Development of a Post-Traumatic Osteoarthritis Model: Studies on the Mechanical and Biochemical Effects of Injury on Cartilage Tissue Analogs

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Articular cartilage can be damaged by traumatic injury, is slow to grow and repair after injury, and can eventually be thinned or completely worn out, resulting in debilitating pain and reduced joint motion. This condition, called posttraumatic osteoarthritis (PTOA), is highly prevalent and affects approximately 6 million individuals with both physical and economic consequences affecting the wellbeing of the patient. Various in vitro, ex vivo, and in vivo models have been developed to better understand different mechanical and biochemical properties of cartilage affected by PTOA. In this work, we create an in vitro model of PTOA by examining the effects of sudden impact and continuous physiologic loading on the structural and biochemical properties of both native and engineered cartilage. We also evaluate the anti-inflammatory and repair-inducing effects of various chemical compounds (anti-apoptotic, inhibitors of matrix loss) in this engineered cartilage model of impact. Our results showed that a few of these compounds can have a positive, therapeutic effects on construct properties after impact or physiological loading. To study these conditions further, combinatorial studies involving the use of both immediate injury and continuous loading, as well as those involving the use of multiple compounds are underway.

Index Terms-articular cartilage, articular joint injury, cartilage tissue analog, cartilage tissue engineering, osteoarthritis, post-traumatic osteoarthritis

INTRODUCTION

With the goal of mimicking the structure and functionality of articular cartilage, many studies have aimed to engineer cartilage using various combinations of cell types (MSCs, chondrocytes) and/or biomaterials (MeHA, agarose) [1-3]. These engineered constructs possess similar matrix composition to that of native cartilage, can maintain the chondrocyte phenotype, and can achieve mechanical properties that approach that of native tissue [3]. While many applications of engineered cartilage focus on repair of injured or degenerated tissue, these constructs can also be utilized for the study of disease pathogenesis, including that which occurs during the progression of osteoarthritis (OA). Specifically, we are interested in a subset of OA that occurs secondary to traumatic injury, post-traumatic osteoarthritis (PTOA). Characterized by joint pain, stiffness and loss of motion,

PTOA is caused by joint trauma that eventually leads to the degradation of articular cartilage [4].

This paper describes a subset of the work done by the Mauck and Dodge labs at the University of Pennsylvania in developing an *in vitro* PTOA model that uses CTAs to study disease mechanisms and evaluate potential therapeutics. In these studies, we used CTAs in combination with a high throughput mechanical screening (HTMS) device to deliver traumatic, compressive injury similar to that which typically leads to PTOA in native cartilage. In addition, we analyzed the effects of continuous pressure loading on various cartilage samples and observed their response both following and independent of injury. Finally, we assessed the effects of potential therapeutics in order to assess the repair response following injury.

I. BACKGROUND

A. Cartilage Damage and PTOA

Previous studies have shown that large strains (75% strain) at high rates (50% strain/s) induce injury in native cartilage [5-9]. Previous in vitro, ex vivo, and in vivo models of PTOA have observed pathological changes which include tissue swelling [5], cell death [8], reduced biosynthesis and loss of proteoglycans [5-6] due to increased matrix metalloproteinase

(MMP) activity [6-7]. and eventual loss of mechanical properties [9]. Traumatic injury initiates this progressive deterioration of articular cartilage that can lead to the development of PTOA, causing debilitating effects on the comfort and health of а patient. According to



Kramer et. al.,

the risk of PTOA after joint injuries ranges from 20% to more than 50% and affects approximately 6 million Americans of various ages [4]. Similarly, Anderson et. al. found that 14% of individuals who had a knee injury during adolescence and

young adulthood developed knee OA whereas only 6% of those who did not have a knee injury developed the condition [10]. As seen in Figure 1, while the immediate effects of joint injury may vary depending on the specific damaged tissues, they all can eventually lead to articular cartilage degradation and destruction [4]. Due to variations in the causes and pathways for PTOA development, further study is required to better understand the condition and possible treatments.

B. High Throughput Mechanical Screening Device



Previous in vitro and in vivo models of cartilage injury have consisted of testing a single sample at a time, making it difficult and extremely timeconsuming to observe the effects of injury

Figure 2: HTMS Device Set Up for Use

on native and engineered cartilage. This also made it more difficult to efficiently screen chemical libraries to find a potential treatment for PTOA.

In an effort to improve testing productivity and for use in previous cartilage impact-related studies, Mohanraj *et. al* of the Mauck lab, developed a custom high throughput mechanical screening (HTMS) device [11]. Seen set up and ready for use in Figure 2, the device is meant to maximize the efficiency of impact testing and minimize the number of individual impact protocols that must be run to complete each experiment. It highly speeds up testing and leads to more comprehensive and consistent results than other methods.

Our studies make use of this HTMS device to evaluate the properties of engineered cartilage and determine potentially beneficial treatments for PTOA.

C. Evaluation of Candidate Therapeutic Molecules

In order to determine which factors are effective at preventing engineered cartilage degradation, various candidate compounds that are thought to promote chondrocyte survival and matrix production, including anti-apoptotic agents and growth factors, were applied to both native articular and engineered cartilage with impact loading. Compounds such as P188 (amphiphile), ZVF (anti-apoptosis), and N-acetylcysteine (NAC, anti-oxidant), have all previously proven to have potentially therapeutic effects and seemed promising in preventing chondrocyte death and matrix degradation. From results of previous studies, such as the ones by Issac et. al. and Martin et. al. [12-13], we chose to analyze the therapeutic powers of these promising molecules. To do so, we performed histological analyses and various biochemical assays, such as those that test for glycosaminoglycan (GAG), collagen, DNA, and nitric oxide (NO). The presence of these substances, allow us to determine the effect of injury on the engineered constructs.

D. Continuous Pressure Loading

In order to measure the effects of repetitive pressure loading on the mechanical and biochemical properties of CTAs, we utilized a bioreactor described by Kraft *et. al.*

As shown in Figure 3, the bioreactor consists of a custombuilt pressure steel chamber that is attached to a 2.5-kip hydraulic piston mounted onto a materials testing machine (Instron Corp., Canton, MA) [14].

Materials Testing Machine



Figure 3: Diagram of the bioreactor used to apply the load

II. MATERIALS & METHODS

A. Preparation of Cartilage Tissue Analogs

More controllable and reproducible than native cartilage, (cartilage tissue analogs) CTAs are a cartilage surrogate that can be used to test the effects of impact. In these experiments, CTAs were prepared using a procedure similar to that described in Novotny *et. al.* [15]. First, we isolated chondrocytes from juvenile bovine cartilage and seeded them into a self-aggregating suspension culture model. Then, on polyhydroxyethylmethacrylate coated 96-well plates (1X10⁶ cells/well), the cells were allowed to coalesce and form a scaffold-free and high cell density biomass with hyaline cartilage chondrocyte phenotype and remarkable matrix productivity [15].



Figure 4: CTA used for injury studies

The resulting CTAs, such as the one seen in Figure 4, are comparable to native cartilage and the production method avoids problems commonly associated with scaffolds. such as degradation and toxicity [15-16]. All CTAs were cultured in DMEM (Mediatech, Inc., Manassas, VA) supplemented with 10% bovine fetal serum, penicillin (100 U/ml). streptomycin (100 µg/ml), amphotericin В (2.5)

µg/ml), 1% MEM vitamin (Mediatech, Inc.), ascorbic acid (50 µg/ml) (Gibco-Invitrogen), and HEPES buffer (25 mM) (Gibco-Invitrogen) [16].

B. High Throughput Mechanical Screening

1) Set-Up

Injurious compression was applied using the custom HTMS device described above. Figure 5 shows the design of the HTMS device, which consists of aluminum housing with linear bearings that guide the sensor-pressing plate and 48 force sensitive resistors (FSRs, seen on right) [11].



Figure 5: HTMS Device Parts

When the FSRs are contacted, the applied load causes them to experience a change in resistance. Measuring the voltage difference, the device which is connected to a DAQ board, can calculate the pressure and/or load applied to various cartilage samples during Instron (5848) controlled displacement of a loading platen (Instron Corp., Canton, MA).

The device can also be used to determine the compressive mechanical properties (equilibrium, dynamic moduli) of engineered tissues. Designed to fit a standard 48-well plate, the device and setup allows for high throughput testing of various cartilage samples and reduces the need for individual impact tests. Instead, each sensor is periodically tested and calibrated and the relationship between load and voltage is determined. This relationship can later be used to determine the load values for each sample being impacted.

In preparation for impact, a hole plate containing 48 PTFE indenters (B and C in Fig. 5) is placed on top of the 48-well plate containing the samples to be tested. During impact, the platen holding the sensor pushes down on the indenters, which in turn pushes down on the cartilage or constructs in the plate. The device measures the pressure caused by these interactions and the stresses can be calculated from these measurements. The results are then analyzed using a custom MATLAB code and the load response of CTAs during compressive injury can be depicted using a three-dimensional histogram, as seen in Figure 6.



Figure 6: Peak voltage recorded using custom LabView and MATLAB software illustrates uniformity of load response of CTAs during compressive injury.

2) Sensor Calibrations



Figure 7: LabVIEW Program Used for Sensor Calibration and Impact Testing

To ensure consistency during each impact session, individual sensors were periodically calibrated. Using the LabView program seen in Figure 7, we were able to observe the voltage differences, and therefore loads, detected by each sensel and save this data for later analysis. Data from each sensor calibration were compared to data from previous calibrations of each sensor, allowing us to calculate the error in our data and determine the efficacy of the HTMS device's data



Figure 8: Example of sensor calibration using sensel 3

collection. An example of the analysis done on each sensor can be seen in Figure 8. After each calibration session, we plotted the sensor's voltage vs. force and fit a curve to the relation. Over various calibration sessions, we compared these fitted curves to see similarities in calibration. Here, sensel 3 shows consistency among various uses with minimal variation until 1 volt; this is the range for which we use the sensor for mechanical testing. Due to this consistency among calibration data, we continued with the use of the device.

C. Cyclical Pressure Loading

While compressive loading with a platen such as the one used in the HTMS device may impede the transport of nutrients, restrict growth, or damage the surface of tissue culture, applying hydrostatic pressure avoids damaging cartilage in such a way [17]. In cases where we do not want to cause traumatic cartilage damage, but instead model loading patterns in physiological situations, we applied hydrostatic pressure to cartilage constructs following a method similar to that described in Kraft *et. al.* [14].



Figure 9: Hydrostatic Loading Bioreactor Set-Up. The pressurized chamber is placed inside a safety box in case of emergencies.

Cultures to be loaded were moved to customized vials that had previously had the top drilled open. The resulting hole in each cap was covered by a flexible silicone semi-permeable membrane that allowed for the transmission of hydrostatic pressure while simultaneously protecting constructs from contamination. These sealed tubes were then placed inside the pressure steel chamber of the custom built bioreactor described previously and shown in Figure 3. The chamber was filled with hydraulic fluid and sealed. All air was then extracted from the chamber. Using a materials testing machine, cyclical loading from 0 to 750 psi was applied for 3 hours 3 times a week for different long-term durations, depending on the experiments. To maintain the temperature, the chamber was warmed during the procedure to 37°C by sitting atop a controlled heating plate. The whole setup can be seen in Figure 9.

After each 3-hour loading session, the tubes were decompressed, removed from the chamber, and transferred back to their respective storage wells with fresh complete medium. Between sessions, the specimens were kept in an incubator at 37° C.

D. Studies on Cartilage Injury

To determine the effects of the of sudden impact, continuous pressure loading, and potentially therapeutic compounds on the properties of engineered cartilage, we conducted a group of studies that fused some of these different aspects together.

1) Impact and Loading in CTAs

The first study applied both single impact loading and continuous pressure loading to CTAs. The purpose of this study was to model the articular environment of an individual who suffers a traumatic injury to the cartilage in the area but continues to go about everyday activities, applying pressure and strain to the area.

CTAs were used for this study. They were divided into 7 groups, as shown in Table 1: impact + loading (group

	1	2	3	4	5	6	7
HTMS Impact	x	x	x				
Bioreactor Loading	x					x	
Harvested after Impact		x		x			
Harvested after 7 days	x		x		x	x	x
Treated with IL-1							x

Table 1: Impact and Loading Study Experimental Set Up

1), impact-no-loading harvested on day 1 (group 2), impact-no-loading harvested on day 7 (group 3), noimpact-no-loading harvested on day 1 (group 4), noimpact-no-loading harvested on day 7 (group 5), noimpact-loading harvested on day 7 (group 6), and interleukin-1-beta (IL-1 β) harvested on day 7 (group 7).

On the first day of the study, the three groups of CTAs that were to be impacted for compressive injury, groups 1, 2, and 3, received the compressive injury through the use of the HTMS device.

Those samples chosen to be impacted were compressed to a 50% final strain at a strain rate of 50%/s for a total compression time of 10 seconds. This protocol has shown to cause injury in CTAs but to not cause serious damage in the construct's structure. It was chosen for this study because of the nature of the type of injury we were trying to observe here, an acute injury in which a patient can continue to move normally after a short rest period.

After impact, all samples were put back into the incubator for four hours. Afterwards, all medium was collected and analyzed for soluble Nitric Oxide (NO), soluble glycosaminoglycan (GAG), and lactate dehydrogenase (LDH) (N=8). CTAs from groups 2 and 4 were harvested and analyzed for histology (N=1) or for

biochemical content (N=3), or frozen in liquid nitrogen (LN_2) for gene expression (N=4).

Immediately after medium collection and CTA harvesting, those constructs in groups 1 and 6 were placed in the bioreactor described by Kraft *et. al* [14] and were put through the pressure loading protocol explained previously. Loading was repeated two more times, after which CTAs in group 7 were treated with IL-1 β (1 µl/ml, 2 days of treatment) to be used as controls. All samples were left to incubate for 48 hours. Medium was then collected from each sample and analyzed for NO, soluble GAG, and LDH (N=9). CTAs from all remaining groups were harvested and analyzed for live/dead image analysis (N=1), histology (N=1), or biochemical content (N=3), or frozen in LN₂ for gene expression (N=4).

2) Poloxamer 188 and Impact in CTAs

We also analyzed the efficiency of Polaxamer 188 (P188) as a potential treatment for those with PTOA. Previous studies have shown that P188 can acutely restore the integrity of damaged chondrocytes [12] and our study aimed to analyze its effects on CTAs that had been affected by injurious compression.

Chondrocytes extracted from a single juvenile bovine donor were used to produce the CTAs using the method previously described. Four groups were created a control group, an impact group, a control + P188 (8mg/ml) group, and an impact + P188 (8mg/mL) group.

To determine peak load and stress for compression tests, a single sample CTA was impacted using the same impact protocol. All experimental samples were then compressed using the set-up determined by the results of the single-CTA test protocol.

The impacted samples were compressed to a 75% final strain at a strain rate of 50%/s for a total compression time of 10 seconds, a protocol previously shown to cause serious damage to CTAs' structural properties. P188 was added to the corresponding groups within 10 minutes of impact for 48 hours of continuous treatment. CTAs were then harvested at 12, 24, and 120 hours post-injury and analyzed for live/dead, histology, and biochemical content.

3) N-Acetyl-Cysteine, z-VAD-FMK, and Impact in CTAs

Since apoptosis is a critical aspect of cartilage degradation after injury, we analyzed the affects of two anti-apoptotic compounds. These two, antioxidant N-Acetyl-Cysteine (NAC) and caspase inhibitor Z-VAD-FMK (ZVF) were chosen because of the promising results of previous studies [13].

14.4 week-old CTAs were used for this study. They were divided into 7 groups: no impact and no treatment, no impact + ZVF (100 μ M), no impact + NAC (2mM), IL-1 β , impact with no treatment, impact + ZVF (100 μ M), and impact + NAC (2mM).

As before, to determine peak load and stress for compression tests, a single sample CTA was impacted using the same impact protocol. All experimental samples were then compressed using the set-up determined by the results of the single-CTA test protocol.

The impacted samples were compressed to a 75% final strain at a strain rate of 50%/s for a total compression time of 10 seconds. NAC or ZVF was added to the corresponding groups immediately after impact to maximize efficacy of apoptosis-prevention. These samples were treated for 48 hours, after which the drug was removed. CTAs were then harvested at 12, 24, and 120 hours post-injury and analyzed for live/dead (N=4), histology (N=4), biochemical content (N=4), NO content (N=4), soluble GAG content (N=4), and LDH content (N=4).

III. ANALYSIS

A. Biochemical Assays

1) DMMB Assay for Glycosaminoglycan Content

Found in the extracellular matrix, glycosaminoglycans (GAGs) are negatively charged polysaccharides secreted by cells. The concentration of GAGs in a sample can be determined using 1,9-dimethelene blue (DMB) dve. DMB dye binds to the negatively charged GAGs and absorbs light at 540-600nm wavelengths. We performed this assay to analyze GAG content in CTAs. In preparation for the assay, the dye was made by dissolving 1 g of sodium formate in 490 ml of deionized water before adding 1 ml of formic acid. The pH was regulated and maintained at about 3.5. A mixture of 8mg of 1,9dimethelene blue and 2.5 ml ethanol was added to the previous solution. Adding an additional 7.5 ml of doubledistilled water brought the total volume to 500 ml. At this point, the dye can be stored at 4°C in the dark until use. Dilutions of chondroitin-6-sulfate at concentrations of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 µg/ml were used as standards. The samples were diluted in each well using serial dilutions and 40µl of the sample was combined with 250µl of the DBM dye. Because of the dye's light sensitivity, the plate was then read immediately. To account for variation in sample size, the amount of GAG was normalized to DNA or wet weight per construct. This assay can be used to determine GAG concentration in a construct as well as the amount released into media (referred to as soluble GAG, sGAG).

2) PicoGreen Assay for DNA

PicoGreen is a fluorescent nucleic acid stain. When bound to DNA, the stain reaches its maximum emission at 530 nm and can be detected using a plate reader. Standards were made by diluting Lambda DNA stock ($100\mu g/ml$) into concentrations of 2, 1, 0.2, 0.15, 0.1, 0.05, 0.02, 0.005, 0.0005, and 0 $\mu g/ml$. The standards and samples were run in triplicate and a working solution of PicoGreen Reagent, 20x TE buffer and doubledistilled water was added to all wells. Then, the plate was incubated and read at wavelengths of 480nm excitation and 520nm emission.

3) Hydroxyproline Assay for Collagen



As seen in Figure 10, insoluble collagen makes up a large part of cartilage's structure and necessary is for cartilage's ability to withstand compressive forces [18]. The levels of

Figure 10: "The chondrocyte and extracellular matrix of articular cartilage showing the underlying collagen fibril

collagen, along with GAG, are a crucial determinant of repair tissue properties [19]. Hydroxyproline is a major component of collagen and is directly related to collagen's stability and insolubility [20]. This assay detects the presence of hydroxyproline, and therefore, of collagen.

Samples were first placed inside glass ampoules, after which 250 µL of 12N HCl was added to each. The ampoules were then sealed and left on a block at 110°C for 16 hours. The ampoules were then opened and placed in a NaOH dessicator or lyophilizer until completely dehydrated. Then, a stock buffer consisting of 50 g citric acid monohydrate, 12 ml glacial acetic acid, 120g sodium acetate trihydrate, 34 g sodium hydroxide pellets, and 1 L double-distilled water was prepared. Assay buffer was prepared by making a 1:10 dilution of this solution; 1 mL was added to each sample for resuspension. Then, 0.3525 g chloramine T was dissolved in 5.175 mL doubledistilled water. A solution of 6.5 mL propanol and 13.325 mL of the previously made stock buffer was made and mixed with the chloramine T solution. DMAB, 3.75 g was then suspended in 15 mL of propanol. Inside a hood, 6.5 mL perchloric acid was added to the solution. Hydroxyproline standards were made in concentrations of 0, 2, 4, 6, 8, 10, 20, 40, 60, 80, and 100µg/mL by mixing assay buffer with a 1mg/ml standard stock. Using a 96-well plate, 150 µL of 1:4, 1:16, 1:64 dilutions of the resuspended samples were made. 75 μ L of the previously made chloramine T reagent was added to each well and mixed. The plate was incubated at room temperature for 20 minutes before 75 µL of the previously created DMAB solution was added. The plate was then floated in a 60°C waterbath for 7 minutes and then in cold water for 5 minutes. At this point, the plate was read and analyzed for collagen content.

B. Live/Dead Analysis

After impact, loading, or treatment of CTAs, the resulting live and dead fractions of the cell population can be observed and analyzed to determine cell viability and construct health. Calcein AM can easily cross the membrane of live cells, where esterases quickly cause it to lose an acetomethyoxy group. At this point, the calcein cannot exit the cell and fluoresces green, indicating a living cell. In dead cells, the acetomethyoxy would not be removed and the calcein would therefore not fluoresce. Similary, ethidium homodimer-1 cannot penetrate live cells but can cross the nublear membrane of dead cells. In these cases, it can bind directly to the nucleic acid, fluoresce red and indicate a dead cell. Using a fluorescence microscope, images of the constructs can be taken and compared to analyze the effects of impact, loading, treatment, etc. on the health of the constructs' cells.

C. AnaSpec Assay for Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) degrade extracellular matrix proteins and are a large part of apoptosis. In this assay, MMPs cleave 5-FAM/QXLTM520 into 5-FAM and QXLTM520 [21]. When located close to one another the QXLTM520 prevents the 5-FAM from fluorescing. When MMPs separate the two components, 5-FAM can fluoresce and be measured using excitation/emission wavelengths of 490nm and 520nm.

D. Griess Assay for Nitric Oxide

A molecular messenger in various cell response pathways, Nitric Oxide (NO) has been identified as an important component of cell stress and inflammation mediated pathways. These pathways are of particular interest in our studies because they regulate cell death and matrix degradation and are the result of injury [22]. By measuring the amount of nitrite, a breakdown product of NO, in a solution, the Griess assay can determine the NO levels in media samples. After removing excess precipitate-producing extracellular components from the media samples using protamine sulfate, the assay could be run. A standard was prepared by diluting 1µl of 0.1M Nitrite Standard with 999µl of complete media. The standard was then diluted to create 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625µM standards. 50µl of the standards were placed in the first 3 columns of a 96well plate. Samples were placed in duplicate or triplicate in the left-over wells. Using a multichannel pipette, 50µl of Griess Reagent (modified, Sigma-Aldrich Co., St. Louis, MO) was added to each well. The plate was incubated at room temperature for 15 minutes and then read at 540 nm.

E. Gene expression

CTAs were flash frozen in LN₂, crushed in Trizol and then stored at -80°C until RNA extraction. A Qiagen RNEasy Mini kit was used during RNA extraction. Using a NanoDrop spectrometer, RNA quality and concentration was determined. Then, cDNA was synthesized using the BioRad Reverse Transriptase Supermix kit. Then, Real time PCR (qPCR) was used to quantify gene expression levels of extracellular matrix proteins, and cell stress molecules in all samples: impacted, loaded, treated, or controls. The particular genes that were observed were, Aggrecan (AGG), Collagen I (COLI), and Collagen II (COLII).

F. Assay for Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme released by cells following cell injury. By measuring the levels of LDH in

media, we can compare the levels of damage each construct undergoes. To prepare the assay reagent, 11 mL of assay buffer is added to 1 vial substrate included in the CytoTox-ONE[™] Homogeneous Membrane Integrity Assay (Promega Co., Madison, WI). In a 384 well plate, 25 µL of sample were mixed with 25 µL of reagent in triplicate. The plate was then shaken, incubated for 10 minutes in the dark at room temperature and read at wavelengths of 560nm excitation and 590nm emission.

G.Statistics

Statistical analysis for various assays was completed using a one-way ANOVA with Tukey's post hoc test with p<0.05.

IV. EXPERIMENTAL RESULTS

A. Impact and Loading in CTAs

1) Soluble GAG

Soluble glycosaminoglycan (GAG) content in media was measured using the DMMB assay previously described. From the data, shown in Figure 11, we



Figure 11: Soluble GAG concentration in impact and loading study. * p<0.05, ** p<0.01, *** p<0.001 vs respective control for same time point; # p<0.05, ## p<0.01, ### p<0.001 vs IL-1

observed that all samples that underwent any type of perturbation (i.e. impact, loading, or both) secreted similar levels of GAG into the media after seven days, showing that any disturbance can cause matrix loss. While these quantities were all significantly higher than those secreted by the controls, they were significantly lower than those found in the media of IL-1 treated samples. Another point to note is the low quantities of soluble GAG at the four-hour point in all groups. At this point, four hours after impact, all groups, impacted or not, showed similar levels of GAG release, showing that GAG loss is not immediate and instead does not begin until a certain period after injury.

2) GAG per Construct

We analyzed the GAG content in each construct and observed, as seen in Figure 12, that the highest GAG per construct was found in samples that were both impacted and loaded, followed by samples that were only impacted and then by those that were only loaded. The impact +

loading group and the impact but no loading groups had GAG concentrations that were significantly higher than



Figure 12: GAG per Construct in Impact and Loading Study * p<0.05, ** p<0.01, *** p<0.001 vs respective control for same time point; # p<0.05, ## p<0.01, ### p<0.001 vs IL-1

those of both the control and IL-1 groups.

3) Live/Dead

Live-dead staining shows the number of dead and living cells in each CTA. Living cells are seen after staining in green and dead cells in red. As seen in Figure 13, those CTAs that underwent both impact and loading experienced the most cell death, followed by those who



Loading Day 7

Figure 13: Live-dead staining allows us to compare the number of dead and living cells in each construct

only underwent impact. While some red is seen in the control image, this can be accounted for by regular controlled cell death (apoptosis) and this group experienced the least amount of cell death.

4) Gene Expression

Gene expression results indicate that there are no significant changes in gene expression for any of the treatment groups with the exception of the loading only group, which showed an increase in type II collagen expression. No other group had any difference in the expression of the type II collagen, aggrecan, or type I collagen genes.



Figure 14: Relative Gene Expression Aggrecan, Collagen 1, and Collagen

B. P188 and Impact in CTAs

Prior to impact loading, CTAs were impacted individually and average peak load and stress were calculated. The average peak load was 120.2 +/- 23.15 N and the average peak stress was 3.47 +/- 0.61 MPa. Since batch impacts consisted of 7-8 samples and to avoid overloading the 1 kilonewton load cell used for impact, the sensor plate was not used during impact.

1) Soluble GAG

GAG assays performed on media samples showed, as seen in Figure 15, that GAG release is similar for nontreated and P188-treated groups when comparing across time points. There were significant differences in GAG release between non-impacted and impacted samples but P188 had no effect on GAG release.



Figure 15: Soluble GAG in P188 study

* p<0.05, ** p<0.01, *** p<0.001 vs respective control for same time point ; % p<0.05 vs +P188 for same time point and condition

2) Percent GAG per Wet Weight

When analyzing GAG in CTAs in the P188 studies, we considered percent GAG per wet weight (Figure 16). We observed that this amount in the injury + P188 groups, compared to the injury only samples at the 120-hour time



Figure 16: Percent GAG per Wet Weight in P188 Study * p<0.05, ** p<0.01, *** p<0.001 vs respective control for same time point ; % p<0.05 vs +P188 for same time point and condition

point, was statistically higher. When comparing the control samples with the control + P188 group at the same time point, P188 caused a significant drop in percent GAG per wet weight.

3) LDH



Figure 17: LDH in P188 study

* p<0.05, ** p<0.01, *** p<0.001 vs respective control for same time point ; % p<0.05 vs +P188 for same time point and condition

Similar to the soluble GAG results, P188 had no significant effect on LDH production when compared to untreated samples in the same time point and condition, as seen in Figure 17. While there are significant differences between samples that underwent impact and those that did not, P188 did not prevent cell membrane disruption in impacted CTAs.

C. NAC, ZVF and Impact in CTAs

1) Percent GAG per Wet Weight

We tested all CTAs for the presence and concentration of GAG and results are shown in Figure 18. Results showed that at the 120-our point, NAC had a GAG content that was statistically significantly higher than the concentration in impact-only CTAs at the same time point. This shows that NAC had a positive effect in



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Figure 18: % GAG per Wet Weight in Anti-Apoptosis Study p<0.05 vs respective control for same time point; # p<0.05 vs IL-1 for same time point, % p<0.05 vs. specified group

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2) Soluble GAG

Using the DMMB assay for GAG, we quantified the amount of GAG in media and observed two cases where treated samples had soluble GAG levels that were significantly different than those of impacted samples, both shown in Figure 19. First, impacted samples treated



Figure 19: Soluble GAG in Anti-Apoptosis Study p<0.05 vs respective control for same time point; # p<0.05 vs IL-1 for same time point, % p<0.05 vs. specified group

with NAC secreted significantly less GAG at the 48-hour point than impacted and untreated samples at the same time point. We also observed a significant increase in GAG release in impacted samples treated with ZVF at the 24-hour time point when compared to the impact group at the same time point.

3) LDH

As seen in Figure 20, the LDH assay showed that ZVF-treated, impacted CTAs, when compared to untreated, impacted CTAs, significantly reduced the quantity of LDH as a percentage of construct wet weight

at the 24-hour point. No other treated sample caused a significant difference in LDH release. We also observed that the percentage of LDH per wet weight levels approached zero in all impacted samples at the 120-hour time point. This occurred at all time points in samples



Figure 20: LDH in Anti-Apoptosis Study p<0.05 vs respective control for same time point; # p<0.05 vs IL-1 for same time point, % p<0.05 vs. specified group that did not undergo impact.

V. DISCUSSION AND CONCLUSION

Our high throughput mechanical screening device and pressure loading bioreactor have shown to be extremely useful in studying the effects of injury and repair on cartilage surrogates.

A. Impact and Loading in CTAs

Injury and loading both increase GAG released to the media, but there doesn't appear to be a synergistic effect when both are applied to constructs. Interestingly, when you do apply either injurious or physiological loading, you see an increase in GAG within the construct, which is contrary to some of the previous results and needs to be further explored. It is possible that although GAG/construct increases, so does the wet weight with injury or loading, and this normalization may then show the same patterns of GAG decrease observed in our previous studies and in those with injury + drug as described below. With regards to gene expression, here we see that there does not appear to be significant changes in expression patterns for any of the matrix proteins tested, with the exception of the loading only group which showed an increase in collagen. It is possible that matrix-related genes do not become up-regulated within such a short time frame, and an extended time-course needs to be conducted in order to upregulation or down-regulation of these genes to be observed. Further analysis of MMPs, iNOS, and other catabolic mediators may further elucidate the pathways and mechanisms of injury and loading. Ongoing work on this project includes further analysis of catabolic mediators in the media (e.g. NO and MMPs), and a time course study of these molecules as they are released following injury and loading. Further avenues of investigation for this study will include loading constructs post-injury for extended periods of time (e.g. 3 weeks) or for extended cycles (e.g. 6 hours) to determine if these parameters affect construct response.

B. P188 and Impact in CTAs

CTAs treated with P188 showed GAG-in-media levels similar to those that did not receive treatment, showing no effect on reducing GAG release in injured constructs. Similar results were observed with concentrations of LDH in media, where there was no reduction of LDH release in those treated with P188. When considering GAG content in a construct through percent of GAG per wet weight however, P188 did seem to have a positive effect in reducing GAG release in impacted samples. This change was significant in the 120-hour time point. The lack of repair shown in the GAG and LDH released to media are inconsistent with previous studies that showed that P188 helped repair chondrocytes that had undergone traumatic injury [12]. Since we treated our CTAs with P188 for 48-hours after impact, it is possible that P188 must be applied for different lengths of time or during impact for it to have a positive effect on them. Future work for this study includes treating samples post-injury for different durations (e.g. 24 hours, 72 hours) and treating samples preand during injury to determine if these parameters affect construct healing.

C. NAC, ZVF and Impact in CTAs

CTAs treated with either NAC or ZVF showed varied levels of repair post-injury. Only NAC made a significant effect at moderating GAG reductions in impacted constructs and only at the 120-hour time point. Inconsistent with the GAG in construct results, the only situations in which either compound reduced GAG release were in 48-hour samples treated with NAC and 24-hour samples treated with ZVF. LDH release was also significantly reduced in only the ZVF-treated, 24hour CTAs. These variances in the NAC and ZVF treatments make it difficult to determine their effectiveness at reducing cell death or CTA degradation. In very few cases did either compound make a significant effect in this study, and in any case that they did, no one group was significantly beneficial in the regulation of all the biochemical compounds we analyzed. Because of this, there is a need for further studies on these anti-apoptotic compounds. Such future studies will include longer-term treatment and longer time points before media collection and CTA harvesting.

VI. FUTURE DIRECTIONS

The HTMS device has proven to be a worthy tool for causing traumatic injury on engineered cartilage constructs. In an effort to continue improving the quality of our data and the efficacy of our experiments, our group is working on ways to improve on the current system. For example, while the device is presently set up to accommodate a maximum of 48 samples, we are considering the scaling up to 96 sensors, a change that would double the efficiency of testing. Other improvements to both the software and hardware used to analyze impact and mechanical testing data are also being considered. Furthermore, the team plans on testing various previously unexplored sensor technologies to determine the one that best fits the device's function.

The Mauck lab is also working on new impact and repair studies dealing with other types of cartilage constructs (e.g. chondrocytes seeded in hyaluronic acid hydrogels). On top of that, other studies that test the effects of two other potentially therapeutic compounds, dexamethasone and TGF- β have begun and are currently ongoing. Lastly, our group is interested in developing an incubator-housed dynamic high-throughput bioreactor that would perform the same actions that our current HTMS device does but do so within an incubator.

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