INJURIOUS MECHANICAL COMPRESSION OF BOVINE ARTICULAR CARTILAGE INDUCES CHONDROCYTE APOPTOSIS

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ABSTRACT

A bovine cartilage explant system was used to evaluate the effects of injurious compression on chondrocyte apoptosis and matrix biochemical and biomechanical properties within intact cartilage. Disks of newborn bovine articular cartilage were compressed in vitro to various peak stress levels and chondrocyte apoptotic cell death, tissue biomechanical properties, tissue swelling, glycosaminoglycan loss and nitrite levels were quantified. Chondrocyte apoptosis occurred at peak stresses as low as 4.5 MPa, and increased with peak stress in a dose dependent manner. This increase in apoptosis was maximal by 24 hours after the termination of the loading protocol. At high peak stresses ($>$20 MPa), greater than 50% of cells appeared to apoptose. When measured in uniaxial confined compression, the equilibrium and dynamic stiffness of explants decreased with the severity of injurious load, though this trend was not significant until 24 MPa peak stress. In contrast, the equilibrium and dynamic stiffness measured in radially unconfined compression decreased significantly after threshold injurious stresses of 12 and 7 MPa, respectively. Together, these results suggested that injurious compression caused a degradation of the collagen fibril network in the 7-12 MPa range. Consistent with this hypothesis, injurious compression caused a dose-dependent increase in tissue swelling, significant by 13 MPa peak stress. Glycosaminoglycans were also released from the cartilage in a dose-dependent manner, significant by 6 MPa peak stress. Nitrite levels were significantly increased above controls at 20 MPa peak stress. Together, these data suggest that injurious compression can stimulate cell death along with a range of biomechanical and biochemical alterations to the matrix and, possibly, chondrocyte nitric oxide expression. Interestingly, chondrocyte programmed cell death appears to take place at threshold stresses lower than those required to stimulate cartilage matrix degradation and biomechanical changes. While chondrocyte apoptosis may therefore be one of the earliest responses to tissue injury, it is currently unclear whether this initial cellular response subsequently drives cartilage matrix degradation and changes in the biomechanical properties of the tissue.

INTRODUCTION

Apoptosis is a normal physiological process involved in immune regulation, the removal of potentially carcinogenic and damaged cells, and during development as evidenced by the apoptosis of hyaline cartilage chondrocytes during endochondral ossification [1]. Aberrant apoptosis, however, can be pathogenic and has been observed in diseases such as Alzheimer's [2] and in neuronal cell death following spinal cord injury [3]. Recent studies of human osteoarthritic articular cartilage [4, 5, 6], along with evidence from an animal model of osteoarthritis (OA) [7] and cartilage wounding experiments [8], have suggested that aberrant apoptosis may play a role in the pathogenesis of OA.

Hypocellularity has been associated with osteoarthritic cartilage and apparently normal cartilage in joints affected with OA . This hypocellularity is believed to be both a risk factor and a contributor to the disease pathogenesis, and the finding of markedly increased levels of apoptotic chondrocytes in diseased tissue has implicated apoptotic cell-loss as a possible cause of OA hypocellularity [5]. The precise role of apoptosis in the pathogenesis of OA is currently unknown, but mechanisms involving calcium precipitation [10] and matrix degradation [6] by apoptotic bodies have been proposed. Further evidence of a role for aberrant apoptosis in articular cartilage comes from correlations of tissue age and percentages of apoptotic chondrocytes found in normal tissue of adult animals [11]. Both animal and human cartilage exhibit age-related decreases in cellularity, and it has been suggested that the inability of hypocellular tissue to maintain and repair itself may contribute to age related degeneration [11]. While this decrease in cellularity with age may be related to apoptosis, a correlation between apoptotic chondrocytes and age in normal human cartilage has not been observed [6].

The biological and physical stimuli that may induce chondrocyte apoptosis in articular cartilage are not well understood. Retinoic acid [13] and antibodies to the CD95 (or Fas) receptor [14] have both been reported to induce chondrocyte apoptosis *in vitro*. Additionally, IL-1 induced nitric oxide in combination with oxygen scavengers has the capacity to induce apoptotic cell death of chondrocytes [15]. However, none of these studies have focused on chondrocytic cell death within

intact cartilage matrix. Proinflammatory cytokines such as TNF-α have also been reported to stimulate cell death [16]; however, it is currently unclear if chondrocyte cell death is directly controlled by these cytokines.

Studies of chondrocyte-mediated matrix turnover in a model for mechanical injury of cartilage have shown that mechanical load can produce non-viable cell populations exhibiting condensed nuclei [17], reminiscent of apoptosis. Additionally, secondary osteoarthritis is commonly associated with mechanical injury of articulating joints [18]. Together, these two observations led us to the hypothesis that injurious mechanical compression of articular cartilage may cause of chondrocyte apoptosis. In the present study, we examined the effects of graded levels of applied injurious compression on the induction of chondrocyte apoptosis in cartilage explants *in vitro*. Our objectives were to quantify threshold levels of mechanical stress that could induce chondrocyte apoptosis as well as to quantify and compare the effects of these injurious compressive stresses on biochemical, biomechanical, and compositional measures of cartilage degradation.

METHODS

Explant and Culture

Articular cartilage disks (3 mm diameter by 1 mm thick, $\sim$8 mg wet weight at time of explant) were obtained from 1-2 week old calves as previously described [19]. Briefly, 9 mm diameter cylindrical disks of cartilage and underlying bone were cored from the femoropatellar groove and inserted into a sample holder of a sledge microtome. The first 100-400 μm of tissue was then removed to provide a flat surface, and the next two 1 mm thick cartilage slices were obtained. From each of these slices, four cartilage disks (3 mm diameter) were cored and maintained in culture medium (low glucose DMEM with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, an additional 0.4 mM proline, 20 μg/ml ascorbate, and 25 μg/ml gentamicin) in a 37° C, 5% CO_2 environment. Conditioned media was frozen for later biochemical analysis.

Injurious Compression

After one to seven days in culture, cartilage disks were placed into individual wells of the base of a polysulfone compression chamber [17] with 0.5 ml media per well. The disks were held between impermeable platens in the chamber base and the overlying chamber lid in uniaxial, radially unconfined compression. The compression chamber (base and lid) was then placed inside a custom built incubator-housed compression apparatus [20] for application of injurious compression. Anatomically matched free-swelling disks served as controls. The injurious compression protocol consisted of six repeated on-off cycles of a displacement controlled ramp (in strain), applied at a strain rate of 1000 μm/s to a final strain level of 30%-50% and maintained at this strain level for 5 minutes, followed by a release of compression for 25 minutes (Fig. 1). Load measurements were recorded during all experiments, and the peak stresses produced during the compression (between 4 and 25 MPa depending on the chosen strain level) were used for comparisons between different experiments. Following compression, injuriously compressed disks and matched controls were returned to free swelling culture.

TUNEL Staining

Injuriously compressed disks and matched controls to be used for the detection of apoptotic nuclei were frozen by direct immersion in liquid nitrogen four days post-compression. Serial cryostat sections (8 μM) were taken through the entire thickness of the disk ($\sim$125 sections/disk). The sections were then immobilized on glass slides, air dried, fixed, and stained for the presence of apoptotic nuclei according to the manufacturer's protocol (ApopTag peroxidase *in situ* apoptosis detection kit, Oncor, Gaithersburg, MD). The peroxidase enzyme label used yielded an insoluble brown stain on positive nuclei.

All $\sim$125 sections/disk were scored blind for the presence of apoptotic nuclei (score from -1 to 3). Sections with positive staining nuclei at their periphery were considered negative (score of -1, 0, or 1), as these apoptotic cells were artifacts of the cutting process [8]. Sections considered positive had apoptotic nuclei away from their edges and were scored according to whether there

were small (score of 2) or large numbers (score of 3) of positive nuclei. The final percentage of positive sections (score 2 or 3) from each disk is reported.

To quantify the number of apoptotic nuclei, apoptotic and non-apoptotic nuclei were counted for a field (60-120 nuclei/field) in the central region of 3-5 sections on each disk using the x40 objective of an Olympus Vanox microscope. The apoptotic nuclei were expressed as a percentage of the total number of nuclei counted.

Biochemical Composition

Sulfated glycosaminoglycans (sGAG) were quantitated in the conditioned medium using the dimethylmethylene blue (DMMB) dye method [21], with shark chondroitin sulfate as the standard. Briefly, 20 μl of medium was mixed with 200 μl of DMMB dye, 20 μl of 70% ethanol was added to remove bubbles, and the absorbance at 520 nm was measured with a microplate reader. Nitric oxide production in the conditioned medium was measured using the Griess reaction with sodium nitrite as the standard [22]. Media were centrifuged at 16,000 g for 1 minute to remove debris. 100 μl of the supernatant was then mixed with 50 μl 1% sulfanilamide in 5% H_3PO_4 and 50 μl 0.1% naphthylethylenediamine dihydrochloride, with 20 μl of 70% ethanol added to remove bubbles, incubated at room temperature for 20 minutes, and measured for optical absorbance at 560 nm in a microplate reader. Wet weights were measured by patting each disk with sterile gauze and weighing inside a preweighed vial.

Biomechanical Characterization of Injury

Immediately after injurious compression, loaded and control disks were placed into PBS containing proteinase inhibitors (leupeptin, pepstatin, PMSF and either E64 or pefablock). The biomechanical properties of each disk were then measured first in uniaxial confined compression and then in radially unconfined compression using a Dynastat mechanical spectrometer, as previously described [23, 19]. For both testing modes, disks were first subjected to sequential increments of strain (to a final strain of $\sim$ 10% based on initial cut thickness) using ramp-and-hold

displacements under displacement control. After stress relaxation following each ramp-and-hold displacement, the measured equilibrium load was normalized to the disk area to obtain the equilibrium stress; the equilibrium modulus was then calculated by fitting a quadratic to the equilibrium stress and strain values. At $\sim$ 10% static offset strain, a series of 5 μm amplitude sinusoidal displacements was then applied to each disk in the frequency range 0.01-1 Hz, also in displacement control [24]. The dynamic stiffness at each frequency was calculated as the measured load normalized to the specimen area and dynamic strain amplitude. The confined and unconfined equilibrium modulus and dynamic stiffness of injuriously compressed disks were normalized to that of matched control disks.

Statistics

Control and experimental groups were compared using either Student's paired or unpaired t test assuming equal variances, with significance at the level $p < 0.05$.

RESULTS

Apoptotic Response

Injurious mechanical compression of bovine articular cartilage disks caused a dose-dependent increase in the percentage of apoptotic nuclei as detected by TUNEL staining (Figs. 2 & 3A). The data in Fig. 3A are reported as the percentage of apoptotic nuclei observed as a function of the peak compressive stress achieved during loading (e.g., Fig. 1). As has been previously reported regarding the cutting of articular cartilage [8], numerous cells at the cut edge in both the loaded and unloaded disks stained positive for apoptosis. In contrast, the central region of the loaded disks showed a dramatic increase in the number of apoptotic nuclei (Fig. 2) compared to unloaded controls, reaching $\sim$ 50% apoptotic nuclei at 20 MPa peak stress. Upon evaluation of all of the sections taken from a disk ($\sim$125 sections/disk), a three-fold increase in the number of sections

judged positive for apoptotic chondrocytes (score of 2 or 3) was seen at a peak compressive stress of just 4.5 MPa (Fig. 3B), although the total percentage of apoptotic cells was low.

The kinetics of the apoptotic response was determined by examining the percentage of cells that were apoptotic in the tissue immediately after (i.e., time 0), 1 day after, and 2 days after a 20 MPa peak compressive stress loading condition (Fig. 4). While there is evidence of some apoptosis immediately after loading, peak levels of apoptosis are clearly observed by 24 hours after loading (Fig. 4).

Changes in Biomechanical Properties

The confined compression equilibrium modulus of injuriously compressed explants showed a non-significant decrease with increasing injurious peak stress; there were no significant changes versus controls for any loading condition (Fig. 5). The confined compression dynamic stiffness at 0.1 Hz also decreased with the severity of injurious load, though this trend was not significant until 24 MPa peak stress. In contrast, radially unconfined compression tests revealed significant reductions in the equilibrium modulus and dynamic stiffness of injuriously loaded tissue. Changes in the equilibrium modulus became significant at 12 MPa and showed a 2.5 fold reduction at 24 MPa peak applied stress. At 0.1 Hz, the loaded tissue exhibited a significantly reduced dynamic stiffness by 7 MPa peak stress, with a nearly 7-fold reduction in stiffness by 24 MPa peak stress. The dynamic stiffness computed at 1, 0.3, 0.03, and 0.01 Hz, showed trends similar to those reported at 0.1 Hz for both confined and unconfined compression modes (data not shown). Together, these results suggested that injurious compression caused a degradation of the collagen fibril network in the 7-12 MPa range.

Changes in Biochemical Composition

Tissue wet weights were not significantly elevated compared to that of control for applied stress levels at and below 8.5 MPa peak compressive stress (Fig. 6). At the 13 and 17 MPa compression levels, the injured tissue swelled significantly compared to control tissue. This increase

arose entirely within the first day following the compression. The cumulative release of sGAG into the medium after injurious compression displayed trends similar to the wet weight data (Fig. 7). For the 13 and 17 MPa peak stress conditions, sGAG release rates were sharply increased during the compression protocol and remained significantly elevated for two to three days before returning to control values. In a separate experiment with a higher sample number, a small but significant increase in the total sGAG released to the medium by day 4 was observed for a 6 MPa peak stress loading condition (6 MPa: 10.1$\pm$.4, Control: 9.3$\pm$.4 μg sGAG/mg initial wet weight, n=12). Cumulative nitric oxide released to the media was significantly increased for a 20 MPa peak stress at four days post compression (20 MPa: .81$\pm$0.08, Control: .59 $\pm$0.02 mM NO_2/mg initial wet weight, n=12), but 6 and 10 MPa peak stress conditions were unchanged compared to control values.

DISCUSSION

The results of the TUNEL staining demonstrate that mechanical compression can induce articular cartilage chondrocytes to undergo apoptosis. A significant dose-related increase in apoptosis was observed at peak stresses as low as 4.5 MPa. While a small amount of apoptosis was observed immediately after loading, there was a significant increase in apoptosis by 24 hours after loading. This time lapse between the completion of the loading protocol and the emergence of an apoptotic response suggests that there are biochemical changes taking place within the chondrocytes or tissue which eventually result in apoptotic cell death.

The biological pathway through which articular chondrocytes are induced to undergo apoptosis is currently unknown, but a variety of hypotheses have been proposed, including binding of CD95 [14], elevated levels of NO [5, 15], and loss of extracellular matrix survival signals [6, 7]. This latter hypothesis is the most consistent with a mechanical origin for the initial apoptotic signal, as a mechanical insult could possibly cause the chondrocytes to be separated from these survival-promoting ECM signals.

Previous studies of bovine explants damaged by cutting [25] have shown that apoptotic

cells remained in the tissue and were detectable by TUNEL staining for at least 20 days following injury. Additionally, there were very few empty lacunae in the sections evaluated for apoptotic cells, suggesting that even after apoptosis the cell remnants remained within the tissue. Based on these observations, the increase in TUNEL staining that we observed with increased peak stress does not appear to be confounded by artifactual loss of apoptotic cells.

Interestingly, a significant increase of apoptotic cells was observed under loading conditions (4.5 MPa peak applied stress) below or near the threshold stress levels required to produce detectable changes in confined (24 MPa) and unconfined (7 MPa) biomechanical properties, sGAG release (6-13 MPa), and tissue swelling in our system. These data suggest that a small percentage of chondrocytes are sensitive to the low peak applied stress. As those cells begin to apoptose, they may in turn degrade the surrounding matrix. This partially degraded matrix, in combination with the increasing load, may further drive apoptosis and the dramatic matrix changes that are observed at the high stresses examined in this study. Based on transgenic mouse studies, it has recently been suggested that both MMP-3 [27] and MMP-9 [26] may play roles in controlling mesenchymal cell apoptosis. In the loaded cartilage system it is possible that as the load is increased, MMP expression also increases [28], resulting in MMP-driven matrix degradation and further stimulation of apoptosis. It will be interesting in this system to determine if MMP expression is in fact elevated and if MMP inhibition can play any role in this process.

The biomechanical characterization of injuriously loaded tissue showed distinct differences in material properties measured in confined versus unconfined compression. Confined compression tests emphasize the role of highly charged aggrecan molecules in resisting compression; unconfined compression tests also emphasize the contribution of the collagen network tensile strength in restraining tissue “bulging” that can occur at the disk periphery in the absence of radial confinement. When measured in uniaxial confined compression, the equilibrium and dynamic stiffness of explants decreased with the severity of injurious load, though this trend was not significant until 24 MPa peak stress. In contrast, the equilibrium and dynamic stiffness measured in radially unconfined compression decreased significantly after threshold injurious stresses of 12 and 7 MPa, respectively. Together, these results suggest that injurious compression caused significant

degradation of the collagen network at peak stress levels in the 7-12 MPa range. Since these biomechanical changes were detected immediately after injurious compression, it is possible that loading caused direct damage to tissue matrix. However, it is not yet possible to rule out cell-mediated collagenolytic activity, such as that associated with elevated MMP activity, based on these data alone. Further studies are in progress to directly assess this possibility.

The results of tissue swelling and sGAG release measurements are also consistent with collagen network damage arising from the more severe compression protocols. When tissue swelling was observed, the increase in swelling relative to controls was most dramatic during the first day following the compression. This swelling most likely occurred from the decreased ability of the damaged collagen network to counteract proteoglycan-induced swelling pressure [24, 29]. The increased rates of sGAG release, most dramatic during two to three days following the more severe compressions, are also consistent with collagen network damage. A damaged collagen network would be expected to have a greater effective pore size and hence increased proteoglycan diffusivity [30]. Indeed, previous studies [17] suggest that this sustained release of sGAG after injurious compression in our system may be associated with increased release of aggregating species in addition to a spectrum of degradation fragments found in controls. The additional role of cell-mediated aggrecan degradation induced by injury is also the subject of further study in this system.

Our results indicate that threshold levels of mechanical stress are sufficient to induce chondrocyte apoptosis in articular cartilage, and suggest that injurious joint loading could cause chondrocyte death even in the absence of other observable biochemical or biomechanical changes to the tissue. The increased biomechanical load could additionally stimulate chondroycte-mediated matrix degradation and inhibit new matrix biosynthesis. Ultimately, apoptosis-mediated cell loss could result in a significant reduction in cell number, leaving too few metabolically active cells to repair the degraded matrix. Thus, a mechanical component may be included in hypotheses relating elevated chondrocyte apoptosis to the pathogenesis of OA. Conversely, OA-related fibrillation of cartilage matrix may expose the chondrocytes to non-physiological levels of mechanical stress and/or an unfavorably degraded pericellular matrix, again leading to apoptosis. In both cases, the induction of apoptosis could be ascribed to mechanical compression, but in the latter case apoptotic

chondrocytes would arise as a secondary result of OA. While chondrocyte apoptosis may therefore be one of the earliest responses to tissue injury, it is currently unclear whether this initial cellular response subsequently drives cartilage matrix degradation and changes in the biomechanical properties of the tissue.

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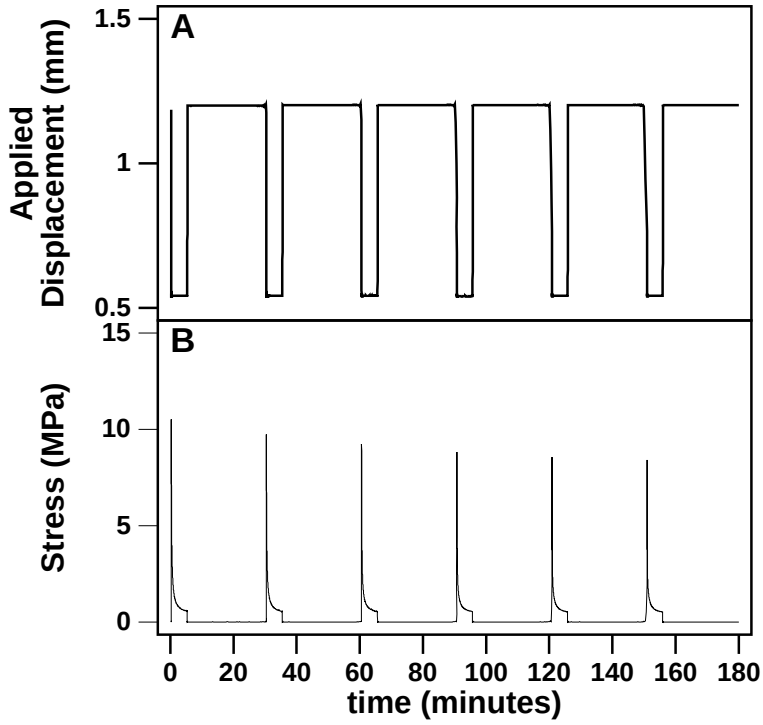

Figure 1: (A) The injurious compression protocol consisted of six repeats of an applied ramp in displacement at 1000 μm/s to a final strain level of 30%-50%, maintained at this strain level for 5 minutes, and then released for a period of 25 minutes. (B) The resulting peak stress achieved for the strain of 45% was 10.5 MPa.

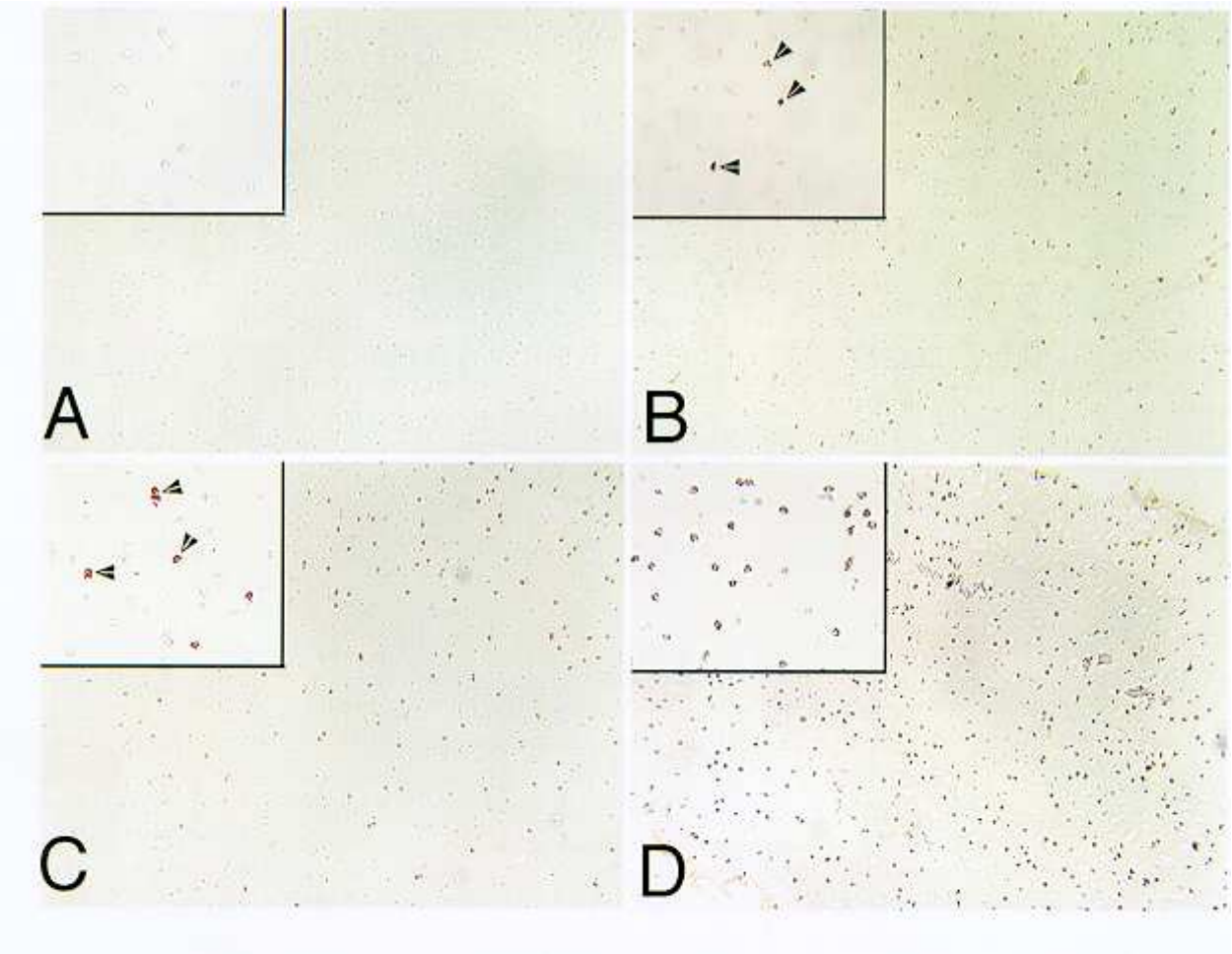

Figure 2: Examples of cartilage tissue stained with TUNEL. Insets are magnified images of a single representative cell. A) Control Tissue B) Tissue loaded with a peak stress of $\sim$6 MPa showing scattered positive TUNEL staining. C) Tissue loaded with a peak stress of $\sim$10 MPa showing increased positive TUNEL staining. D) Tissue loaded with a peak stress of $\sim$21 MPa showing a large number of positive staining cells.

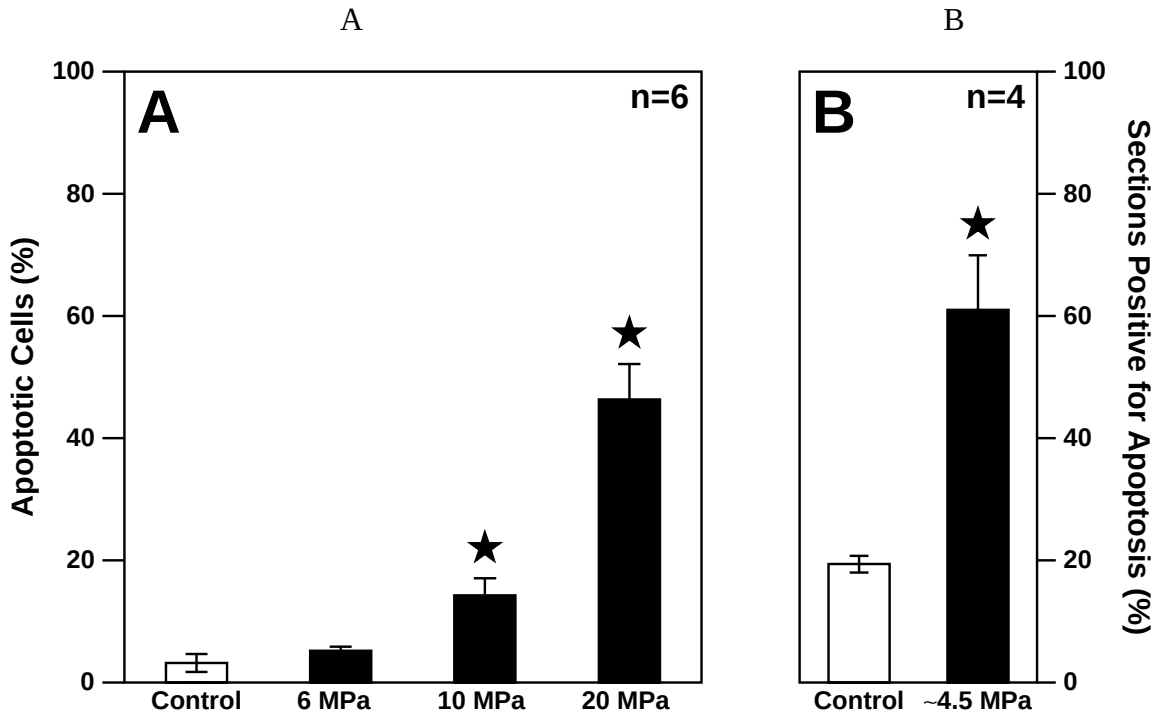

Figure 3: A) Dose Response: Cartilage explant disks subjected to peak compressive stress of 0 (control), 6, 10, and 20 MPa, were subsequently incubated for 4 days, and then analyzed for apoptotic nuclei using the TUNEL method on frozen sections of the tissue. B) Threshold Response: Results of TUNEL staining of $\sim$4.5 MPa peak stress loaded cartilage tissue. Results are shown as the percentage of sections considered positive for apoptosis (score of 2 or higher). Data are mean$\pm$SEM; $\star$ indicates $p < 0.05$ by paired t test.

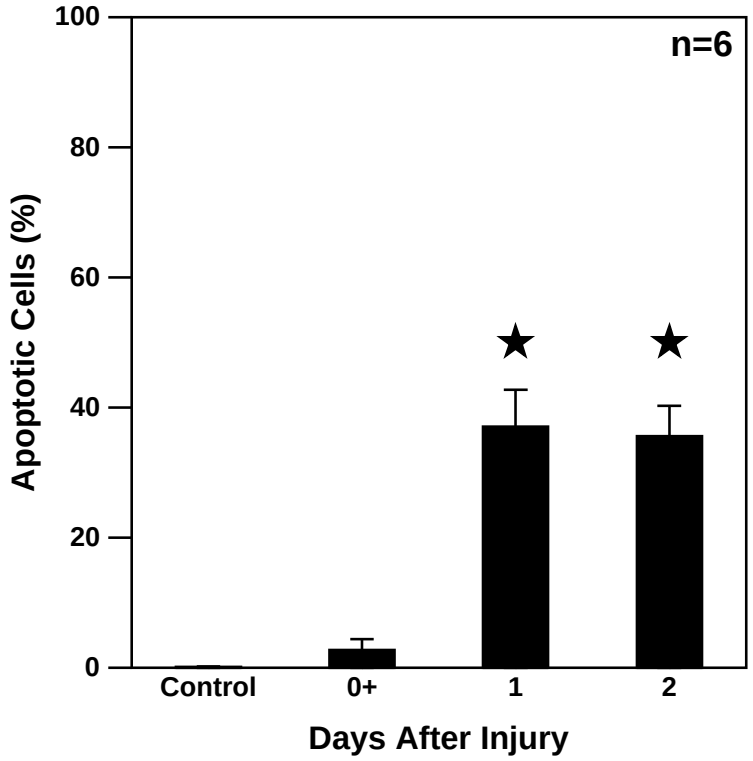

Figure 4: Cartilage explant disks were analyzed for apoptotic nuclei using the TUNEL assay immediately after (0+), 1 day after (1), and 2 days after (2) a 20 MPa peak compressive stress loading condition and compared to unloaded control. Data are mean$\pm$SEM; $\star$ indicates $p < 0.05$ by paired t test.

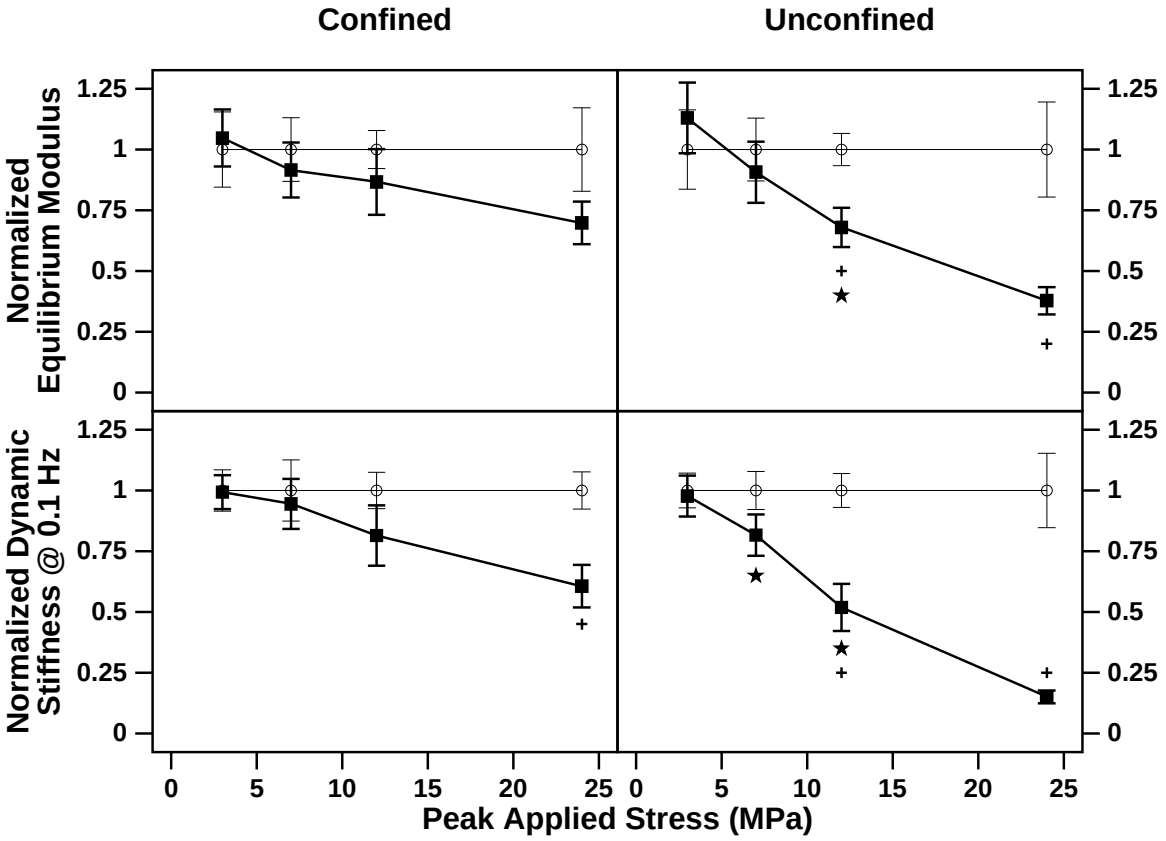

Figure 5: Groups of explants were subjected to peak stresses of 3 MPa (n=16), 7 MPa (N=11), 12 MPa (n=8) and 24 MPa (N=4/8 control/experimental) ■, with free-swelling anatomically matched tissue serving as control ◯. Directly after compression, the tissue was analyzed for changes in equilibrium modulus and dynamic stiffness for both uniaxial confined and unconfined compression. The dynamic stiffness shown was measured with a 5 μm amplitude 0.1 Hz sinusoid. Data are normalized to the values of the control tissue and are shown as mean$\pm$SEM; $\star$ indicated $p < 0.05$ by paired t test, + indicates $p < 0.05$ by t test with equal variances.

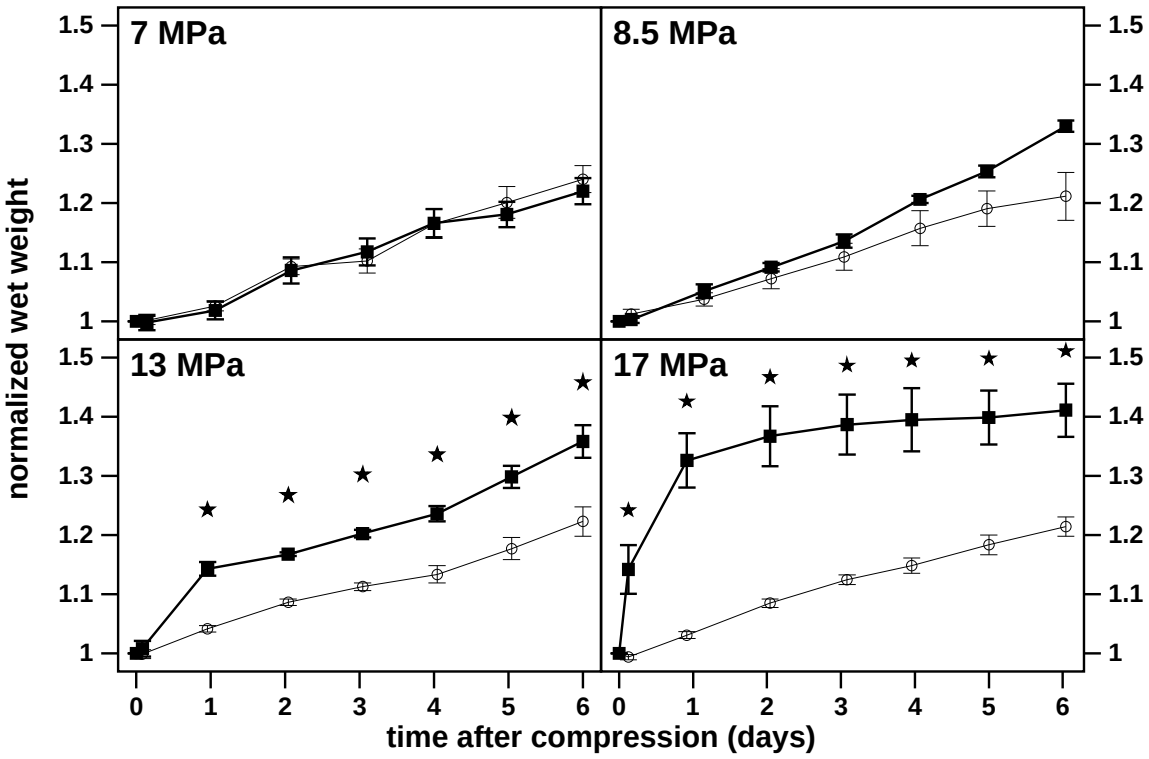

Figure 6: Tissue wet weights were recorded immediately before and immediately after compression to 7, 8.5, 13, and 17 MPa peak stress and every day afterwards for six days, ■ Loaded, ◯ Control. Data are normalized to the initial measured wet weight before compression;mean±SEM, n=6; ⋆ indicates $p < 0.05$ by paired t test.

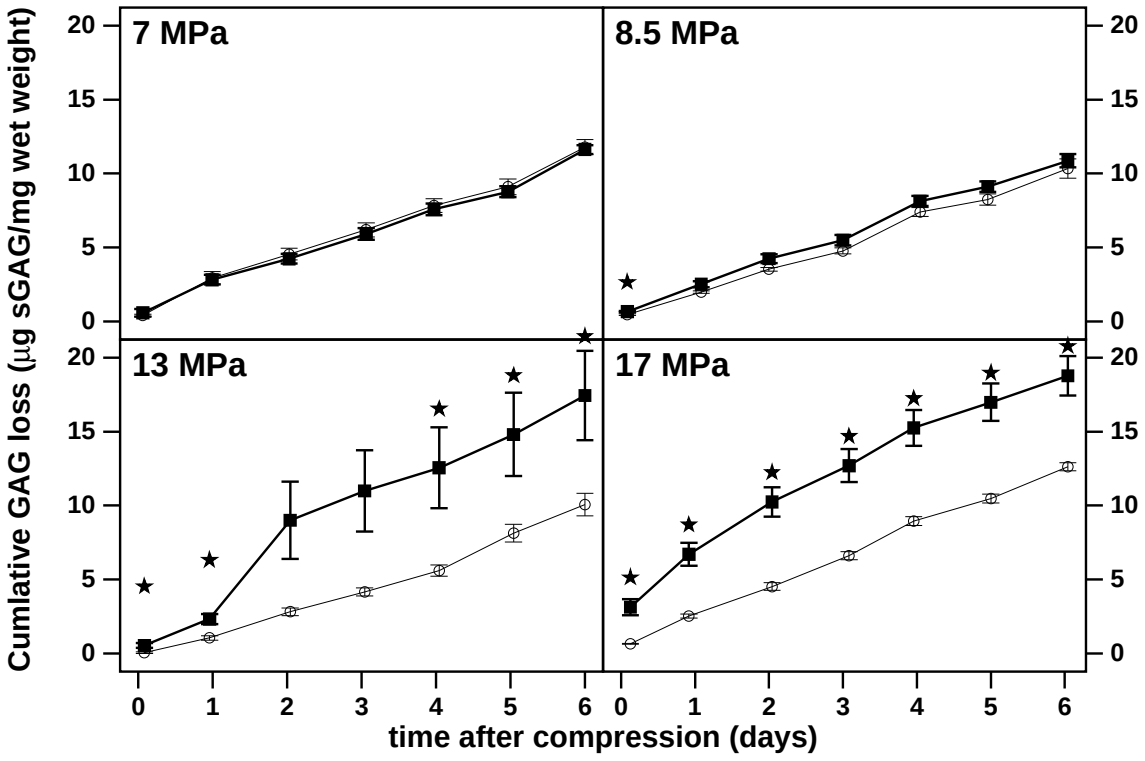

Figure 7: Cumulative GAG loss from groups of cartilage explants were subjected to peak stresses of 7, 8.5, 13, and 17 MPa, with free-swelling anatomically matched tissue serving as control. Conditioned media was stored directly after compression and every day thereafter and analyzed for sGAG. Results were normalized to the initial measured wet weight before compression. ■ Loaded, ◯ Control. Data are mean±SEM, n=6; ⋆ indicates $p < 0.05$ by paired t test.