

Temporal Effects of Impact on Articular Cartilage Cell Death, Gene Expression, Matrix Biochemistry, and Biomechanics

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Abstract—Articular cartilage injury can cause post-traumatic osteoarthritis, but early processes leading to the disease are not well understood. The objective of this study was to characterize two levels of impact loading at 24 h, 1 week, and 4 weeks in terms of cell death, gene expression, extracellular matrix biochemistry, and tissue biomechanical properties. The data show cell death increased and tissue stiffness decreased by 24 h following High impact (2.8 J). These degradative changes persisted at 1 and 4 weeks, and were further accompanied by measurable changes in ECM biochemistry. Moreover, following High impact at 24 h there were specific changes in gene expression that distinguished injured tissue from adjacent tissue that was not loaded. In contrast, Low impact (1.1 J) showed little change from control specimens at 24 h or 1 week. However, at 4 weeks, a significant increase in cell death and significant decrease in tissue stiffness were present. The constellation of findings indicates Low impacted tissue exhibited a delayed biological response. The study characterizes a model system for examining the biology of articular cartilage post-impact, as well as identifies possible time points and success criteria to be used in future studies employing intervention agents.

Keywords—Post-traumatic osteoarthritis, Mechanical injury, Glycosaminoglycan, Collagen, Tissue mechanics.

INTRODUCTION

Impact loading of articular cartilage leads to post-traumatic OA through effects on the cells and extracellular matrix (ECM) of the tissue. In such cases, cartilage function is believed to deteriorate as a result of chondrocyte death,⁵ changes in the biochemical characteristics of the ECM,³³ and weakening of the tissue's biomechanical properties.¹⁹ Several studies have identified similar characteristic responses of articular cartilage to mechanical injury, showing the level of impact or injurious compression correlates

with increasing cell death and degradative changes in the ECM,^{24,34} which ultimately manifest as detrimental changes in tissue biomechanical properties.^{13,14,28} The present study employs an *ex vivo* system of impact loading applied to full thickness, mature bovine articular cartilage, radially confined by surrounding tissue with underlying bone attached.

Several *in vitro* explant studies have investigated the nature and degree of cell death following mechanical injury, though the majority has looked only at early times, typically less than 7 days. Studies employing impact loading to chondral explants have demonstrated that cell death increases with increasing impact energy.^{22,24} Onset of cell death has been shown at stresses as low as ~3–6 MPa.^{11,15} In contrast, other studies suggest a threshold of 15–20 MPa for the initiation of cell death, with further increase in peak stress causing both increased amount and depth of cell death from the surface.^{34,48} In a relatively long *in vitro* study, canine cartilage explants were cyclically loaded at 0.3 Hz to 5 MPa at a rate of 60 MPa s⁻¹ and subsequently cultured for 3 weeks.³⁰ Cell death was found to increase with time in culture, be highest inside the impact area, and spread to adjacent tissue, demonstrating that damaged tissue can affect adjacent tissue. However, to our knowledge, no *in vitro* study of mechanical injury to articular cartilage has investigated cell death beyond 3 weeks.

Studies measuring the biochemical content of the ECM post-injury have found impact causes glycosaminoglycan (GAG) release and proteoglycan synthesis to decrease. In one study, a drop tower was used to apply several levels of impact to bovine articular cartilage explants to assess matrix loss and synthesis over 2 weeks of culture.²⁵ Results indicated both GAG and collagen were lost to the culture media, and an initial decrease in ECM synthesis recovered, with recovery time depending on intensity of the impact load. Sub-impact loading of bovine osteochondral explants also resulted in GAG release to the media over 4 days, with

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proteoglycan synthesis decreasing with increased loading.³⁸ GAG release has been shown to consist of both aggregating proteoglycans and degradation fragments.³⁹ Finally, a differential response of injured tissue compared to adjacent tissue has been observed for the ECM in terms of damage²³ and proteoglycan synthesis.³⁸

To begin to understand chondrocyte molecular biological responses to mechanical trauma, gene expression patterns have been examined up to 24 h post-injury. One study used gene chip technology to investigate expression changes following cyclical loading, and found 172 genes showed significantly different expression compared to controls.⁷ Using a custom-made bovine cDNA array and qRT-PCR, Chan *et al.*⁸ identified several genes with significantly altered expression 3 h post injurious compression to a peak stress of 30 MPa, some of which were matrix metalloproteinases. Another study used qRT-PCR, demonstrating increased expression of aggrecan, matrix metalloproteinase-1, tissue inhibitor of matrix metalloproteinase-1, and collagen type I after injurious compression.²⁹ However, no data on gene expression following injurious loading are available past 24 h.

Collectively, the studies described above underscore the detrimental effects mechanical injury can produce. Though cell death and matrix damage have been widely studied, only one *in vitro* study has looked at changes in mechanical properties, showing decreased stiffness following injurious loading. However, that study was only taken out to 3 days.²⁸ To further understand post-traumatic OA, better characterization of the biomechanical behavior of articular cartilage post-impact loading is needed to see if there is continued loss of tissue stiffness. Moreover, spatio-temporal characterization of gene expression profiles may aid in understanding early stages of the disease process. In this study we investigated, simultaneously, changes taking place in articular cartilage cell viability, gene expression, matrix biochemistry, and biomechanical properties at 24 h, 1 week, and 4 weeks following impact loading at two levels. Our goals were to (1) characterize established responses of articular cartilage to mechanical injury, as occurring in our system, and (2) investigate differential gene expression and matrix biochemical content in injured, compared to surrounding, tissue. We hypothesized that tissue stiffness would continue to decrease over time in culture following impact injury, which would be paralleled by increased cell death and GAG loss. Further, based on the differential response of the ECM from injured compared to adjacent tissue reported in the literature, we expected spatial variation in gene expression. The comprehensive approach of this study enables multiple correlations between different assays to be drawn, and a description of events taking place

post-impact from the molecular level to the tissue level to be made.

MATERIALS AND METHODS

Tissue Harvest and Articular Cartilage Impact

Tissue harvest and impact were performed as previously described.⁴⁴ Briefly, bovine elbow joints from 24 skeletally mature heifers were acquired from a local abattoir within 4 h of slaughter and harvested within 18 h. The distal portion of the proximal ulna was cut from the specimen parallel to the articular surface using a reciprocating saw (Ryobi, Hiroshima, Japan) leaving ~1 cm of attached underlying bone. Specimens were washed with sterile PBS and transferred *in situ* onto a sterile specimen clamp. The specimen clamp was then positioned in the impact instrument. The impact instrument consists of linear bearings with an attached sliding plate to which variable weight can be added. The plate slides down the bearings until striking an impact interface attached to an impact tip that is contact with the tissue surface. The tissue surface is rendered immobile by its positioning in the specimen clamp. The accelerometer is attached to the underside of the impact interface.⁴⁴ For this study two levels of impact were employed, which will hereon be designated as 'Low' (6 cm drop for an 18.4 N tup weight, 1.1 J) and 'High' (10 cm drop for a 27.8 N tup weight, 2.8 J). These levels were chosen such that the Low level does not visibly damage the cartilage, whereas the High level causes immediate grossly identifiable damage.⁴⁴ For this study, the impact area was defined as the cartilage tissue immediately below the 5 mm diameter impact tip. Impact was performed in the middle of the medial compartment with underlying bone still attached and 2–3 radii of separation between each impact area. For each joint, tissue was randomly assigned to the control, Low, or High impact levels.

Explant Culture and Processing

After impact, 8 mm diameter explants centered about the 5 mm impact area (described above) were removed using a dermatological punch and a #10 scalpel blade. Baseline explants (tissue without impact or time spent in culture) were randomly taken from six animals and processed on the same day as tissue harvest. For each animal the control, Low, and High impact explants were placed into individual wells of a 6-well tissue culture plate with 3 mL of media containing Dulbecco's modified eagle medium (DMEM) with GlutamaxTM (Invitrogen, New York), supplemented with 100 units/mL Penicillin (Biowhittaker, Maryland), 100 µg/mL Streptomycin (Biowhittaker),

50 $\mu\text{g}/\text{mL}$ Gentamycin (Invitrogen), 50 $\mu\text{g}/\text{mL}$ Kanamycin (Sigma, Missouri), 2.5 $\mu\text{g}/\text{mL}$ Fungizone (Biowhittaker), 0.1 mM non-essential amino acids (Invitrogen), 50 $\mu\text{g}/\text{mL}$ ascorbic acid, and 10% Fetal Bovine Serum (FBS) (Gemini Bioproducts, California). The explants were cultured for 24 h, 1 week, or 4 weeks with media changes at 24 h and every 2–3 days thereafter. Spent media were combined to yield collections corresponding to 0–24 h, 0–7 days, 8–14 days, 15–21 days, and 22–28 days and stored at $-20\text{ }^{\circ}\text{C}$ for later analysis.

After 24 h, 1 week, or 4 weeks in culture, explants were processed for qRT-PCR, matrix biochemistry, and biomechanics assays. First, from the center of the 8 mm explant, a 5 mm diameter punch was taken yielding the tissue area that was subjected to the impact load plus an outer ring (designated outside the impact area). Half of the outer ring was stored in RNeasy[®] (Ambion, Austin, TX) at $-20\text{ }^{\circ}\text{C}$ until RNA isolation. The rest was weighed and lyophilized for at least 48 h. Second, from the center of the 5 mm punch corresponding to the impact area, a 3 mm diameter punch was taken yielding biomechanics specimens plus an inner ring (designated inside the impact area). The 3 mm diameter portion of tissue was wrapped in gauze soaked with normal saline plus protease inhibitors (10 mM N-ethylmaleimide, 5 mM benzamide, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and stored at $-20\text{ }^{\circ}\text{C}$ until creep indentation biomechanical testing. The remaining inner ring was cut in half and processed in the same manner as the outer ring.

Viability Staining

Cell viability was assessed on a separate set of explants. Staining (Live/Dead[®] assay; Molecular Probes, Eugene, Oregon) was performed on 0.5 mm thick sections of 8 mm diameter baseline tissue or explants taken directly from culture at each time point, allowing assessment of impacted and adjacent tissue areas. Sections were incubated in 0.5 μL calcein-AM and 2 μL ethidium homodimer-1 per mL of complete media for 20 min at $37\text{ }^{\circ}\text{C}$. Viability pictures were taken with an epi-fluorescence microscope (Zeiss, New York), and images were analyzed using ImageJ (National Institutes of Health, Maryland). Percent viability was measured over a square area defined by the full thickness of the explant.

RNA Isolation and qRT-PCR

RNA isolation was performed simultaneously for all 16 groups: (1) baseline, (2–4) 24 h, 1 week, and 4 week culture controls (CCs), (5–7) 24 h, 1 week, and 4 week

Low impact inside the impact area (LI), (8–10) 24 h, 1 week, and 4 week Low impact outside the impact area (LO), (11–13) 24 h, 1 week, and 4 week High impact inside the impact area (HI), (14–16) and 24 h, 1 week, and 4 week High impact outside the impact area (HO). Tissue was homogenized in TriZol (Invitrogen, Carlsbad, CA) using a Polytron homogenizer (Kinematica, Switzerland), and RNA was isolated following the manufacturer's protocol. The RNA concentration of each sample was determined using a spectrophotometer (Nanodrop, Wilmington, DE), and 300 ng of RNA was used in the reverse transcriptase (RT) reaction. The RT reaction consisted of incubating 1 mM dNTPs, 1 mM random hexamers, RNase Block, and Stratascript RT enzyme (Stratagene, Inc. LaJolla, CA) at $42\text{ }^{\circ}\text{C}$ for 60 min, followed by termination at $90\text{ }^{\circ}\text{C}$ for 5 min. All samples were RT'd simultaneously.

qRT-PCR was performed for the following genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen type I (Col1), collagen type II (Col2), aggrecan (AGC), superficial zone protein (SZP), matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of MMP-1 (TIMP-1). The sequences for the primers and probes (Table 1) have been previously developed in our lab and were used with the Multiplex Master Mix kit (Qiagen, Valencia, CA).^{9,21} Briefly, the qRT-PCR consisted of 25 μL containing 1 μL cDNA from the RT reaction, 0.025 U/ μL HotStar-*Taq* polymerase, 3.5 mM MgCl_2 , 0.2 mM dNTPs, buffer, and primer (synthesized by Sigma-Genosys, Woodlands, TX) and probe (synthesized by Biosearch Technologies, Novato, CA) concentrations ranging from 50 to 125 nM depending on the triplex. The reaction conditions were optimized to yield efficiencies in the range 0.9–1. qRT-PCR was performed using a Rotor-Gene[™] (Corbett Research, Sydney, AU) with a 15 min denaturing step followed by 45 temperature cycles (15 s at $90\text{ }^{\circ}\text{C}$, 30 s at $60\text{ }^{\circ}\text{C}$). Since the amount of RNA used for each RT reaction was equal, it was possible to calculate abundance values and thereby compare gene expression quantitatively. The abundance level for the gene of interest (A_{GOI}) was calculated using the take-off cycles for the gene of interest (C_i) according to the following equation:

$$A_{\text{GOI}} = \frac{1}{(1 + E_{\text{GOI}})^{C_i}}$$

where E_{GOI} is the reaction efficiency for the gene of interest.¹

Biochemical Characterization

Biochemical assays were performed for sGAG, total collagen, and DNA content. For all assays, samples were digested in 2 mL papain solution² for 18 h, or

TABLE 1. qRT-PCR target gene primers and probes and dyes and quenchers.

Gene (accession number, product size)	Forward primer (5'–3') Reverse primer (5'–3') Probe (5'–3')	Dye Quencher
Glyceraldehyde-3-phosphate dehydrogenase (U85042, 86 bp)	ACCTCAAGATTGTCAGCAA ACGATGCCAAAGTGGTCA CCTCCTGCACCACCACTGCTT	FAM BHQ-1
Aggrecan (U76615, 76 bp)	GCTACCCTGACCCTTCATC AAGCTTTCTGGGATGTCCAC TGACGCCATCTGCTACACAGGTGA	Quasar 670 BHQ-2
Collagen type II (NM_174520, 69 bp)	AACGGTGGCTTCCACTTC GCAGGAAGGTCATCTGGA ATGACAACCTGGCTCCCAACACC	ROX BHQ-2
Superficial zone protein (AF056218, 77 bp)	CACCATCAGGATTCACTACACA TCACITTAACCTTCATTATGGAGGA CCCCTCAGAGTCCCTTATCAAGACA	ROX BHQ-2
Collagen type I (X02420, 97 bp)	CATTAGGGGTCACAATGGTC TGGAGTTCATTTTCACCAG ATGGATTTGAAGGGACAGCCTTGGT	Quasar 670 BHQ-2
Matrix metalloproteinase I (X74326, 82 bp)	CAAATGCTGGAGGTATGATGA AATCCGGGAAAAGTCTTCTG TCCATGGATGCAGGTTATCCCAA	Quasar 670 BHQ-2
Tissue inhibitor of matrix metalloproteinase I (NM174471, unknown)	GAGATCAAGATGACTAAGATGTTCAA GGTGTAGATGAACCGGATG AGGGTTCACTGCCTTGAGGGATG	ROX BHQ-2

until no tissue remained. sGAG content was tested using a 1,9-dimethyl-methylene blue colorimetric assay (Blyscan™ Sulfated GAG Assay kit, Accurate Chemical and Scientific Corp., Westbury, NY). sGAG released to the media was normalized to tissue volume, with volume calculated knowing the explant area (8 mm Ø) and thickness. Collagen content was measured using a modified chloramine T and dimethylaminobenzaldehyde colorimetric assay⁴¹ using SIRCOL Collagen Assay (Accurate Chemical, Westbury, NY) as the standard. Total DNA content of each sample was also assayed (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes, Eugene, Oregon). Cell number for a sample was calculated assuming 7.8 pg of DNA per chondrocyte.²⁶

Creep Indentation Biomechanical Properties

Prior to mechanical testing, the 3 mm diameter samples (see explant processing above) were thawed for 1 h at room temperature in normal saline with protease inhibitors. A creep indentation apparatus was used to determine the compressive creep and recovery behavior of the cartilage explants.⁴ Each sample was attached to a flat stainless steel surface with a thin layer of cyanoacrylate glue and equilibrated for 20 min in normal saline with protease inhibitors. The sample was then placed into the creep indentation apparatus, which automatically loaded and unloaded the specimen while recording the tissue's creep and recovery behavior. A tare load of 0.005 N (0.5 g), followed by a

test load of 0.02 N (2 g), was applied to the sample with a 0.8 mm diameter, flat-ended, rigid tip (50% porosity, 50 µm pore diameter). Specimen thickness was measured using a needle probe, force transducer, and linear variable differential transformer.⁴⁶ To calculate the specimen's material properties, a semi-analytical, semi-numeric, linear biphasic model was used.³⁶

Statistical Analysis

A sample size of $n = 4-6$ was used for gene expression. For cell viability, sGAG release and tissue content, total collagen content, DNA content, and creep indentation measurements, a sample size of $n = 5-6$ was used. Sample size was based on a power analysis ($\alpha = 0.05$ and $\beta = 0.2$) of pilot gene expression and creep indentation data allowing detection of a 40% change. Standard deviations associated with the other analyses (cell death and matrix biochemistry) were substantially less than those of gene expression or tissue stiffness, so the magnitude of change detected was less than 40%. We chose an $n = 6$ for each assay based on the power analysis, though some gene expression groups had $n = 4$ and some biomechanics groups had $n = 5$. The decreased sample number in these groups was due to the samples not being of sufficient quality to test, for example, samples with RNA isolations not meeting the recommended A260/A280 ratio. For gene expression data, each group (combination of time and impact/tissue area) was first normalized to the baseline abundance value of that gene to

yield fold change from native expression. Subsequently, a 2-way ANOVA was performed. For all other assays, a 1-way ANOVA was performed on all data, including baseline values (StatView, Abaqus Concepts, Berkeley, CA). If significance ($p < 0.05$) was found, a Student–Newman–Keuls post-hoc test was performed.

RESULTS

Impact Measurements

The impact instrument consistently reproduced two distinct levels of loading. Table 2 characterizes the Low and High impact levels in terms of the following metrics: peak stress, time to peak stress, and duration of impact. Comparing the two impact levels, peak stress and duration of impact were significantly different as seen previously.⁴⁴ Of note, these impacts satisfy the definition put forth by Aspden *et al.*³ in that they occur in on the order of ms at stress rates greater than 1000 MPa s⁻¹.

Gross Morphology and Cell Viability

As previously shown,⁴⁴ High impact caused immediate grossly identifiable damage that persisted over 4 weeks in culture. Low impact did not cause immediate damage, nor did any noticeable damage develop in culture. As expected, non-impacted CCs also showed no gross damage throughout the experiment. Figure 1 shows 100× magnification pictures of specimens stained for viability. Dead cells fluoresce red and living cells fluoresce green. Inside the impact area, cell death increased both with time in culture and level of impact. At baseline, almost all of the chondrocytes were alive (Fig. 1a), similar to what was seen in the non-impacted CC specimens at 24 h and 1 week (Figs. 1b and 1e). However, the 4 weeks non-impacted CCs began to show cell death in the superficial zone (Fig. 1h). An increased amount of cell death due to time in culture was also seen following Low (Figs. 1c, 1f, and 1i) and High impact (Figs. 1d, 1g, and 1j). Further, increasing impact level also caused increased cell death. At 24 h following Low or High impact cell

death was limited to the superficial zone. At 1 and 4 weeks cell death in the Low and High impact groups increased in the deeper zones of the tissue (Figs. 1f, 1g and 1i, 1j). Note the surface damage characteristic of High impact. There was little to no cell death noted outside the impact area at 24 h or 1 week for control, Low, or High impact. At 4 weeks, some cell death appeared in the superficial zone of adjacent tissue for Low and High impact, similar to the 4 week non-impacted CC described above.

The qualitative amount of cell death observed due to impact or time in culture was reflected quantitatively. Figure 2 shows a graph of percent cell death inside the impact area. Percent cell death in baseline specimens (2.6 ± 0.9) and the 24 h (1.8 ± 0.8) and 1 week (4.9 ± 3.1) CCs was not significantly different. However, at 4 weeks cell death in the non-impacted CC (10.5 ± 4.0) was significantly increased over baseline and 24 h CCs, though similar to the 1 week CC. At 24 h, both Low (9.9 ± 1.1) and High impact (18.8 ± 3.7) resulted in significantly increased cell death compared to baseline and the 24 h CC. Further, cell death following High impact was significantly increased compared to Low impact at 24 h. This trend was also seen at 1 and 4 weeks. Following Low impact, cell death did not significantly increase between 24 h and 1 week (11.4 ± 2.1), but, at 4 weeks (35.6 ± 5.1), Low impacted specimens showed significantly more cell death than was present at 1 week. In contrast, High impact resulted in significantly increasing cell death at each time point (27.8 ± 6.1 and 47.3 ± 7.7 for 1 and 4 weeks, respectively). Though cell death in controls did increase 9 percentage points from 24 h to 4 weeks, both Low and High impact boasted increases of 30 percentage points. Thus, the vast majority of cell death following impact was due to the injury and not time in culture.

qRT-PCR

GAPDH abundance showed no significant change for any group in this study ($p = 0.18$). Though quantitative, abundance does not have any physical meaning. Because of this, abundance values for each gene assayed were normalized to the gene's native expression (measured from baseline explants) to reflect

TABLE 2. Impact measurements for the Low and High levels.

Impact level	Tip weight (N)	Drop height (cm)	Energy (J)	Peak stress (MPa)	Time to peak stress (ms)	Duration (ms)
Low	18.4	6	1.1	3.1 ± 0.7^A	0.7 ± 0.3	1.6 ± 0.4^A
High	27.8	10	2.8	9.1 ± 4.1^B	0.5 ± 0.3	1.0 ± 0.3^B

Data given as mean \pm SD ($n = 10$ per impact level). Within a column, numbers with different superscripted letters ('A' and 'B') are significantly different ($p < 0.05$).

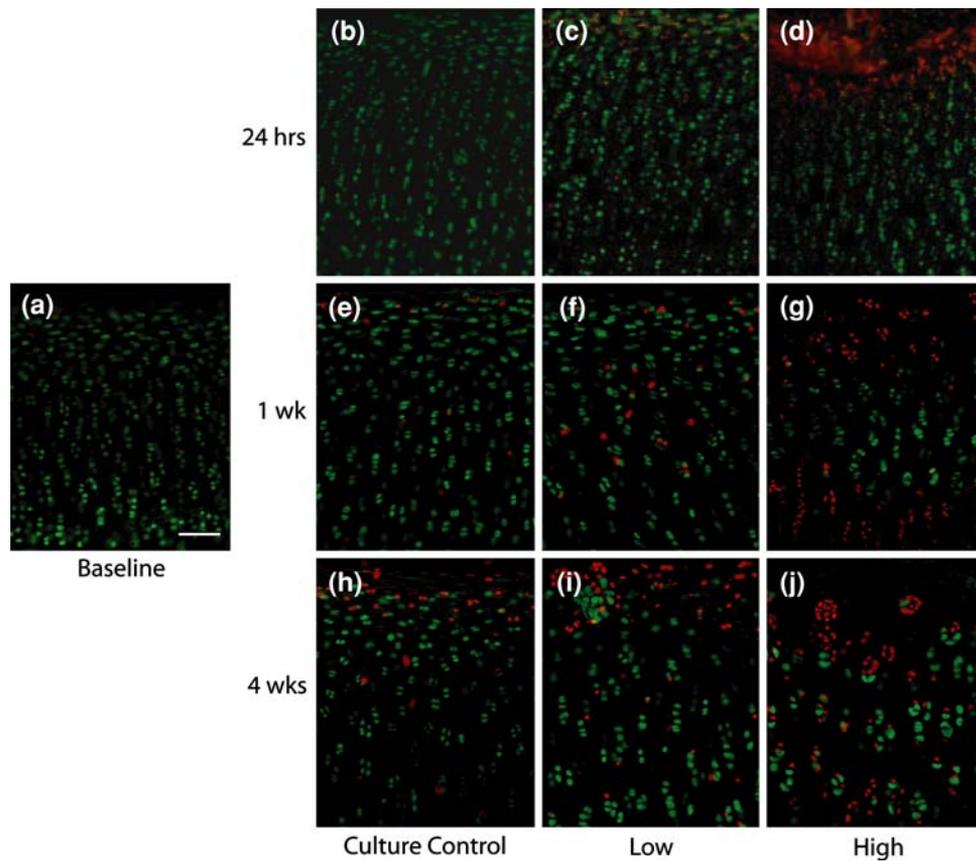


FIGURE 1. Viability staining. (a) Baseline; (b) 24 h culture control; (c) 24 h Low impact; (d) 24 h High impact; (e) 1 week culture control; (f) 1 week Low impact; (g) 1 week High impact; (h) 4 week culture control; (i) 4 week Low impact; (j) 4 week High impact. All images are from inside the impact area. Red stain indicates dead cells and green indicates living cells; scale bar 100 μm . Baseline explants were neither impacted nor cultured. Culture controls were not impacted. At 24 h cell death was limited to the superficial zone following Low and High impact. Cell death increased and spread to deeper regions of the cartilage over time.

fold change. This also enabled a 2-way ANOVA to be performed on time in culture and tissue area/impact level. Mean native abundance values were 2.1×10^{-4} , 2.2×10^{-6} , 1.9×10^{-6} , 3.9×10^{-7} , 3.0×10^{-9} , and 5.2×10^{-12} for Col2, SZP, AGC, TIMP-1, Coll, and MMP-1, respectively. This order for abundance agrees reasonably with prior work,²⁹ for which any differences may be due to the age of the animals used.

For each gene investigated, time was a significant factor, while only for SZP was tissue area/impact level a significant factor. Several additional differences were found at 24 h following High impact. Figure 3 shows the temporal expression patterns inside the impact area for AGC, Col2, and SZP (Fig. 3a) and Coll, MMP-1, and TIMP-1 (Fig. 3b). At 24 h AGC expression had increased 2.4-fold from its native value. Further, 24 h AGC expression in HO tissue was significantly increased over the 24 h CC, LI, and LO groups (Fig. 4a). AGC expression remained increased at 1 week, but significantly decreased expression at 4 weeks. Col2

expression at 24 h was 75% of its native expression level and then significantly decreased at 1 and 4 weeks. Similar to AGC, SZP expression was increased 2.8-fold at 24 h and expression in HO tissue was significantly increased over CC, LI, LO, and HI groups (Fig. 4b). However, between 24 h and 1 week SZP expression decreased significantly to an expression level only 4% of its native value. This decrease recovered to 40% at 4 weeks, though not significantly.

Coll expression changed most dramatically in this study compared to the other genes, so much so that the Coll data in Fig. 3b have been scaled down by a factor of 10 (i.e., the ~37-fold increase at 4 weeks is really a 370-fold increase) to fit on the same graph as MMP-1 and TIMP-1. Coll expression at 24 h dropped to 4% of its native expression. At 1 week, this drop recovered to exceed native expression 35-fold, though not significantly. Notably, while expression at 1 week in the non-impacted CC, LI, and LO averaged only a 1.8-fold increase, the average increase for HI and HO was

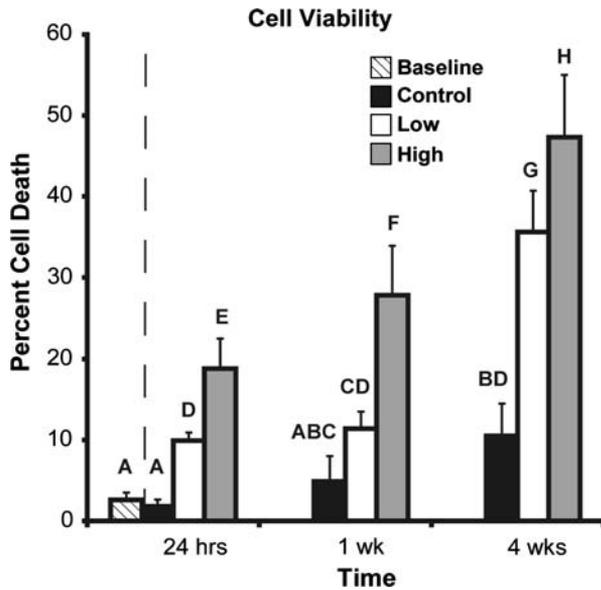


FIGURE 2. Percent cell death quantified from viability staining expressed as mean \pm SD. Groups not connected by the same letter are significantly different from one another ($p < 0.05$). To the left of the vertical dotted line is the baseline group, representing immediate characterization of native tissue. Cell death significantly increased at 24 h following Low and High impact. Further, there was a temporal increase in cell death which, following Low impact, was magnified between 1 and 4 weeks.

78-fold. Finally, all tissue area/impact groups at 4 weeks demonstrated a minimum 323-fold increase, with the average being 378-fold. MMP-1 expression at 24 h was 19 times its native expression level. Further, 24 h MMP-1 expression in HI tissue was significantly increased over the 24 h non-impacted CC (Fig. 4c). MMP-1 expression significantly decreased at 1 and 4 weeks. TIMP-1 expression paralleled MMP-1 expression with 24 h expression being significantly increased compared to both 1 and 4 weeks. Also, at 24 h, TIMP-1 expression in HO tissue was significantly increased over the 24 h CC, LO, and HI groups (Fig. 4d). There were no significant changes observed for the MMP-1/TIMP-1 ratio.

Biochemistry

Table 3 shows the results from characterization of matrix biochemical content at 1 and 4 weeks. Specifically, sGAG, collagen, and DNA content as percentage of wet weight, as well as %H₂O of the explants are presented. In terms of sGAG content, no significant changes were noted at 1 week. However, by 4 weeks LI and both HI and HO experienced significant decreases in sGAG content from baseline. Collagen content trended down at 1 week

compared to baseline for all groups. At 4 weeks, the downward trend was reversed with all groups showing slightly increased total collagen content compared to baseline. Notably, collagen content for HI was significantly increased from its respective 1-week value. The DNA content inside the impact area was significantly decreased for both impact levels compared to outside the impact area and the non-impacted CC at 1 week. There was a decrease in the number of cells present as time in culture increased. This was significant for the 4 week CC, and both LO and HO, but not for LI and HI. Percent water, reflecting tissue hydration and swelling, was not significantly different for any group.

Figure 5 shows cumulative sGAG release from the explants. Increased culture duration caused an increase in sGAG release. With respect to impact level, at 24 h there were no significant differences (1.8 ± 0.5 , 3.7 ± 1.1 , and $3.5 \pm 1.2 \mu\text{g mm}^{-3}$, for CC, Low, and High, respectively). However, at both 1 and 4 weeks High impact (23.9 ± 12.7 and $55.7 \pm 11.6 \mu\text{g mm}^{-3}$, respectively) resulted in a significant increase in sGAG release compared to non-impacted CCs (11.6 ± 4.2 and $32.5 \pm 7.2 \mu\text{g mm}^{-3}$, respectively) and Low impact (14.4 ± 5.1 and $38.2 \pm 4.4 \mu\text{g mm}^{-3}$, respectively). Finally, sGAG release showed a significant linear correlation with cell death ($R^2 = 0.68$, $p = 0.006$).

Biomechanics

Tissue stiffness (Fig. 6), as measured by aggregate modulus, was significantly affected by impact level and time spent in culture. The aggregate moduli of baseline specimens (687 ± 65 kPa) and the 24 h (654 ± 128 kPa) and 1 week (696 ± 202 kPa) non-impacted CCs were not significantly different, but, at 4 weeks tissue stiffness in the CC (392 ± 83 kPa), was significantly decreased compared to baseline and the 24 h and 1 week CCs. In terms of impact level, at 24 h High impact (402 ± 32 kPa) resulted in a significant decrease in aggregate modulus compared to the CC and Low impact (560 ± 156 kPa) specimens. This was also observed at 1 week (266 ± 68 and 503 ± 192 kPa for High and Low impact, respectively). It is notable, however, that at 4 weeks both Low (183 ± 37 kPa) and High (156 ± 59 kPa) impact explants experienced a significant decrease in aggregate modulus compared to the 4 week non-impacted CC, and were not different from each other. To help understand the causes for the loss of tissue stiffness observed, the aggregate modulus was correlated with cumulative sGAG loss and percent cell death. Significant linear correlations were found with $R^2 = 0.67$ ($p = 0.007$) and $R^2 = 0.87$ ($p = 0.0002$), respectively. There were no significant differences in tissue permeability (range $0.32\text{--}11.32 \times 10^{-15}$

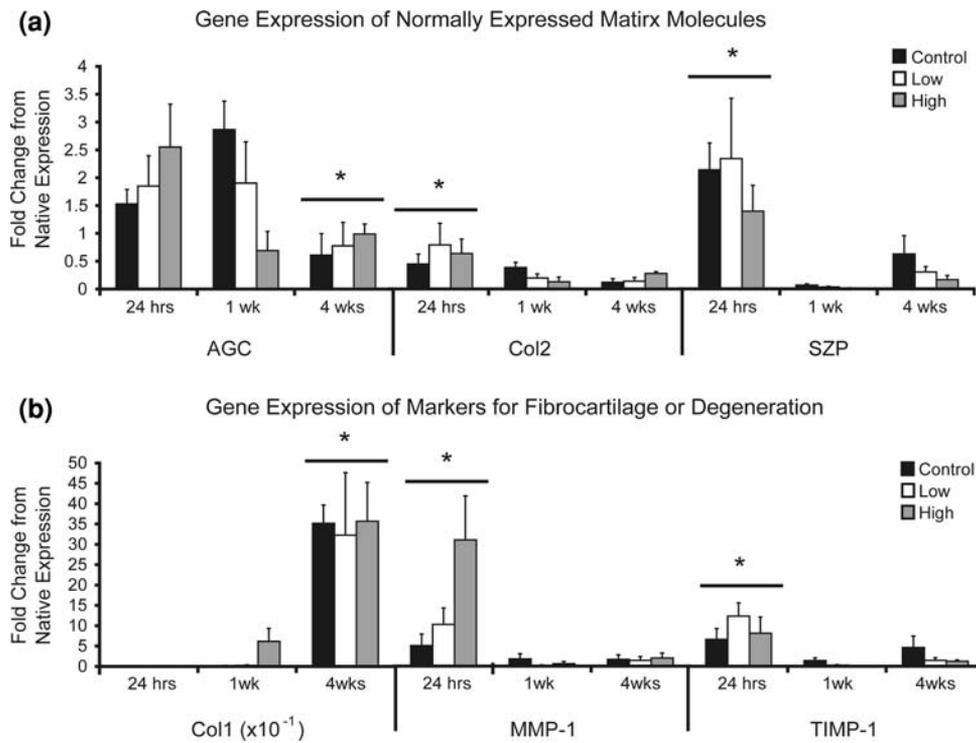


FIGURE 3. Fold change in expression from native values with respect to time for the various genes examined in this study. Data for Low and High impact are from tissue inside the impact area. A value of one implies no change from native expression. AGC = aggrecan, Col2 = collagen type II, SZP = superficial zone protein, Col1 = collagen type I, MMP-1 = matrix metalloproteinase-1, and TIMP-1 = tissue inhibitor of MMP-1. Values are mean ± SEM. * indicates significantly different from other time points for the gene of interest ($p < 0.05$). Note, to accommodate scale, the actual values for Col1 presented in the figure are one tenth of what was actually measured. Col2, SZP, MMP-1, and TIMP-1 all experienced significantly decreased expression at 1 and 4 weeks compared to 24 h. AGC expression significantly decreased between 1 and 4 weeks whereas Col1 expression significantly increased.

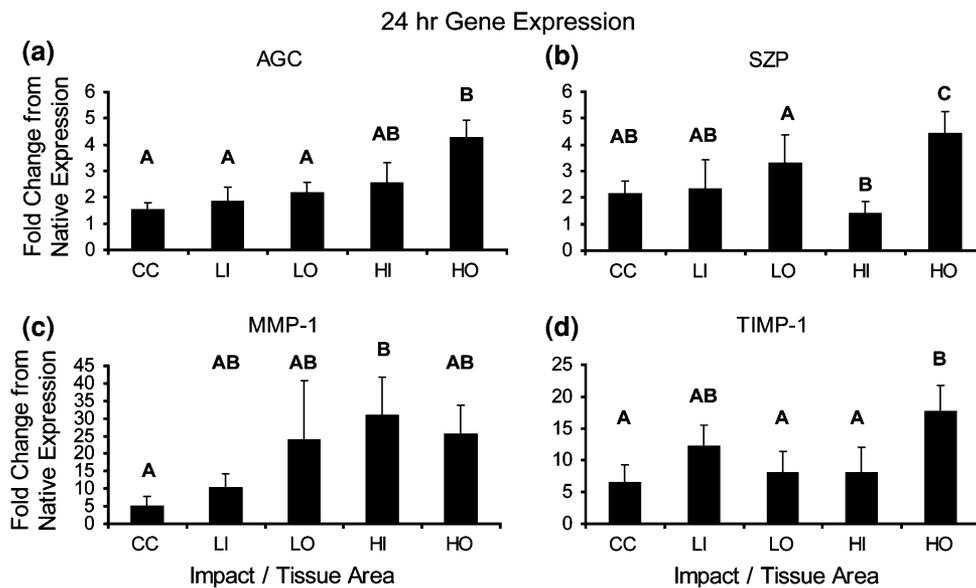


FIGURE 4. Fold change in expression from native values at 24 h for AGC (a); SZP (b); MMP-1 (c); and TIMP-1 (d) for all impact levels and tissue areas. CC = Culture Control, L = Low impact, H = High impact, I = Inside impact area, O = Outside impact area. Values are mean ± SEM. Within a panel, groups not sharing a similar letter are significantly different from one another ($p < 0.05$). AGC, SZP, and TIMP-1 expressions in tissue adjacent to High impact were significantly increased compared to the culture control. Also, SZP expression for High impact inside the impact area was decreased compared to outside the impact area. Finally, MMP-1 expression was significantly increased over culture control in tissue subjected to High impact. There were no significant differences among the groups for Col1 or Col2 at 24 h.

TABLE 3. Data for tissue matrix biochemical content.

Group	GAG content (% WW)	Collagen content (% WW)	DNA content (Cells × 10 ⁶ /g WW)	% H ₂ O
Baseline	5.5 ± 0.5 ^A	13.6 ± 1.6	55.7 ± 7.2 ^{A,B,C}	75.0 ± 1.1
1 week				
CC	4.7 ± 1.4	11.8 ± 1.8	61.8 ± 15.8 ^A	77.5 ± 3.1
LI	4.7 ± 0.5	12.9 ± 3.6	38.4 ± 6.4 ^D	77.6 ± 1.5
LO	5.0 ± 0.6	11.7 ± 1.2	59.6 ± 10.0 ^{A,B}	76.9 ± 2.6
HI	4.1 ± 0.6	10.8 ± 1.2 ^A	37.2 ± 5.5 ^D	76.3 ± 2.4
HO	4.8 ± 0.8	11.4 ± 1.1	58.7 ± 5.7 ^{A,B}	76.3 ± 2.6
4 weeks				
CC	4.5 ± 0.6	14.0 ± 1.2 ^B	46.2 ± 4.9 ^{B,C,D}	74.9 ± 1.8
LI	3.7 ± 0.9 ^B	14.0 ± 1.8	38.4 ± 12.5 ^D	72.3 ± 6.3
LO	4.0 ± 0.8	14.4 ± 1.7 ^B	44.0 ± 10.5 ^{C,D}	75.0 ± 2.0
HI	3.7 ± 1.6 ^B	14.0 ± 1.4 ^B	31.9 ± 10.6 ^D	73.4 ± 5.1
HO	3.5 ± 1.3 ^B	14.4 ± 1.5 ^B	42.7 ± 5.4 ^{C,D}	73.9 ± 3.0

Values in table are mean ± SD (*n* = 6). WW = wet weight, CC = Culture Control, L = Low impact, H = High impact, I = Inside impact area, O = Outside impact area. Within a column, groups not sharing a similar letter are significantly different from one another (*p* < 0.05). GAG content significantly decreased from baseline in the 4 week LI, HI, and HO groups. Collagen content significantly increased from 1 to 4 weeks in HI tissue.

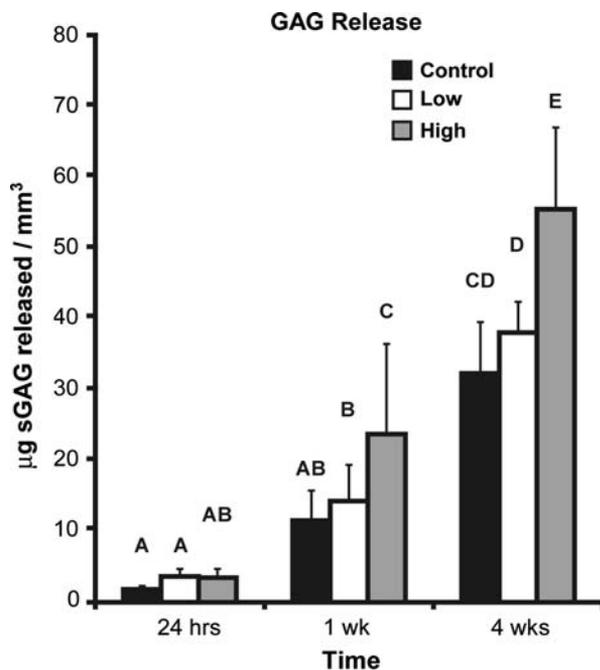


FIGURE 5. Comparison of cumulative GAG released to the media for the impact levels and time points examined in this study. Values are mean ± SD. Groups not connected by the same letter are significantly different from each other (*p* < 0.05). GAG release significantly increased with time in culture. Further, GAG release following High impact was increased over non-impacted controls at the 1 and 4 week time points.

$m^4 N^{-1} s^{-1}$). Analysis of Poisson's ratio (range 0.0–0.32) showed that 1 week Low impact was significantly decreased from baseline but recovered by 4 weeks.

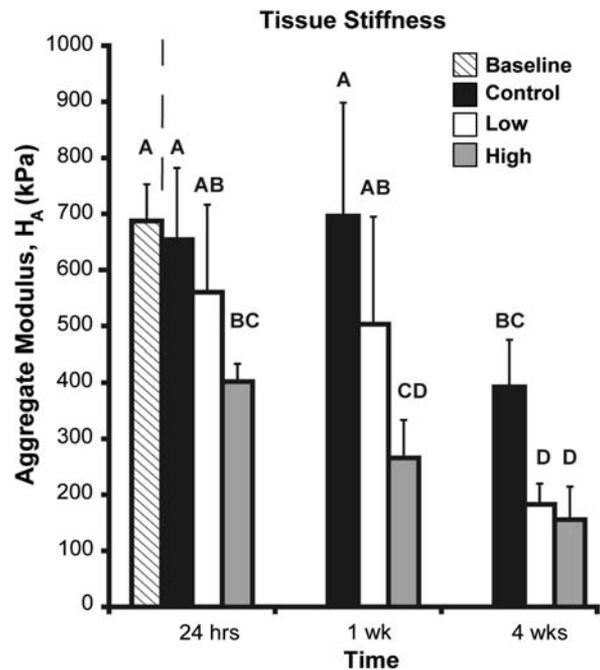


FIGURE 6. Comparison of aggregate moduli for the impact levels and time points examined in this study. Values are mean ± SD. Groups not connected by the same letter are significantly different from each other (*p* < 0.05). To the left of the vertical dotted line is the baseline group, representing immediate characterization of native tissue. At 24 h, the stiffness of High impacted tissue was significantly decreased compared to the non-impacted culture control. This trend remained at 1 and 4 weeks. Though similar to controls at 24 h and 1 week, Low impacted tissue at 4 weeks had significantly decreased stiffness compared to controls and was similar to High impacted tissue.

DISCUSSION

In this study we characterized the response of articular cartilage following two levels of impact loading at 24 h, 1 week, and 4 weeks post-impact. While High impact resulted in early degenerative changes, Low impact exhibited a delayed biological response. Further, to the authors' knowledge, this study is the first to examine gene expression following mechanical injury at time points greater than 24 h (including injured and adjacent tissue sites) and to simultaneously assess gene expression and protein levels. The data demonstrate that a High impact load of 2.8 J resulted in cell death and decreased tissue stiffness at 24 h. At 1 and 4 weeks, these degenerative changes remained, and further tissue degradation was manifested by increased GAG release from, and an accompanying decrease in GAG content measured in, the tissue. In contrast, a Low impact of 1.1 J caused little change from the non-impacted culture controls at either 24 h or 1 week, but, at 4 weeks, Low impact showed similar detrimental changes as seen following High impact. This was most noteworthy for a

measurement of tissue functionality, where the aggregate moduli following Low and High impact declined 73% and 77% from baseline, respectively. Moreover, there were specific changes in gene expression resulting from High impact that manifested at the early time points. Finally, results from cell viability, matrix biochemical content, and gene expression indicated differences between tissue subjected to the mechanical load compared to adjacent tissue.

The impact levels in this study meet the definition set forth by Aspden *et al.*³ in that they occur in less than 30 ms and are greater than a stress rate of 1000 MPa s⁻¹. Other studies have used higher peak stresses but report the impact measurements in various ways. Repo and Finley⁴² found the peak stress threshold for gross damage of articular damage in the presence of underlying bone to be 25 MPa at strain rates of 500 and 1000 s⁻¹, whereas Haut²⁰ found 25 MPa to cause subchondral bone fracture. Further, Duda *et al.*¹¹ found cell death at peak stresses as low as 3 MPa, though others have suggested 15–25 MPa as a threshold for the initiation of cell death.^{42,48} Thus, while the peak stresses (3.1 and 9.1 MPa) measured in this study were not as high as some studies, the differences with respect to gross damage and cell death are likely due to the very high stress rates (~4420 and 18,200 MPa s⁻¹ for Low and High, respectively). In a similar drop tower experiment to cartilage without underlying bone,²³ stress rates of ~15,000 MPa s⁻¹ were found, but with a peak stress of 22 MPa. The difference between peak stresses between the experiment just described and ours is indirect evidence that underlying bone attenuates the peak loads experienced by cartilage, thus protecting it from injury, a function supported by previous work.^{24,27,40}

In this experiment, the amount of cell death increased with time and impact level, as well as exhibited a spatio-temporal pattern. It has been previously shown that loading rate affects cell death such that increased rate causes less cell death,^{12,34,35} though these studies explored rates less than or equal to 1000 MPa s⁻¹. The present study found increased death with increased impact energy. In support of this, other very high stress rate experiments have also shown increased cell death with increasing energy of impact.^{22,24} At 24 h, cell death was limited to the superficial zone inside the impact area. Cell death then spread to deeper regions of the cartilage over time in culture, but there was no noticeable radial spread. These findings are similar to previous work that loaded cartilage from 0 to 65 MPa at 35 MPa s⁻¹ without underlying bone.⁴⁸ In that study, cell death immediately following a 10 MPa injury was limited the superficial zone. At 24 h, cell death did not extend into the unloaded region, but appeared to extend

throughout the depth of the explant. This is in contrast to work by Levin *et al.*³⁰ who cyclically loaded canine articular cartilage and observed direct evidence for the spread of cell death from injured to non-injured tissue, suggesting that loading regiment may alter the spatio-temporal pattern of cell death. This possibility is supported by another single impact loading study that showed little cell death in tissue adjacent to the impact site and no spread over 5 days in culture.³¹ Lastly, similar to cell viability staining, in the present study DNA content measured in the tissue showed injured tissue contained significantly fewer cells than control or adjacent tissue at 1 week, and, at 4 weeks, changes in the adjacent tissue were comparable to changes in controls.

The gene expression data show that High impact (2.8 J) resulted in significantly different expression from non-impacted culture controls and there is a temporal profile of gene expression changes resulting from explanting the tissue. Compared to native expression levels, AGC, SZP, and TIMP-1 all showed increased expression in the tissue adjacent to the impact site at 24 h following High impact. This observation may represent a decreased ability of the impact area to attempt repair, or the surrounding area attempting to heal itself. Additionally, MMP-1 expression increased in the injured tissue at 24 h. This finding is similar to work by Lee *et al.*²⁹ who showed that AGC, TIMP-1, and MMP-1 were increased in loaded tissue 24 h post injurious compression to 50% strain at 1 s⁻¹ (resulting in a ~20 MPa load). Further, increased MMP-3 expression has been observed for the same loading protocol just described, as well as following injury at 30 MPa at a rate of 600 MPa s⁻¹.^{8,37} Interestingly, in the present study SZP expression was significantly decreased in HI tissue compared to HO and Coll had dramatically increased expression in both HI and HO tissue at 1 week. Both of these changes are signs of chondrocyte de-differentiation.⁹ The constellation of de-differentiation, decreased expression of normally expressed matrix molecules, and increased expression of several MMPs suggests, at least in these *in vitro* studies, that a degenerative process is initiated early post-injury. Moreover, these results are similar to *in vivo* work that has looked at osteoarthritis and cartilage injury in humans,^{43,47,50} such as CollA1 and CollA2 having increased expression in damaged cartilage taken from OA joints.⁴³ It is briefly worth speculating on molecular mechanisms that may transduce the impact load and lead to changes in gene expression. Impact loading of cartilage generates streaming potentials, stress/strain fields, and hydrostatic pressure fields within the tissue, all of which are experienced at the cellular level. It has been shown that chondrocytes respond to mechanical load

through integrin–cytoskeletal interactions, as well as ion and stretch activated channels located in the cell membrane.⁴⁹ Indeed, hydrostatic pressure applied to chondrocytes has been shown to affect ion transporters^{6,18} and cause changes in collagen type II and aggrecan gene expression.⁴⁵ Further characterization of temporal gene expression profiles may aid in discovery of clinical tools to diagnose or monitor treatment of post-traumatic OA.

Turning to matrix biochemistry, at 4 weeks GAG concentration significantly decreased and total collagen concentration trended upwards. GAG concentration decreased in injured tissue following Low and High impact as well as in tissue adjacent to High impact. Notably, changes in tissue GAG took 4 weeks to become significant, while GAG release to the media was significant at 1 week (Table 3 and Fig. 5). Thus, it appears GAG release into culture media is more sensitive than GAG in the tissue to detect ECM degradation. While the amount of GAG in the tissue is due to a balance of loss and production, decreased synthesis in response to injurious loads has been reported.^{25,28,38} Though we observed increased AGC expression, increased mRNA levels may not translate to increased protein production. An alternative explanation for the increased GAG release to the media is that increased enzymatic activity caused more collagen matrix breakdown, freeing GAG, either whole or degradation fragments, from the tissue. Such a mechanism is supported by DiMicco *et al.*¹⁰ who showed MMP inhibition reduced cumulative sGAG loss from injured tissue while inhibitors of biosynthesis had no significant effect. Though total collagen concentration for any group at 4 weeks was not significantly different from baseline, collagen concentration trended upwards from 1 week to 4 weeks, significantly so for HI tissue. This trend may reflect the increase in Col1 gene expression and/or the decrease in tissue stiffness seen for control, Low, and High impact.

Tissue stiffness of cartilage post-injury was observed to decrease with time. Compared to non-impacted culture controls, High impact resulted in a significant decrease in tissue stiffness by 24 h that increased with subsequent culture. In contrast, tissue stiffness following Low impact was similar to culture controls at 24 h and 1 week, but by 4 weeks, became similar to High impacted tissue. The drop in tissue stiffness between 1 and 4 weeks following High impact paralleled culture controls (44% and 41% decreases for control and High, respectively), reflecting the fact that the majority of change in tissue stiffness following High impact occurred during the 1st week of culture. However, during the same time, stiffness of Low impacted tissue significantly decreased 64%, a full 20% points more than culture controls. Moreover, tissue

stiffness correlated well with GAG release and percent cell death, and the gene expression data showing a decrease in Col2 and an increase in Col1 indicate a more fibrocartilaginous collagen matrix in all groups at 4 weeks,^{16,17} possibly contributing to the decreases in tissue stiffness observed. Changes in mechanical properties of cartilage are well documented in OA,¹⁹ but not extensively studied for acute injury. In a 3 day *in vitro* study of injurious compression (loaded to 50% strain at a rate 0.01, 0.1, or 1 s⁻¹ with resulting peak stresses of 12, 18, and 24 MPa, respectively), the dynamic compressive stiffness of injured tissue was shown to decrease with increasing strain rate. Additionally, two *in vivo* studies have shown decreased stiffness in rabbit patello-femoral cartilage beginning 4.5 months post-injury.^{13,14} Though *in vivo* studies may be a better representation of the human condition, an advantage of *in vitro* models is the ability to create the same observation in a shorter time period, a fact that may become important in screening potential therapeutic interventions.

We have previously shown in the same system that High impact results in an immediate decrease in tissue stiffness resulting from purely mechanical phenomena and correlating to the presence of surface damage.⁴⁴ Considering this fact, the present results suggest that mechanical insult initiates a detrimental biological response that causes continued loss of tissue stiffness, cell death, and ECM degradation. As a corollary, the delayed loss of tissue stiffness and cell death in Low impacted tissue is potentially explained by a delayed biological response occurring some time between 1 and 4 weeks, perhaps with similar changes in gene expression that were seen following High impact at 24 h.

In summary, the results of this explant study characterize the temporal effects of two levels of impact injury to articular cartilage in terms of cell death, gene expression of pertinent cartilage markers, ECM biochemistry, and tissue biomechanical properties. Our results parallel established changes from other cartilage injury studies, as well as provide new data in the form of spatial and temporal gene expression patterns and show tissue subjected to a Low level of impact exhibits a delayed biological response. It has been suggested that some cases of joint injury with normal arthroscopic, radiologic, and laboratory findings go undiagnosed at early time points³² and said that cartilage without visible damage is considered intact, thus neglecting initial damage occurring at the cellular level.¹¹ Though both Low impact and baseline tissue were not different in terms of gross morphology or aggregate modulus at 24 h, Low impact explants at 4 weeks exhibited a tableau in terms of cell death and loss of tissue stiffness comparable to that of High impact, supporting the notion of clinically silent injuries

developing into identifiable disease. Ultimately, understanding the temporal effects of impact loading will help to elucidate the processes that occur in post-traumatic OA and point to future avenues of inquiry to prevent or reverse this disease.

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