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Mechanical characterization of articular cartilage degraded combinedly with MMP-1 and MMP-9

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ABSTRACT

Matrix metalloproteinases (MMPs) degrade the extracellular matrix (ECM) subsequently damaging cartilage and altering biomechanical properties. Collectively, MMPs cleave every ECM macromolecule. However, MMPs present complex substrate interactions and digest differing ECM components making it difficult to understand the individual role each MMP plays in cartilage degradation. To understand the combined impact MMPs have on cartilage biomechanical properties, MMPs from two subfamilies: collagenase and gelatinase were investigated. Three ratios of MMP-1 (c) and MMP-9 (g), c1:g1, c1:g0 and c0:g1 were considered. Cartilage plugs (n = 30) were collected from the femoral condyles of 3 bovine stifle joints. In groups of 10, samples were treated with MMP-1, MMP-9, or a combination. Samples were subjected to indentation loading up to 20% bulk strain and were assessed mechanically and histologically to determine the degradative impact. Young's modulus and peak load were compared between the control and degraded explants. In comparison to samples degraded by MMP-1 or MMP-9 individually, cartilage degraded with both enzymes resulted in a 9–15% greater reduction in stiffness and peak load. Individually, MMP-1 and MMP-9 have a minimum effect on cartilage micromechanical properties, but synergistically the two enzymes digest ECM components and a much greater degradative effect is observed.

1. Introduction

Degradation of articular cartilage is a prime hallmark of osteoarthritis (OA) and other arthritic diseases (Yuan et al., 2017). Proteases play a key role in the degradation of cartilage extracellular matrix (ECM) components leading to a loss of cartilage, a main feature in OA pathology (Murphy and Lee, 2005; Smith and Marshall, 2010; Troeberg and Nagase, 2012). The breakdown of major ECM macromolecules, such as type II collagen and proteoglycans, are triggered by enzymatic activity in which matrix metalloproteinases (MMPs) play a dominant role (Grenier et al., 2014). While a healthy balance of MMPs is essential to the cartilage remodeling and repair process, overexpression of MMPs has been studied as a potential indicator of the onset of OA as well as rheumatoid arthritis (RA) as the degradative enzymes facilitate damage to articular cartilage associated with arthritic disease pathways (Burrage et al., 2006; Heard et al., 2012). While the degradation of proteoglycans is considered an early and reversible process, the loss of tensile properties and structural integrity associated with the breakdown of collagen fibrils is believed to be irreversible (Kempson, 1979). Collagen damage at the articular surface lead by degradative enzymes has long been recognized as one of the earliest stages of disease progression (Buckwalter and Mankin, 1998; Panula et al., 1998; Weiss and Mirow, 1972) and is believed to be the point of no return (Buckwalter and Mankin, 1998).

In the traditional view, MMPs are collectively capable of degrading all components of the ECM, and the breakdown of ECM constituents by different MMPs is widely accepted as a fundamental of arthritic disease (Billinghurst et al., 1997; Mehana et al., 2019; Näkki et al., 2016). Upregulation of MMPs-1, 2, 3, 9, 13 and others has been detected in the synovial fluid in the pathways to cartilage destruction and is considered to be responsible for significant consequences in the progression of OA (Son et al., 2017; Zeng et al., 2015). Typically known as collagenases and gelatinases, these enzymes act in two steps; first collagenases (MMPs-1, 8 or 13) cleave and bind triple helical collagen molecules and denature the collagen fibrils (Powell et al., 2019; Rosenblum et al., 2010; Sarkar et al., 2012). Afterwards, gelatinases (MMPs-2, 9) digest the denatured fibrils (Atkinson et al., 2001; Collier et al., 2011; Rosenblum et al., 2010). In the present study, two key members of the MMP family, MMP-1 (interstitial collagenase) and MMP-9 (gelatinase B) were predominantly considered as representatives from each protease classes.

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However, MMP-13 (collagenase 3) has often been studied (Knäuper et al., 1996; Rose and Kooyman, 2016; Shiomi et al., 2010) as the major catabolic effector in OA and has been assumed to be more active than MMP-1 on type II collagen; but the level of MMP-1 expression is typically 10-fold higher than that of MMP-13 expression (Elliott et al., 2003), suggesting that the sheer amount of MMP-1 can overcome its comparative lack of efficiency in degrading the load-bearing type II collagen fibers. On the other hand, MMP-9, which acts as both collagenase and gelatinase (Laronha and Caldeira, 2020), can individually degrade non-collagen matrix components. Therefore, the current study aims to compare the role of two classes of MMPs, MMP-1 and MMP-9, when they act either independently or in synergy on cartilage degradation mechanism.

Enzymatically medicated changes in the ECM facilitated by the combination of MMPs-1 and 9 specifically can induce profound effects on the mechanical properties of the cartilage tissue. These changes are tied with diminishing function and altering mechanical loading in the joint. A limited number of *in vivo* studies based on contact forces was conducted on OA patients' knee joints to understand the correlation between the biochemical changes and mechanical loading (Kumar et al., 2013; Marouane et al., 2016; Meireles et al., 2017). Clinical cartilage testing is conducted utilizing long-term indentation tests (post-mortem) as a method of evaluating degenerative changes, such as OA, along with normal age associated changes observed in cartilage. Alternatively, transient indentation testing is of interest for *in vitro* studies to measure compressive stiffness along with mild cartilage degeneration (Bae et al., 2006, 2007). The connection between degraded matrix constituents and cartilage's mechanical integrity was discreetly investigated *in vitro* through enzymatic degradation (Grenier et al., 2014; Saarakkala et al., 2004; Schmidt et al., 1990; Töyräs et al., 1999; Wang et al., 2008). A variety of enzymes have been used in the past to simulate ECM degradation *in vitro* in the context of osteoarthritis (Grenier et al., 2014; Saarakkala et al., 2004; Wang et al., 2008), but these models are limited. Furthermore, the combined catabolic effect MMP-1 and MMP-9 have on the structural integrity (stiffness or load-bearing ability) of cartilage tissue has not been explicitly studied. Hence, the current *in vitro* study is designed to investigate the aggregate mechanical properties of cartilage treated with MMP-1 (c) and MMP-9 (g) independently and in combination to unfold the mutual effect of MMPs on cartilage biomechanics.

Unique MMP expression profiles and combinations of specific MMPs are the subject of interest in distinguishing between normal and arthritic cartilage. The primary goal of this study was to investigate *in vitro* the influence of MMP-1 (collagenase) and MMP-9 (gelatinase) on cartilage tissue to unfold the synergistic effect of the two selected MMPs on the cartilage pathology. Accordingly, an *in vitro* cartilage degradation model with ECM degeneration at the articular surface treated with three different ratios of MMP-1 (c) to MMP-9 (g) as 1:1, 1:0 and 0:1 was developed. Mechanical integrity such as the intrinsic, equilibrium Young's modulus (E) and the peak load at 20% bulk strain of the cartilage tissue was computed and compared under indentation loading for both intact and enzymatically degraded cartilage explants. This study enables a better understanding of the pathomechanics of degenerated cartilage tissue in the context of arthritic disease and may eventually pave the way for the development of targeted MMP inhibitors for therapeutic interventions.

2. Materials and methods

For this study, bovine articular cartilage plugs with subchondral bone attached were harvested from 3 different fresh stifle joints obtained from a local abattoir. All the stifle joints were collected from mature cows approximately ~2 years in age. The stifle joints were stored frozen at −50 °C until the time of sample extraction.

2.1. Sample preparation

Prior to sample extraction, the frozen stifle joints were transferred to a −20 °C freezer for 24 h, and the joints were equilibrated to room temperature following the 24 h-time period. The stifle joints were dissected at room temperature to isolate the femur and expose the sample extraction location—the medial and lateral femoral condyles (Peng et al., 2015). Using a mosaicplasty tubular chisel (Smith & Nephew 7209234) a total of 30 articular cartilage plugs of diameter 3.5 mm and 5 mm thickness with attached subchondral bone were harvested from the femoral condyles of the 3 stifle joints (Fig. 1). Throughout the tissue extraction process, the articular surface was kept moist with phosphate buffered saline (PBS) to prevent the articular surface from drying out. Cartilage quality was carefully assessed before and after the sample extraction through visible inspection, and cartilage with any surface defects or fissures were excluded from the current study. Until the time of testing, the extracted cartilage plugs were stored frozen at −20 °C in PBS moistened gauze and equilibrated to room temperature for 1 h at the time of testing (Ghassemi et al., 2019). Previous studies have shown that freeze-thaw cycles have a negligible impact on articular cartilage explant mechanical properties (Athanasiou et al., 1994; Riemenschneider et al., 2019; Szarko et al., 2010). Prior to testing, the thickness of each cartilage plug was individually assessed using a needle probe of the micromechanical tester, Mach-1 v500c (Biomomentum Inc.) Following assessment, the average articular cartilage thickness was measured 1.3 ± 0.08 mm (Fig. 2).

2.2. Enzymatic degradation of articular cartilage

The extracted articular cartilage plugs were individually placed inside a standard well plate for enzymatic treatment. A mixture of 2% agarose gel was prepared using solid agar (Sigma-Aldrich) and TAE buffer (Sigma-Aldrich). Using a pipette, warm agarose gel was carefully placed around each sample until only the top of the articular surface was exposed to the enzyme solution to simulate *in vivo* conditions. The agarose gel was allowed to solidify for 30 min before the samples were subjected to enzymatic treatment. To induce downward degradation starting at the articular surface, each sample was loaded with either individual activated human recombinant MMP-1, MMP-9 (BioVision, Milpitas, CA), or both depending on the treatment group. In groups of 10, samples were treated with MMP-1, MMP-9, or a combination of the two as shown in Table 1. The concentration scale of MMPs used for sample degradation was chosen based on studies utilizing immunoassays (ELISA) to detect the pro-form of the enzymes within the synovial fluid of both osteoarthritis and inflammatory arthritis patients. The level of proMMP-1 detected did not exceed 6 ng/mL in trials including over 300 patients (Tchetverikov et al., 2005).

2.3. Indentation testing

During the testing procedure, each sample was individually placed inside of an osteochondral core holder to secure the sample and prevent displacement. Samples were submerged in a PBS bath throughout the testing process to prevent drying of the articular surface (Riemenschneider et al., 2019). Articular cartilage explants were subjected to compressive stress-relaxation tests using a spherical indenter (1 mm dia.) in the Mach-1 testing apparatus. Each sample was compressed at a strain rate of 0.1%/sec until the total strain reached 20% (Patel et al., 2019), which typically falls under normal physiological conditions. At this time, samples were allowed to relax for 2 h before the test was repeated (Korhonen et al., 2002; Mixon et al., 2021). The change in the sample results between the control tests was monitored. After the relaxation period, samples experienced a negligible amount of change (≤2%). Each articular cartilage sample was tested in a control state prior to the introduction of degradative enzymes (MMP-1 and MMP-9 separately or combinedly) to establish a baseline. Following enzymatic

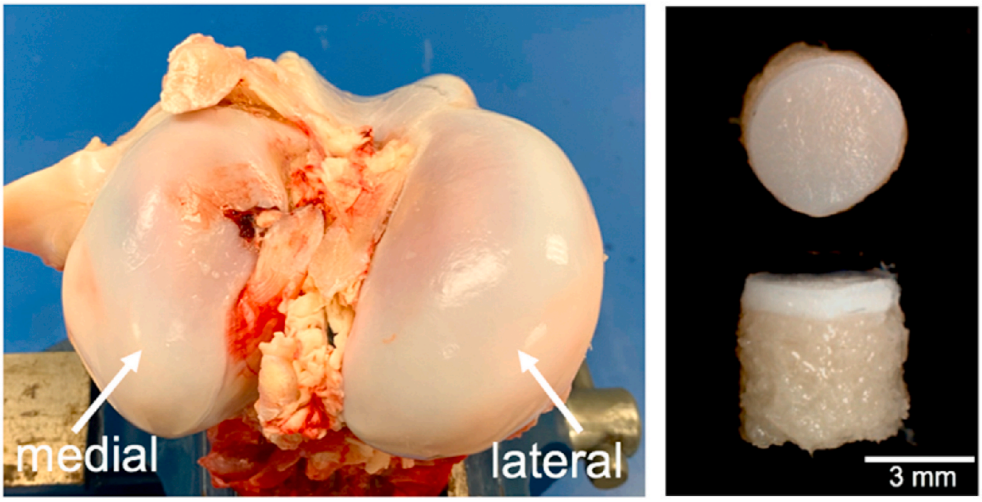

Fig. 1. **(a)** Medial and lateral femoral condyle locations used for sample extraction; **(b)** articular cartilage plug with attached subchondral bone.

Fig. 2. Micromechanical tester, Mach-1 v500c (Biomomentum Inc.) used to preform indentation testing of cartilage samples.

treatment, the same tests were repeated for each sample (i.e., 4 tests total per sample), which allowed each sample to serve as its own control. To investigate the differences in mechanical properties between healthy and degraded states, the peak load required to strain the sample and the Young's modulus (E) were determined from the indentation test.

Table 1
Combinations of enzymes MMP-1 and MMP-9 used to degrade articular cartilage samples.

MMP-1 (c) Concentration (ng/mL)	MMP-9 (g) Concentration (ng/mL)	c:g Ratio
4	4	1:1
4	0	1:0
0	4	0:1

2.4. Histological analysis

Prior to histological assessment, cartilage was carefully removed from the subchondral bone using a scalpel. Both healthy and enzymatically treated specimens were fixed at 4 °C in 10% phosphate buffered formalin for 24 h. Since decalcifying agents such as EDTA, nitric, and hydrochloric acids have shown the potential to extract or destroy proteoglycans present in cartilage resulting in diminished binding of Safranin O/Fast Green stain (Callis and Sterchi, 1998; Schmitz et al., 2010), these reagents were not considered in this study. After formalin fixation, samples were rinsed in running tap water for 10 min to prevent exposure to formalin pollution during the dehydration process. Dehydration was achieved through a series of graded ethanol changes (50%–100%); samples were cleared with xylene prior to being embedded in Paraplast Plus paraffin for histological analysis. A rotary microtome was used to create 12 μm thick sections that were mounted on plain glass slides for assessment. Mounted sections were then deparaffinized and rehydrated for either Safranin-O/Fast Green or Picrosirius Red staining using standard protocols (Schmitz et al., 2010). The intensity of the Safranin-O stain is proportional to the proteoglycan content present in the cartilage sample, while the Picrosirius Red intensity is proportional to the collagen content. The reduction of the histochemical staining in the degraded samples will confirm the loss in proteoglycan and collagen content compared to controls, thus providing evidence of effective enzymatic degradation of the cartilage.

To observe the degradation, micrographs of both control and degraded stained samples were obtained using AmScope biological microscope XSG Series under 10x magnification. The acquired images were subjected to a qualitative analysis led by three independent observers who were asked to grade the staining intensity as minimal (0), very

weak (1), weak (2), moderate (3), strong (4), and very strong (5) (Sun et al., 2012).

2.5. Statistical analysis

Statistical analyses were performed using OriginPro 2019b (OriginLab Corporation, Northampton, MA) (OriginPro 2019b). Numerical data are presented as *mean* ± *SD* and graphical results are *mean* with standard deviation (*SD*) as error bars unless otherwise stated. To assess the relationship between histological scoring and loss of mechanical integrity, the Pearson correlation was used in the current study.

3. Results

3.1. Histological analysis

Images of Safranin O and Picrosirius Red stained cartilage sections for each treatment group along with the control group are shown in Fig. 3. The untreated control groups show a very strong affinity for both Safranin O and Picrosirius Red staining indicating normal collagen and proteoglycan content (Fig. 3a and e). The c1:g0 treatment group has a strong uptake in Safranin-O stain while having a moderate uptake in Picrosirius Red as determined by the independent observers (Fig. 3c and g); this is indicative of a loss in collagen content only. The strong uptake of Safranin O suggests that the proteoglycan content for samples treated with the c1:g0 ratio is normal (Fig. 3c). In contrast, the c0:g1 treatment group portrays the opposite behavior where there is very strong affinity for Picrosirius Red stain and a weakened affinity for Safranin O uptake (Fig. 3d and h). The qualitative assessment resulted in the c0:g1 group receiving a grade of 3 meaning independent observers believe the Safranin O staining intensity to be moderate (Fig. 3d). This result depicts a loss of proteoglycan content for samples in the c0:g1 treatment group. The c1:g1 treatment group also received a moderate grade for Safranin O intensity but received a weak grade of 2 for Picrosirius Red intensity (Fig. 3b and f). In both the c1:g1 and c0:g1 treatment groups, an intensity gradient is visible spanning for the superficial zone, where the treatment was applied, to the deep zone for Safranin O stained sections (Fig. 3b and d). In comparison, this gradient can also be seen in sections stained with Picrosirius Red from the c1:g1 and c1:g0 treatment groups (Fig. 3f and g). Additionally, the Pearson correlation was employed to analyze the relationship between the loss in mechanical integrity and histological scoring. The resultant correlation coefficient of −0.97 implies that as loss of mechanical integrity increases, histological scoring decreases with the two instances being highly correlated.

3.2. Mechanical characaterization

Indentation testing has shown potential for assessing the mechanical properties of articular cartilage (Bae et al., 2006, 2007) and was utilized in this study to characterize the mechanical integrity of enzymatically degraded cartilage.

The load vs. time analysis shown in Fig. 4 indicates a pattern of reduced load bearing ability throughout the duration of the indentation tests when comparing degraded cartilage from each treatment group to their respective control. Out of the three treatment groups, articular cartilage explants degraded with a 1:1 ratio of MMP-1 to MMP-9 show the greatest reduction in load capacity over time. The c1:g0 and c0:g1 groups show a very slight decrease in load over time, with the greatest point of difference being the peak load. Each treatment group was evaluated in comparison to its control state to detect changes in both peak load and stiffness resulting from enzymatic degradation facilitated by isolated MMP-1, MMP-9, and a combination of the two enzymes in an equal ratio.

The violin plot in Fig. 5 shows the distributions of changes in Young's modulus of degraded cartilage for three treatment groups c1:g1, c1:g0, and c0:g1 as a continuous approximation of the probability density function, computed using kernel density estimation (KDE). The approximate frequency of data points in each region is indicated by the width of each curve. The violin plot portrays higher probabilities in wider sections, while narrow sections portray lower probabilities. Distribution differences are displayed in the violin plot through KDE (smoothed histograms) of each dataset. From the plot in Fig. 5, it is apparent that the distribution of the c1:g1 treatment group is unimodal, while the c1:g0 and c0:g1 groups display bimodal distributions.

The bar graph in Fig. 6 depicts the changes in peak load (*mean* ±*SD*) experienced by samples in each of the three treatment groups: c1:g1, c1:g0, and c0:g1. Throughout all three treatment groups, a consistent pattern has been shown as all 30 samples used in this study experienced a weakened peak load after subjected to enzymatic treatment. Articular cartilage explants in the c0:g1 degraded with only MMP-9 experienced a minimal decrease, on average ~4%, in peak load when compared to their control baseline data. In their respective control state, under 20% of applied compressive bulk strain, samples belonging the c0:g1 group experienced a peak load of 0.23 ± 0.08 N, which decreased to ~0.22 ± 0.07 N after enzymatic digestion by MMP-9. In their control state, samples belonging to the c1:g0 treatment group reached a peak load of

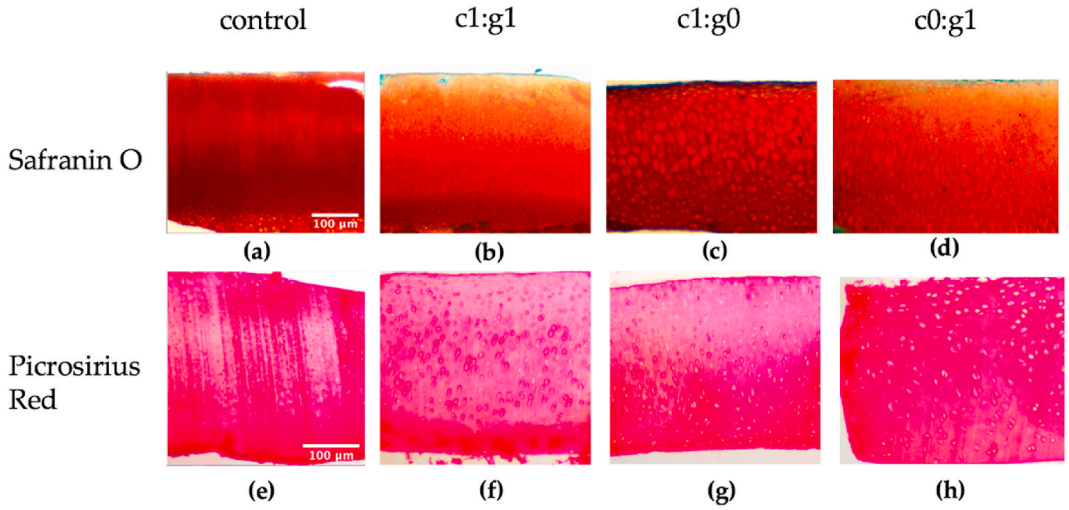

Fig. 3. **(a, e)** Change in staining intensity due to enzymatic degradation compared to controls; **(c, g)** in representative samples degraded exclusively with MMP- 1; **(d, h)** MMP-9; **(b, f)** or an equal combination of the two enzymes. **(a–d)** Staining was performed with Safranin O to observe proteoglycans; **(e–h)** and Picrosirius Red to observe collagen. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

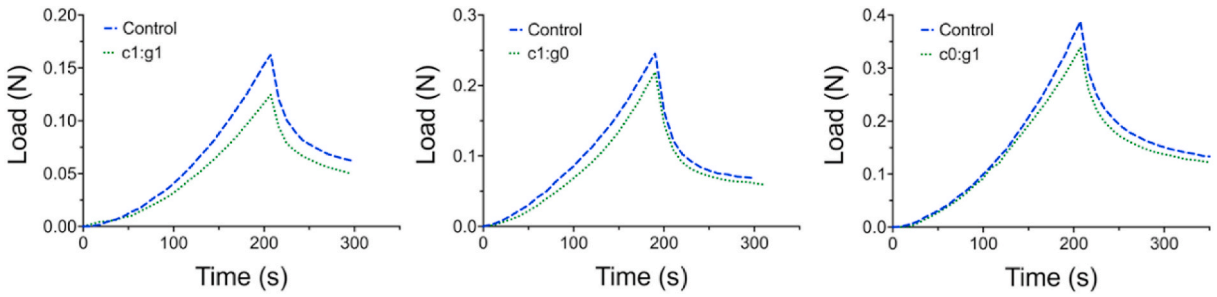

Fig. 4. Characteristic examples of the variation in load over time during indentation testing experienced by articular cartilage samples in their control and treated states for c1:g1, c1:g0, and c0:g1 treatment groups.

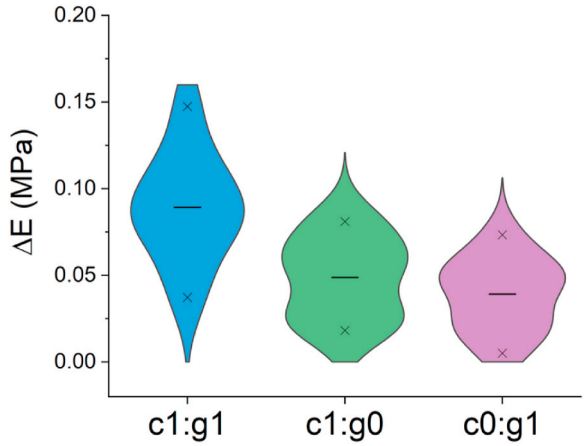

Fig. 5. Distribution of changes in Young's modulus (ΔE) of degraded cartilage for c1:g1, c1:g0, and c0:g1 treatment groups.

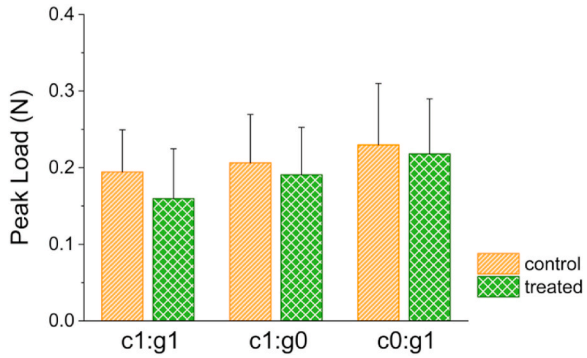

Fig. 6. Comparison of average peak load (*mean* ±*SD*) at 20% of applied bulk strain experienced during indentation by both healthy and enzymatically degraded articular cartilage samples.

0.21 ± 0.06 N. Following enzymatic treatment by MMP-1 exclusively, the samples belonging to this group experienced a ~10% reduction in the peak load subsequently resulting in a degraded peak load of 0.19 ± 0.06 N. Out of all three treatment groups, samples degraded with the combination of MMP-1 and MMP-9 in the c1:g1 group experienced the greatest reduction in peak load reached during indentation testing. The samples treated with a combination of both enzymes showed a ~19% reduction in peak load decreasing the peak load from 0.20 ± 0.05 N in their healthy state to 0.16 ± 0.06 N in their degraded state at 20% of applied bulk strain.

Fig. 7 shows the differences in stiffness between the control and enzymatically degraded states for each treatment group. Similar to the results observed in the peak load data, the c1:g1 treatment group

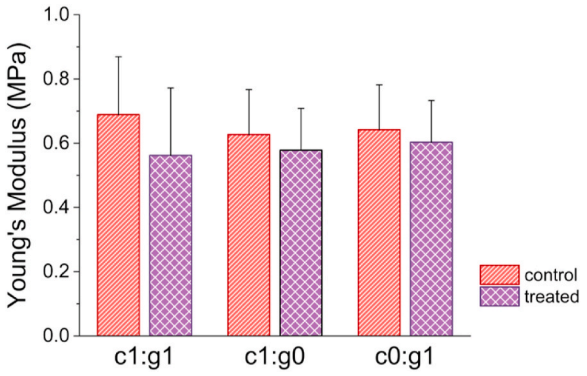

Fig. 7. Comparison of average Young's Modulus (*mean* ±*SD*) determined from indentation testing of both healthy and enzymatically degraded articular cartilage samples.

experienced the greatest reduction in Young's modulus ~19%. Prior to enzymatic treatment with both MMPs-1 and 9, the stiffness of the samples belonging to the c1:g1 group was 0.69 ± 0.18 MPa; after treatment the Young's modulus decreased to 0.56 ± 0.21 MPa. Respectively, samples in the c1:g0 and c0:g1 groups experienced an ~8% and ~6% decrease in stiffness following enzymatic degradation by either MMP-1 or MMP-9 exclusively. The c1:g0 group decreased in stiffness from 0.63 ± 0.14 MPa to 0.58 ± 0.13 MPa after collagen digestion by MMP-1 while the c0:g1 group decreased in stiffness from 0.64 ± 0.14 MPa to 0.60 ± 0.13 MPa after proteoglycan digestion by MMP-9.

4. Discussion

The matrix metalloproteinase family comprises a highly diverse group of enzymes, which are essential in regulating cartilage extracellular matrix remodeling; collectively, MMPs are able to cleave every ECM macromolecule (Mott and Werb, 2004). However, MMP combinations present complex substrate interactions, and differing ECM components are susceptible to digestion by multiple MMPs while not all ECM macromolecular components are vulnerable to cleavage by every MMP (Löffek et al., 2011; Mott and Werb, 2004). In human tissue, there are 23 MMPs (Nagase et al., 2006) that work in combination to facilitate a wide spectrum of both physiological and pathological processes. Due to the complex nature of the MMP family, it is necessary to investigate their precise role in tissue-specific context as well as their role in different stages of disease development or progression (Djurj et al., 2017). In arthritic diseases such as osteoarthritis and rheumatoid arthritis, two subfamilies of MMPs (collagenases and gelatinases) are the subject of interest for their destructive ability to denature collagen fibrils at the molecular level, decreasing the load bearing capabilities of the affected joint (Powell et al., 2019). In the current study, MMP-1 (c) and MMP-9 (g) were selected as representatives from the collagenase and

gelatinase family respectively to develop a meaningful *in vitro* cartilage degradation model to evaluate the synergistic effect of the two enzymes in the context of degenerative joint disease.

To investigate the degradative effect of the two MMPs in combination, cartilage samples were treated with either only MMP-1 (c1:g0), MMP-9 (c0:g1), or an equal combination of the two (c1:g1). Histological assessment revealed a greater degradative impact on cartilages treated with enzymes in combination with equal potency. The reduced intensity of both Safranin O (Fig. 3b) and Picrosirius Red (Fig. 3f) supports the fact that MMP-9 not only digests the denatured collagens (due to the action of MMP-1) but also plays a significant role in proteoglycan digestion. Because of proteoglycan digestion by MMP-9, more collagens are exposed in the middle and deep layers, which typically have more proteoglycan macromolecules, and MMP-1 can degrade the collagens in those layers. In comparison, the stain intensity and visible gradient from the surface downward (Fig. 3g) suggests that MMP-1 (c1:g0) can similarly degrade collagen content at the superficial layer. However, the degradative action of MMP-1 has been diminished spatially due to the increase of proteoglycan content in the subsequent layers, where the proteoglycans play a stabilizing role (Faisal et al., 2019) if they are not compromised. Proteoglycan digestion is also minimal (Fig. 3d) due to the isolated action of MMP-9 (c0:g1) at the superficial layer, where lesser amounts of proteoglycans are typically observed. The staining intensity gradient depicts the aggregate degradation of cartilage depends on enzyme penetration, which increased largely when both MMPs acted in synergy in the degradation mechanism.

The variability in stiffness changes (ΔE) for the combinations of enzyme demonstrates a central tendency for c1:g1. In contrast, the other two treatment groups show a greater difference in distributional shape (Fig. 5), illuminating susceptibility of cartilage damage due to MMPs being used in combination rather than individually. Resultantly, cartilage mechanical integrity was compromised (Figs. 6 and 7) when samples were treated with both MMP-1 and MMP-9 in the c1:g1 treatment group. Morphologically, the collagen fibrils connect to form a fibrillar network providing cartilage with tensile strength, which is highest in the superficial zone where tightly packed collagen fibrils are parallel to the articular surface (Smith, 1999). The collagen fibrillar network also endows cartilage with the ability to resist proteoglycan swelling, which contributes to cartilage compressive stiffness (Kempson et al., 1973; Pool, 2001).

Prior *in vitro* studies modelling degraded cartilage also resulted in a decrease in cartilage compressive modulus following treatment with type II bacterial collagenase (Grenier et al., 2014; Neidlin et al., 2019), which is able to cleave the collagen fiber network and decrease the proteoglycan content subsequently altering tissue biomechanical properties as well as inducing fibrillation at the articular surface (Grenier et al., 2014; Neidlin et al., 2019). Bacterial collagenase preparations contain both collagenolytic and proteolytic enzymes that effectively denature both cartilage ECM structural components. However, a much greater degradative effect was observed because bacterial collagenase initially cleaves all three chains at multiple domains along the triple helix, in comparison to the human collagenase used in this study, which cleaves only a single site at each chain (Grenier et al., 2014). The reduction in compressive stiffness following treatment with both MMPs-1 and 9 indicates similar findings; in combination MMPs-1 and 9 may cleave the type II collagen network and denature proteoglycans to reduce cartilage compressive stiffness in a mechanism similar to bacterial collagenase. Collagenases cleave the collagen triple helix, effectively denaturing the type II collagen network that provides the tissue with its compressive stiffness (Burrage et al., 2006). Gelatinases are responsible for cleaving the proteoglycan core protein-aggrecan; however, enzymes included in the gelatinase family will further degrade collagen after collagenase has cleaved the collagen triple helix (Burrage et al., 2006). In healthy tissues, aggregating proteoglycans that are occupying the interfibrillar zone may act as a barrier to protect the cross-linked collagen fibrils from enzymatic denaturation (Smith, 1999). With the aggregating proteoglycan barrier compromised by the presence of MMP-9, collagenase receives access to the type II collagen fibrils paving the way for further degradation of denatured collagen by gelatinase. Overall, implicating a greater destructive force when collagenase and gelatinase work in combination compared to their individual degradative impact on cartilage mechanical integrity.

Investigating the effect of degraded collagen fibrils on cartilage mechanics is experimentally challenging. The limited availability of fresh animal or human articular cartilage and the lengthy experimental protocols often require the cartilage to be stored before or during an experiment and undergone freeze-thaw cycle. Although it is not fully proven but several prior studies (Kempson et al., 1971; Kiefer et al., 1989; Swann, 1988) and anecdotal observations (Athanasiou et al., 1991; Elmore et al., 1963) suggests that no changes in biomechanical properties are observed due to freezing. However, freeze-thaw cycle may lead to minor degradation to the ECM, which can be detected by sensitive electromechanical measurements, but not by biomechanical or histological assessment methods (Changoor et al., 2010). The results from this study provide a promising insight into how the combination of MMP-1 and MMP-9 may impact the biomechanical response of articular cartilage in comparison with isolated effect. However, the results exhibit a large amount of variance in the control states of the samples, which may stem from the utilization of samples from multiple bovines. All the samples were harvested from mature bovines (~2 years in age) and a visual inspection was carefully performed to exclude any damaged cartilage. However, even small variations in bovine age could imply large differences in proteoglycan content as with increasing age, proteoglycan aggregates become shorter and have fewer attached monomers introducing differences in cartilage biomechanical properties (Buckwalter and Mankin, 1998). Gender related changes in the bovines may also account for the large variance between control groups. Recent studies show variations in the response of chondrocytes that are gender specific as knee tissues are mediated by sex hormones during the tissue remodeling and repair processes in both males and females (Boyan et al., 2013; Kinney et al., 2005). In the current study, a sample size of ($n = 30$) was used to investigate the biomechanical changes in cartilage treated with MMP-1 and MMP-9 in combination. The small sample sized used in the current study along with larger variance may be a limiting factor in observing statistical difference in the results.

While MMP mediated degradation of articular cartilage is widely accepted as a pillar of OA disease progression, experimental research investigating the combined influence of collagenase and gelatinase is limited. The degradative effect of isolated enzymes has been commonly measured (Toyoshima et al., 2001; Töyräs et al., 1999), and the experiments to measure the cumulative effect of enzymes on cartilage is very few but more appropriate for understanding degenerative diseases (Laasanen et al., 2003). Specifically, the synergistic role of MMPs on the aggregate tissue level properties has not been observed in any prior works to the best of our knowledge. The results of the current study provide insight into the combined degradative effect MMP-1 and MMP-9 have on articular cartilage biomechanical properties *in vitro*. Evidently, from the results it can be concluded that when MMP-1 and MMP-9 are combined, a greater degradative force is observed than when the enzymes are present individually. It can be assumed that alone MMP-1 cannot access the type II collagen that is protected by the proteoglycan aggregates; but when combined with MMP-9's ability to degrade proteoglycan core proteins both enzymes may effectively degrade the ECM and alter the cartilage macro-mechanical properties. In addition, aggregate degradation and the associated mechanical properties of cartilage largely depend on enzyme diffusivity that differs significantly when acted in synergy rather than individually. As MMPs have been studied extensively for their ability to degrade the components of the cartilage ECM, they are also the subject of interest as potential therapeutic targets (Cawston and Young, 2009). Investigating the effect of how combining MMPs impacts cartilage mechanical integrity provides necessary insights into potential therapeutic interventions through

direct MMP inhibition to prevent the progression of cartilage destruction associated with arthritic diseases such as osteoarthritis and rheumatoid arthritis.

Author contributions

Tanvir Faisal (T.F.), Ahmed Suparno Bahar-Moni (A.B.), and Allison Mixon (A.M.) conceived the idea, and T.F. and A.M. designed the study. A.M. performed the experiments and conducted histologic assessment. A.M. and T.F. conducted the statistical analysis and interpretation of data. All authors discussed the results and contributed to drafting this manuscript. All authors reviewed and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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