down new matrix synthesis at the transcriptional level (Henry et al. 2012). Given the well-known nature of Sox9’s key roles, we interpret the positive Sox9 labeling as indications of changes in chondrocyte potential and/or functions that align with these aforementioned anabolic activities. Conversely, metabolically inactive or hypertrophic chondrocytes cannot express Sox9 (Zhao et al. 1997).

**Temporal Differences**

Our results show that Sox9 labeling is highly sensitive to mechanical loading, and perhaps increased impact energy density further stimulates the master transcription factor Sox9. In the SZ we found increased impact energy density correlated with increased Sox9 labeling. Thus, if a catabolic threshold exists in the SZ it is beyond 3.2 mJ/mm³.

No studies in the cartilage literature probe Sox9 with treatments comparable to our mechanical impact model. Multiple studies confirm transient expressions of Sox9 under compression loading (Fitzgerald et al. 2004), including after single 20-25 MPa injurious compressions (at least ten times greater than the maximum second P-K stresses in our experimental model) (Lee et al. 2005, McCulloch et al. 2014). Chan et al. (2005) found 30 MPa injurious compression repressed expression of cell adhesion molecules three hours post-injury, which can influence processes such as chondrogenic differentiation. However, we achieved maximum loads in less than 20 ms with our custom drop tower while these studies applied loads over longer periods of time. Differences in the duration of loading make direct comparisons among these experiments difficult. Since we found increased Sox9 expression, it is likely our low-energy impact loads did not repress cell adhesion molecules.

**Zonal Differences**

In healthy human cartilage the percentage of Sox9 labeling increases from the SZ to the DZ (Fukui et al. 2008). We found similar trends at two hours in the 1.5 mJ/mm³ post-impact group. Microcracks initiate in the SZ in the same range of impact energy densities used in
Can impact loading produce positive (anabolic) responses?

this study, and typically extend from the SZ into the MZ (Santos et al. 2019). Immediately after impact, we saw significantly greater Sox9 labeling in the SZ of the 3.2 mJ/mm$^3$ impact group, then a delayed increase to Sox9 labeling parity by the 1.5 mJ/mm$^3$ impact group at 24 hours post impact. After 24 hours, we saw significantly greater Sox9 labeling in the 3.2 mJ/mm$^3$ impact group in the SZ. At 3.2 mJ/mm$^3$ microcracks initiate in the network of collagen with a 1.6$\times$ greater probability than that at 1.5 mJ/mm$^3$ (Kaleem et al. 2017).

**Chondroprogenitor Activity**

Due to its avascular nature cartilage may contain a large proportion of chondroprogenitor cells that may be identified using Sox9 (Grogan et al. 2009). Chondroprogenitors may be found in all three through-thickness zones, but they concentrate primarily in the two most upper zones (Grogan et al. 2009). Overall, we found greater percentages of Sox9 labeling in the MZ than the others, but more significant increases in Sox9 labeling in the SZ with increased impact energy density. This may indicate progenitor cell migration from the MZ to the SZ as a result of increased loading at the articular surface. Studies show regional chondroprogenitors migrate towards the impact site after $\sim$70 mJ/mm$^3$ blunt impact (Seol et al. 2012) (> 45$\times$ our impact loads) and after $\sim$8 mJ/mm$^3$ (Riegger et al. 2018) (3−5$\times$ greater than our impact loads). Additionally, chondroprogenitors may cluster at the articular surface, highlighting their involvement in potential matrix remodeling as a result of loading or damage (Grogan et al. 2009).

**3.5.3 Effects of Low-Energy Impacts on pEGFR and Signaling Pathways**

EGFR is a tyrosine kinase receptor with multiple roles in development, homeostasis, and disease (Scaltriti and Baselga 2006). EGFR signals regulate cartilage development and growth (Zhang et al. 2013), and EGFR activation triggers both anabolic and catabolic tissue
3.5 Discussion

responses (Shepard et al. 2013, Jia et al. 2016, Bellini et al. 2017). Hence joint health likely requires a balance of EGFR signals for maintenance. In healthy tissue, pEGFR labeling indicates activation of the EGFR signaling pathway (Shepard et al. 2013), and presents in all three zones. In OA-like tissue, this antibody presents only in the SZ (Jia et al. 2016). Our pEGFR labeling occurred in only the SZ and MZ, which may indicate the overall health of the tissue after loading.

We did not see simultaneous increases in both pEGFR and Ki67 labeling at the same timepoint and zone, except for at 24 hours post-impact in the MZ. Thus, under our loading conditions, EGFR activation (as indicated by pEGFR labeling) via impact loading did not mediate cell proliferation. It is possible that inhibiting the EGFR signaling pathway may cause an increase in cell proliferation (He et al. 2016), though this is beyond the scope of our study. Additionally, increases in Sox9, which we saw at two hours post-impact in the SZ, may lead to changes in signaling pathway mediators (Lefebvre and Dvir-Ginzberg 2017). This was the only time point where we saw significant differences between our loading groups in Sox9 labeling and pEGFR labeling in the same zone.

3.5.4 Linear Regressions as Predictors of Cell Labeling

Using linear regressions, we found impact energy density to be a more consistent predictor of percent positive cell responses, having the greatest number of statistically significant fits (nine) across all time points. Measures of strain relate only to deformations while measures of stress relate only to forces. Energy density, however, combines measures of both deformation and force. This may be why engineering strain and first P-K stress showed less predictive power than impact energy density. Su et al. (2018) and Kaleem et al. (2017) also found impact energy density to be a significant predictor of articular cartilage response, where Kaleem et al. (2017) found impact energy density to predict the occurrence of microcracks in the network of collagen within cartilage better than measures of strain or stress. These studies both quantified micro-mechanical damage to cartilage, not
chondrocyte responses to loading. Some studies looked at genetic markers as predictors of chondrogenic differentiation (Giovannini et al. 2010, Kanawa et al. 2018), but none look at chondrocyte responses due to mechanical loading, such as chondroprogenitor cell activity and EGFR signaling or pEGFR labeling.

### 3.5.5 Limitations and Outlook

Though we did not perform live/dead cell viability assays, our preliminary results investigating C3 protein after low-energy impacts indicate it is possible, but highly unlikely that a significant number of cells died during our experiment. We assume our extraction method (using cylindrical punches to extract cylindrical plugs) caused minor structural damage at the edges of the specimens. Thus we did not analyze images near to the vertical edges of the specimens. To determine horizontal boundaries parallel to the articular surface for each zone during image analysis, we manually determined transitions based changes in cellular shape and arrangement. We attempted to minimize human-bias by selecting boundaries in the middle of transition zones. Finally, we experienced minor discrepancies with our immunohistochemical staining likely due to environmental conditions and human factors.

Our study demonstrates that low-energy mechanical impacts (1.5 – 3.2 mJ/mm³) generally stimulate time-dependent anabolic responses in the superficial zone of articular cartilage and catabolic responses in the middle and deep zones. We also found that impact energy density is a more consistent predictor of cell responses to low-energy impact loading. These spatial and temporal changes in chondrocyte behavior result directly from low-energy mechanical impacts, revealing a new level of mechanotransductive sensitivity in chondrocytes not previously appreciated. Additional future work includes investigating the cellular response after a continuum impact energy densities in order to increase the robustness of regression modeling.
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. KR prepared specimens and conducted the experiments; analyzed and interpreted data; and gave final approval of the version submitted. MCF analyzed and interpreted data; participated in drafting the article and revising it critically; and gave final approval of the version submitted. CND 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. 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.

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Samolyk, Faith Sporbert, and Brianna Westenfield for assistance analyzing images and organizing data. We also thank Dr. James Grady and Rong Wu from the Connecticut Institute for Clinical and Translational Science for consulting on statistics.
Appendix A

Figure 3.6: Box Plots by Antibody. We found significant differences between the 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impact groups occurred in all zones, with longitudinal differences in zonal activity. Longitudinal distributions of percent positive cell labeling within the superficial zone (SZ), middle zone (MZ), and deep zone (DZ) after both 1.5 mJ/mm$^3$ and 3.2 mJ/mm$^3$ impacts for (a) Ki67, (b) Sox9, and (c) pEGFR. Here * indicates differences with statistical significance ($p < 0.05$) and $p$-values indicate marginally significant differences (0.05 < $p$ < 0.10).
4 Can we prevent microcrack formation and/or propagation?

4.1 Abstract

Objective We recently demonstrated that we could initiate microcracks (cracks in the network of collagen with widths < 30 µm) using low-energy impact loads, and found that they tended to propagate during unconfined cyclic compression by increasing in length and width. In this study, we aimed to determine the effect of: (1) genipin as a “preventative” treatment mitigating initiation of microcracks under impacts; (2) genipin as a treatment mitigating propagation of microcracks under cyclic, unconfined compression; and (3) timing and number of genipin treatment on propagation of microcracks.

Design We performed mechanical testing on bovine osteochondral explants, and imaged specimens using second harmonic generation prior to and after all mechanical treatments. We assigned specimens to one of four combinations of chemical treatments: with or without genipin prior to mechanical impact and with or without genipin prior to cyclic compression. Genipin treatments comprised 11 mM of genipin for 24 hours, while no treatment comprised phosphate buffered saline (PBS) for the same time. We initiated microcracks using a drop tower and an impact energy density of 2.5 mJ/mm³, and propagated microcracks using unconfined cyclic compression at 1.44 Hz and an amplitude of 10% the initial thickness of the specimens.
4.2 Introduction

Macroscale and microscale injury naturally occur in the knee joint, due to consistent complex and/or compound motions such as flexion, extension and rotation (Peña et al. 2007). Damage to the articular cartilage serves as one precursor to the cascade of degenerative effects that load to post-traumatic osteoarthritis (OA), a painful disease that afflicts nearly 20% of adults in the United States, and is the second most costly health condition (first being diabetes) according to the Center for Disease Control. This damage may occur to any of the depth-dependent constituents, including the collagen fibers, which comprise of 68-85% of the wet weight, and chondrocytes, which comprise of less than 4%.

Cracks or fissures visible on the surface of articular cartilage by eye or by optical tools often manifest due to breaks in the network of collagen that resulted from large tensile stresses (Workman et al. 2017), or cross-link and/or collagen fiber ruptures (Santos et al. 2019). These cracks may be on the millimeter scale (Repo and Finlay 1977, Thambyah et al. 2008) or the sub-millimeter scale (Kaleem et al. 2017). Recently, we initiated and propagated microcracks, or cracks with widths smaller than that of lacunae (30 µm), in bovine articular cartilage (Santos et al. 2019) using single low-energy impacts and cyclic unconfined compressive loads. After finding that microcracks propagated primarily by
Can we prevent microcrack formation and/or propagation?

Micro-damage to the collagen matrix and loss of cartilage integrity suggests repair strategies that mitigate microcrack initiation and growth. The use of low-energy impact that creates and grows micron-scale cracks in the collagen network (Kaleem et al. 2017, Kaplan et al. 2017a, Malekipour et al. 2013) suggests a poor outlook for joint health even during routine physical activities following injury. The expectation of extended deterioration and degradation of the extracellular matrix, including microcrack growth, coupled with catabolic responses from cells, may define some of the key early stages of osteoarthritis pathogenesis (Aigner et al. 2007). Consequently, we sought to investigate possible therapeutics that would slow or arrest microcrack growth, and minimize the possibility of increased cartilage and joint pathology.

Crosslinking of collagen has been utilized to improve the mechanical stiffness and structural rigidity of (especially monomeric) networks, through chemical, mechanical, or combined radiative means (Novakofski et al. 2015, Novak et al. 2016, Wollensak et al. 2003). In particular, carbodiimide crosslinkers (e.g. 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride, or EDAC) are effective to improve the collagen network modulus, but can create a toxic cellular environment (Lee et al. 2001). In contrast, genipin is a well-known natural cross-linker for proteins, including collagen, and has been utilized for improved strength of collagen networks (Uquillas et al. 2012). It functions by stimulating intra- and intermolecular cross-links of the amino residues on tropocollagen or proteoglycan molecules (Lima et al. 2009). While genipin cannot repair large (millimeter scale) fissures (Bonitsky et al. 2017), its ability to repair sub-millimeter scale fissures remains unclear.

In this study, we aimed to determine the effect of: (1) genipin as a “preventative” treatment mitigating initiation of microcracks under impacts; (2) genipin as a treatment mitigating propagation of microcracks under cyclic, unconfined compression; and (3) timing and number of genipin treatment on propagation of microcracks. Understanding these
aims contributes to interrupting the progression of damage in the network of collagen, which may lead to improved clinical treatment targets. We hypothesize that treatments with genipin will improve the damage resistance of cartilage, specifically reducing the initiation and propagation of microcracks in the network of collagen under impact loading. To these ends, we initiated microcracks using a single low-energy mechanical impact, and propagated microcracks using unconfined cyclic compression (c.f. Santos et al. (2019)), with our chosen inputs previously yielding the greatest crack density (5.7 cracks/mm³) compared to other mechanical treatment combinations. We incorporated single or multiple doses of genipin in between mechanical treatments and second harmonic generation (SHG) imaging phases, and quantified changes in crack morphology and orientation.

### 4.3 Materials and Methods

In total 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 crosslinker dosing groups (Table ??). We compared results to specimens undergoing the same mechanical treatments but no crosslinking solution in a previous study, which served as our control group ($n = 10$) (Santos et al. 2017). We applied the same impact energy density and unconfined cyclic compression to all specimens. We also performed Second Harmonic Generation (SHG) imaging (Carl Zeiss LSM 510) after three experimental phases (pristine, post-impact, and post-cyclic compression). In Fig. 4.1 we show a summary of the experimental protocol.

<table>
<thead>
<tr>
<th>Dose A</th>
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<td>10</td>
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<td>−</td>
<td>+</td>
<td>14</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>12</td>
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<tr>
<td>+</td>
<td>+</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 4.1: Specimen distribution by dose groups. − indicates no genipin treatment, + indicates 11 mM genipin treatment.
4 Can we prevent microcrack formation and/or propagation?

4.3.1 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 previously described in Santos et al. (2017). Briefly, we extracted cylindrical osteochondral plugs from visibly pristine load-bearing regions on both the lateral and medial condyles while noting the local split-line direction (Athanasiou et al. 1991, Neu et al. 2007). Using a scalpel, we removed a majority of the subchondral bone while ensuring that the remaining subchondral bone (approx 1-2 mm) 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 (Schindelin et al. 2012). To store specimens not immediately tested, we immersed them in Phosphate Buffered Saline (PBS, pH 7.4) and stored them at -80°C (Athanasiou et al. 1991, Szarko et al. 2010). On the day of testing we thawed the specimens and mounted them to custom, ultra-wear-resistant nylon platens using cyanoacrylate adhesive for subsequent imaging and mechanical testing.

4.3.2 Collagen Crosslinking

We received the chemical compound genipin (Adipogen Life Sciences, San Diego, CA) on ice, and stored the genipin as a 200 mM stock solution in anhydrous DMSO at -20°C. We prepared working solutions of 11 mM (Pinheiro et al. 2016) genipin in PBS. For every
24 hour incubation (Elder et al. 2017, Bonitsky et al. 2017, Pinheiro et al. 2016) we placed specimens in 300 μL of the 11 mM genipin solution and incubated at 37°C. After the incubation period, we rinsed the specimen for at least 30 seconds in PBS prior to mechanical testing.

### 4.3.3 Mechanical Tests

**Low-Energy Impact**

To apply low-energy impact loads, we used our testing device and protocol as previously described in Santos et al. (2017). Briefly, we impacted the articular surface of unconfined pristine specimens with an impact energy density of 2.5 mJ/mm³ using a custom drop tower with a 12.4 mm diameter flat metal indenter. Post-impact, we submerged the specimens in PBS at 37°C for at least one hour to equilibrate prior to subsequent imaging, dosing, and mechanical testing (Kaleem et al. 2017).

**Cyclic, Unconfined Compression**

Post-impact and a 24 hour incubation period, we conducted unconfined cyclic compression tests, a technique well-established in the literature (Mow et al. 1999, Korhonen et al. 2002, Park et al. 2004), using a Bose LM1 Electroforce linear motor with WinTest 7 software (Bose, Eden Praire, MN). We used a protocol as previously described in Santos et al. (2017). Briefly, after a force-controlled 0.2 N compression and equilibration in 37°C for 3000 seconds, we applied a pattern of cyclic compression including 0.69 sec sinusoidal compression with an amplitude of 10% of the cartilage thickness, followed by 0.67 sec recovery (total cycle time equals 1.36 sec or 0.74 Hz), cf. Zhang et al. (2015). Post-cyclic compression, we submerged the specimens in PBS at 37°C for at least one hour prior to subsequent imaging (Kaleem et al. 2017).
4.3.4 Images via Second Harmonic Generation

As with our previous work cf. Santos et al. (2017), we performed SHG imaging using 850 nm excitation at three separate experimental phases (pristine–\(P\), post-impact–\(PI\), and post-cyclic compression–\(PC\)). For all images we used a water-immersion objective (W Plan-Apochromat 20x/1.0) and a \(600 \times 600 \mu m\) (512 \times 512 pixel) field of view. For each specimen, we acquired a \(7 \times 7\) tile grid (100 \(\mu m\) 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 50-200 \(\mu m\) (slice increment of 2.5 \(\mu m\)) from a \(3 \times 3\) tile grid centered on the articular surface (to avoid edge effects).

4.3.5 Analyses of Images

We stitched together our SHG images using Fiji’s Grid/Collection Stitching Plugin (Preibisch et al. 2009) for ImageJ (National Institutes of Health, Bethesda, MD) to generate images of the full circular cross section at a resolution of 1.2 \(\mu m/pixel\). Using only the \(3 \times 3\) tile grid centered on the articular surface \((3618.8 \times 3618.8 \mu m, 3093 \times 3093\) pixels), independent observers measured the length, width, and principal angle (relative to the split-line direction) of each microcrack in each image (parallel to the articular surface) manually, using the measurement tools in Fiji. We 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 between the last two imaging phases. For each specimen, we also calculated the overall microcrack area density using the total number of microcracks in the \(3 \times 3\) tile grid centered on the articular surface.
4.3.6 Statistical Analyses

We used separate mixed regression modeling using SAS 9.4 (SAS Institute, Inc., Cary, NC) to evaluate the effects of genipin dose on microcrack density, and on the length, width, and depth of microcracks. We included 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 analyze microcrack propagation, we analyzed the subset of our data where we tracked individual cartilage microcracks over the course of the experiment using the same mixed-model regressions, but with specimen included as additional random factors (to account for non-independence at those scales).

4.4 Results

First, we confirmed our protocol successfully caused crosslinking in the network of collagen. After a 24 hour incubation, the cartilage transformed from its normal white and glossy state to fully saturated as black. The dark color is produced when genipin reacts with the amino groups, and is associated with with oxygen radical-induced polymerization of genipin, as found by Muzzarelli (2009).

Additionally, we viewed untreated and treated specimens via Raman Spectroscopy (Witec Alpha 300 Raman Spectrometer) at a wavelength of 785 nm and a laser power of 60 mW. We saw normal Raman signals in untreated specimens, and encountered fluorescence at 785 nm in treated specimens. This shows that crosslinking created structural changes; however, we were unable to quantify these changes due to the signal interference caused by the fluorescence phenomenon.

4.4.1 Microcrack Initiation

We present our measurements and statistical results in Table 4.2.
Can we prevent microcrack formation and/or propagation?

<table>
<thead>
<tr>
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<th>Measure</th>
<th>Mean</th>
<th>StdDev</th>
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<td></td>
<td>Length (µm)</td>
<td>135.21</td>
<td>114.23</td>
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<td>Width (µm)</td>
<td>7.59</td>
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<td>Depth (µm)</td>
<td>37.47</td>
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</tr>
<tr>
<td></td>
<td>Depth (µm)</td>
<td>54.58</td>
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</table>

Table 4.2: (Top) Crack density and dimensions for microcracks initiated by impact loading. (Bottom) p-values and estimates from the mixed model comparing treated and untreated specimens. We denote statistically significant differences ($p < 0.05$) with a *.

Genipin has no detectable effect on microcrack density and microcrack width, as shown in the box plots in Fig. 4.2. We found statistically significant differences in microcrack length, such that adding a dose of genipin causes increased length in initiated microcracks. Adding genipin did not cause a significant increase in microcrack width, but did show the same pattern of negative influence in microcrack depths. Therefore, microcracks initiated in specimens treated with genipin tended to be longer and penetrate deeper into the cartilage tissue.

4.4.2 Microcrack Propagation

We present our measurements and statistical results in Table 4.3.

We found that two doses of genipin caused significantly greater propagation than one dose of genipin, as shown in Fig. 4.3. Overall, one dose seemed to very marginally cause less propagation than non-treated specimens, but these were not statistically significant. We found trends indicating a decrease in the change of microcrack width compared to the
Figure 4.2: *Microcrack Initiation comparing treated and untreated specimens.* Box plots show the interquartile ranges (black lines) and median (red line) by different dose groups for microcrack density, length, width, and depth.
Figure 4.3: Microcrack Propagation using tracked microcracks post-impact to post-cyclic loading. Box plots show the interquartile ranges (black lines) and median (red line) by different dose groups for microcrack density, length, width, and depth.
4.5 Discussion

In this study, we induced microscale damage to the network of collagen using low-energy impacts, and propagated the microcracks in unconfined, cyclic compression. Microcracks may continue to propagate and become macroscale fissures that greatly disrupt the network of collagen and deteriorate the mechanical function of cartilage. Cartilage degradation is associated with OA; however it is unclear whether microcracks are a precursor to OA. Nonetheless, arresting or mitigating microcracks would provide insight in understanding the damage mechanisms behind cartilage degradation by means of damage to the network control. However, these changes were not statistically significant.

Lastly, we saw no change in propagation by depth among any of the treatment groups.

Table 4.3: (Top) Crack density and dimension for microcracks post-impact (PI) and post-cyclic (PC) for the different dose groups. (Bottom) p-values for each statistical comparison. We denote statistically significant differences ($p < 0.05$) with a *.

<table>
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<td></td>
<td></td>
<td>Length ($\mu m$)</td>
<td></td>
<td>142.29</td>
<td>101.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Width ($\mu m$)</td>
<td></td>
<td>7.41</td>
<td>16.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Depth ($\mu m$)</td>
<td></td>
<td>38.69</td>
<td>31.75</td>
</tr>
</tbody>
</table>

Table 4.3: (Top) Crack density and dimension for microcracks post-impact (PI) and post-cyclic (PC) for the different dose groups. (Bottom) p-values for each statistical comparison. We denote statistically significant differences ($p < 0.05$) with a *.

<table>
<thead>
<tr>
<th>Length</th>
<th>Width</th>
<th>Depth</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.12</td>
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<tr>
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<td>0.494</td>
<td>0.999</td>
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<td>0.968</td>
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<tr>
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<td>- +</td>
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<td>- +</td>
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<tr>
<td>0.311</td>
<td>0.411</td>
<td>0.2946</td>
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<tr>
<td>+ -</td>
<td>+ -</td>
<td>+ -</td>
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<tr>
<td>0.026†</td>
<td>0.029†</td>
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</table>
Can we prevent microcrack formation and/or propagation?

of collagen.

Genipin, a crosslinker derived from plants, has received attention as a means to enhance mechanical properties (Bonitsky et al. 2017, Lima et al. 2009), particularly in tissue engineered cartilage constructs, and as a potential repair for collagen damage. Its low cytotoxicity makes genipin an appealing option to stimulate structural changes that may potentially improve outcomes after severe mechanical loading.

In this study, we aimed to evaluate the efficacy of genipin as a treatment against microcrack initiation and propagation.

Our results do not support our hypothesis that treatments with genipin will improve the damage resistance of cartilage, specifically reducing the initiation and propagation of microcracks in the network of collagen under impact loading. We determined the natural crosslinker genipin is not an effective treatment for preventing or repairing damage within the network of collagen, at least in the doses tested.

4.5.1 Microcrack Initiation

We initiated microcracks using an impact energy density of 2.5 mJ/mm$^3$, and a velocity of 0.5 m/s. At this impact energy density, there is approximately a 40% probability of initiating microcracks in the network of collagen in human cartilage (Kaleem et al. 2017). The morphology of cracks initiated in this study matched those initiated in Santos et al. (2019) at similar impact energy densities.

Considering genipin as a “preventative” dose, adding a dose of genipin had no detectable effect on the total number of microcracks initiated as a result of low-energy impact loading. However, adding genipin did negatively alter the morphology of initiated microcracks, specifically in the length and depth of the resulting cracks. Genipin treatments to cartilage can significantly increase stiffness, as demonstrated in specimens treated with 10 mM for 24 hours by Bonitsky et al. (2017), and confirmed by increases in Young’s moduli in genipin-treated specimens (Lima et al. 2009). We aimed to improve the damage resistance
of the collagen network by crosslinking, the resulting increase in stiffness reduced damage resistance. Thus, the increased stiffness likely caused a reduction in ductility, which caused the cartilage to be less resistant to damage under impact.

Damage mechanisms in cartilage include cartilage fibril breaking (Henzgen et al. 1996) and/or peeling (Lewis and Johnson 2001). Genipin causes bonding between amino residues both between collagen fibrils via intermolecular crosslinking, and within collagen fibrils via intramolecular crosslinking (Lima et al. 2009). For microcracks forming parallel to the split-line direction, a common crack initiation direction (Santos et al. 2019), breaks in the network of collagen likely occurred between fibrils. Since crosslinking with genipin did not prevent microcrack initiation, the additional crosslinks formed by genipin were insufficient. We were unable to determine whether these were inter- or intra-molecular crosslinks.

4.5.2 Microcrack Propagation

After initiating microcracks using impact loading, we propagated them using unconfined cyclic compression at 1.44 Hz and an amplitude of 10% of the cartilage thickness. Under these conditions, microcracks typically tend to propagate by length and width, but not depth (Santos et al. 2019).

We found that treatment with genipin did not mitigate propagation of microcracks via unconfined cyclic compression. This contrasts results where genipin improved cartilage’s response to wear against 316L stainless steel at a rate of 4 mm/sec, with a stroke of 70N applied at a rate of 150 N/sec for a 10 mm path, and no load for an additional 8 mm (Bonitsky et al. 2017). These mechanical tests are fundamentally different, but do suggest that load rate and amplitude may affect genipin’s response to long-term cyclic loading.

We confirmed that genipin cannot heal macrocrack fissures, and determined it cannot heal or repair microscale fissures either.
Effects of One Dose

We found no statistical difference in propagation in non-treated specimens compared to specimens treated with one dose; however, we did observe different trends. We found signs of lessened propagation with respect to microcrack length and width between untreated and specimens treated with one dose ($p > 0.05$), regardless of the time of dose application. Therefore, applying one dose of genipin before mechanical loading as a “preventative” dose or after impact loading causes no difference in the propagation response.

Effects of Two Doses

Two doses caused significantly greater propagation compared to one dose. Adding a second dose of genipin seemed to cause further damage, which suggests the stiffness further increased with the second dose. Kaplan et al. (2017b) found that 12,000 cycles at 1.44 Hz causes an increased effective stiffness due to compaction of the specimens. Therefore, it is possible that the combination of these influences, namely potential increases in stiffness from compaction, coupled with changes in structure and stiffness from multiple doses of genipin, cause the severe increases in crack propagation we saw in the two-dose treatment group.

4.5.3 Limitations and Outlook

We were unable to quantify the degree of crosslinking in the network of collagen. Thus, our assessment of crosslinking was dependent on qualitative measures which indicated structural changes, but not the quantity nor mechanism.

We performed our experiments on cartilage explants and did not include cell culture or other measures to maintain cell viability. Our findings therefore only address the passive mechanical responses of the collagen network to external loads.

In some cases, we found edge cracks due to the specimen extraction process. Thus we
analyzed only a central region away from the edges to reduce edge effects, as in Santos et al. (2019).

Unfortunately treatments with genipin, and the resulting mechanisms of crosslinking, do not provide resistance to damage. There are other crosslinkers available such as carbodiimide or riboflavin/Ultraviolet-A treatments, which may enhance the damage resistance of cartilage and the collagen therein.

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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. 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.
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