RICE UNIVERSITY

Interferometry of Chondrocytes and Impact of Articular Cartilage

by

Charles Corey Scott

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APPROVED, THESIS COMMITTEE:

Kyriacos A. Athanasiou, Ph.D., P.E.
Karl F. Hasselmann Professor
Committee Chair
Bioengineering

Margaret M. French, Ph.D.
Research Fellow
Bioengineering

Michael C. Gustin, Ph.D.
Associate Professor
Biochemistry & Cell Biology

Antonios G. Mikos, Ph.D.
J.W. Cox Professor
Bioengineering

Houston, Texas
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ABSTRACT

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Osteoarthritis and the subset of post-traumatic osteoarthritis both represent the end-stage of a degenerative process that can result from an initial tissue insult, particularly from a single mechanical impact. No current treatment has been shown to slow or stop its progression. Here, two approaches are taken to understand the physiology and pathology of articular cartilage. A cellular approach develops and uses a novel imaging technique for single cells and bioactive surfaces, while a tissue approach consists of understanding the acute and temporal effects of mechanical impact. Thus, the goals of this study are two-fold: 1) to develop vertical scanning interferometry (VSI) to obtain all salient features of chondrocytes and characterize bioactive surfaces, and 2) to develop methodologies for protecting diarthrodial joints from pathologic impact loading. VSI was validated and developed to obtain three-dimensional chondrocyte and fibroblast geometries, as well as to characterize protein-coated surfaces. VSI can now be applied to an array of studies involving single cell biomechanics, surface characterization, and cell adhesion and spreading. To examine pathology, an impact instrument was built and validated to apply repeatable impacts to articular cartilage. An explant model was characterized to understand the physiologic changes articular cartilage tissue experiences in culture over four weeks. Then, the acute and temporal effects of two levels of impact were characterized, consisting of a low level impact that did not show initial gross
damage and a high level impact that caused immediate surface disruption. These studies illustrated that clinically undetectable impact injuries immediately show some subtle changes in extracellular matrix (ECM) glycosaminoglycan release and gene expression, but otherwise resemble the culture controls, while the high impact level caused gross damage. However, over a four week culture period, the subclinical impact proved to have started a degeneration cascade that significantly affected the biomechanical integrity, gene expression profile, biochemical makeup of the ECM, and chondrocyte viability. Therefore, impact injuries may account for a substantial proportion of the primary osteoarthritis cases. Further, if the start of the degeneration cascade of the low impact level can be stopped or reversed when only subtle changes are occurring, osteoarthritis prevention would be possible.
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INTRODUCTION

This research represents an attempt to further understand, and to develop innovative tools for future research into the physiology and pathology of human articular cartilage. This work ultimately attempts reduce the incidence and ensuing personal and financial burden of osteoarthritis to individual patients and society, especially post-traumatic osteoarthritis. The large number of traumatic and impact injuries to articular cartilage combined with a minimal regeneration capacity of this tissue lead to irreversible damage and disability. In order for clinicians to develop alternative therapies for these patients, a more comprehensive understanding of articular cartilage, and specifically its response to injury, is needed.

This thesis contains a body of work that consists of two distinct approaches. The first approach utilizes vertical scanning interferometry (VSI), a novel technique, to allow imaging of single cells, as well as extracellular matrix (ECM) protein coatings on surfaces. The objective of the first approach was to develop VSI for imaging chondrocytes and other single cells to obtain their three-dimensional geometry, as well as to image protein-coated surfaces. Development of this method would allow more precise manipulation of cell attachment to substrata and improved single cell mechanical models to enhance tissue engineering and further comprehend mechanotransduction. This method would also allow for more accurate characterization of protein-coated surfaces, which can be used for chondroinduction of fibroblasts. The hypothesis of this objective was that interferometry can be an effective tool to acquire all salient features of threedimensional chondrocyte geometries and characterize protein-coated surfaces.
The second distinct approach involves understanding how articular cartilage responds to impact loading. The objective of this approach was to establish methodologies for protecting diarthrodial joints from injury following traumatic impact. Articular cartilage was exposed to different levels of impact to identify detrimental biomechanical milieux and their acute and temporal effects in cartilage pathophysiology. This objective also involves building and validating an impact instrument. These investigations ascertained how articular cartilage responds to impact loading in terms of gene expression, biochemistry, biomechanics, as well as grossly and histologically. The cartilage impact instrument applied and recorded consistent, measurable impacts on the surface of articular cartilage. Diarthrodial joints were subjected to three different levels of direct impact (zero, 'Low', and 'High') using the impact instrument. The effects of impact on articular cartilage were examined in and around the impact site immediately following trauma using a comprehensive array of assessment tests. These included a gross morphology scoring system, creep indentation to measure tissue biomechanical properties, histology to identify immediate changes in tissue architecture, quantitative biochemistry to determine changes in tissue composition, and changes at the messenger level to examine chondrocyte differentiation.

The objective of the mechanical impact study was to elucidate the progression of traumatic effects on cartilage as a function of time. Joint explants were cultured for 'short' or 'long' durations (1 week and 4 weeks, respectively) and changes in the tissue's structure-function characteristics were quantified. The hypothesis of this objective was that different impact levels would result in different sequelae. Specifically, 'High' impact compressive forces would result in immediate changes in the tissue's properties, as
evidenced morphologically, biomechanically, histologically, and biochemically. On the other hand, 'low' impact would not result in changes in morphology, biomechanics, and quantitative biochemistry immediately after, but in a delayed fashion, long after the trauma; however, low impact would result in changes at the gene expression or messenger level upon the traumatic episode. The expectation was that low and high impact would likely result in degenerative changes and the degree of severity would mirror the impact level. Figure 1 illustrates this overall hypothesis.

Figure 1. Hypothesis of the effects of impact on cartilage

The specific aims of this thesis were 1) to develop vertical scanning interferometry as a method for acquiring the three dimensional geometries of single chondrocytes and for characterizing protein coated surfaces; 2) to design, fabricate, and validate a drop tower to perform repeatable and measurable impacts on articular cartilage; 3) to elucidate the
effects of trauma on articular cartilage immediately following impact; and 4) to examine temporally under explant culture conditions the effects of trauma on articular cartilage.

The first three chapters of this thesis address the first objective and specific aim of the thesis. Chapter 1 is a survey of the literature that explores the effect of shear stress on chondrocytes, including methods of applying shear to single cells, as well as populations of cells and the ensuing effects. In particular, techniques to measure cell adhesion and to obtain the mechanical properties of single cells are covered, as well as studies exploring the effects of shear stress on chondrocytes. In Chapter 2, which is an original research article, VSI is developed and validated as a technique for imaging single cells. This chapter also explores future possibilities for this technology applied to cellular studies, including the study of cell dehydration. Chapter 3 is an original research article that further explores VSI as a tool to characterize bioactive coatings and how these coatings can induce fibroblasts toward a chondrocytic phenotype.

The next six chapters consist of one review paper and five original research investigations exploring the acute and temporal effects of impact on articular cartilage. Chapter 4 is a comprehensive review of the literature concerning the effects of impact on articular cartilage. Specifically, the chapter describes the significance of impact on cartilage, a definition of mechanical impact and how that translates to experimental work. A definition of impact is suggested, in vitro and in vivo models of impact on articular cartilage are presented, and treatments that have been employed in an attempt to reverse the negative consequences of impact on articular cartilage are discussed.
Chapters 5 through 9 are original research articles that correlate to the second through fourth specific aims and the second objective of this thesis. Chapter 5 is an original research article that investigates the effect that enzymatic digestion has on articular chondrocytes (a necessary component for tissue engineering). This study aids in understanding which enzymatic digest protocols are best for attaining large cell numbers and which protocols are better for having a gene expression profile closer to native tissue. This chapter is part of the tissue level approach to understanding articular cartilage physiology. Chapter 6 explores explant culture of articular cartilage from the gene expression level, to the biochemical and biomechanical effects that occur over a four week period. This study illustrated that the articular cartilage explant model was beneficial for investigating the effects of impact because the changes to the explants in culture over four weeks was modest. Chapter 7 details the design, fabrication, validation, and use of the articular cartilage impact instrument. Repeatable impacts were demonstrated, as well as the effects in terms of morphology and biomechanics. In chapter 8 the acute effects of impact on cartilage are explored within 24 h of the event. Chapter 9 is examines the effects on impact on cartilage temporally, after 1 and 4 wks. Chapters 8 and 9 comprehensively cover the effects of impact using gene expression, histology and viability staining, biochemical assays, and creep indentation biomechanical testing. For the first time, these studies investigate the acute and temporal effects of mechanical impact on articular cartilage from the level of gene expression to the ECM biochemistry to tissue biomechanics to the gross morphological properties of the tissue. The results from these investigations are synthesized into several conclusions, which are summarized in the Discussion, along with recommended future studies. Also, as
education is one of the goals of doctoral training, an original education paper is in Appendix A that describes a teaching project that was developed and evolved at Rice University for BIOE 575: Continuum Biomechanics.
CHAPTER 1: SHEAR STRESS AND CHONDROCYTES*

ABSTRACT

The application of shear forces in chondrocytes has significant ramifications, both in terms of cell adhesion and mechanotransduction. Cell adhesion studies have generated information about chondrocyte membrane receptors and cellular interactions with different biomaterials and proteins, as well as the levels of shear that detach cells from various substrata. The techniques used to study cell adhesion of chondrocytes can also be used to obtain cell material properties through modeling or to apply a known amount of shear stress to a single cell or an average shear to a population of cells. In terms of mechanotransduction, shear stress has a multitude of effects on the regenerative and degenerative processes of articular cartilage. Studies characterizing the effect of shear on chondrocytes usually look at an average shear over a population of monolayer cells and have shown that shear increases pro-inflammatory cytokines, such as metalloproteinase-3, interleukin-6, nitric oxide, and prostaglandin E2. Shear also induces protective cytokines, such as interleukin-4 and tissue inhibitor of metalloproteinase, and increases proliferation, glycosaminoglycan size and production, and proteoglycan size. Recent work found that shear also decreases collagen type II and aggrecan production. From the

perspective of tissue engineering, the effects of shear are both beneficial and harmful, and separation of the effects of shear stress and mass transport is difficult. Increased understanding of how shear affects chondrocytes on a single cell level and deeper elucidation of the specific mechanotransduction pathways should allow more intelligent tissue engineering strategies, possibly taking advantage of the beneficial effects of shear and blocking the potentially degenerative effects.
INTRODUCTION

Significance

Articular cartilage failure is currently a cause of much morbidity and patient suffering, and few solutions currently exist for any but the smallest defects. Though articular cartilage is relatively thin, avascular and aneural,\(^1\) replacement through tissue engineering still remains elusive. However, the field of tissue engineering has entered an exponential phase of growth and discovery, in particular in terms of elucidating the effects of the mechanical and biochemical environment on chondrocytes, as well as the synergy of the two.\(^2\)

Osteoarthritis (OA), while affecting millions, is not fully understood. OA affects over a fifth of Americans over age 45, almost half of Americans over age 65 and affects women more often than men.\(^3\) Predisposing factors include previous injury, fracture, ligament tear, and many other causes of misaligned or abnormal force transduction across a joint.\(^4\) Also, heavily trained horses showed increased cartilage fibrillation and decreased stiffness compared to lightly trained horses.\(^5\) Thus, mechanical forces are critical in cartilage pathology. Patients who have had their joints immobilized, thus much lower mechanical loading, eventually had their cartilage start to thin and alter in composition in addition to bone loss.\(^6\text{-}^8\) These studies illustrate the critical importance of mechanical loading to normal cartilage maintenance, as well as pathology.

Applying different forces to cartilage or chondrocytes has been shown to result in diverse genetic and metabolic effects. Compressive forces and hydrostatic pressure on
cartilage and chondrocytes are known to be beneficial in specific amounts at certain frequencies and can be synergistic with certain growth factors, although some specifics are still unknown.\textsuperscript{9-12} Shear stress, another form of mechanical loading on chondrocytes and cartilage, is not as fully understood.

\textit{Shear Stress}

Shear stress has been shown to have diverse effects on chondrocytes through mechanotransductive mechanisms and cell adhesion studies. The difficulty of applying a known and uniform shear stress to cells adds complexity to the issue of shear and chondrocytes.

One method of exploring the effect of shear stress on chondrocytes involves cell adhesion or the force of detaching a cell from a substrate, particularly if detached by fluid flow or a force parallel to the substrate surface.\textsuperscript{13-17} Also, cell adhesion is vital for cells and tissues to function, particularly for connective tissues.\textsuperscript{18} Many techniques have been used to measure the detachment force or strength of cells on different surfaces, either individually, or in monolayer culture. In addition to knowing the adhesive strength of cells on various materials in different media, these experiments have led to some understanding of the surface characteristics and cytoskeletal behavior of chondrocytes with respect to resisting shear stress. The same techniques used for cell adhesion can be employed to apply a known amount of shear to chondrocytes, gage the genetic and phenotypic response, and gather the mechanical properties. Cell adhesion techniques are used to understand chondrocyte metabolism and mechanotransduction, but the material properties must be known for the cell’s mechanical environment to be characterized.
To obtain the shear mechanical environment experienced by the cell, the material properties of the chondrocyte must be identified.\textsuperscript{19} Many models of the techniques used to study cell adhesion, such as micropipette adhesion\textsuperscript{20,21} and microplates\textsuperscript{22} allow the material properties of the chondrocytes to be calculated. Other techniques, such as cytoindentation,\textsuperscript{23} magnetic microbeads,\textsuperscript{24-26} or atomic force microscopy\textsuperscript{27,28} are also used to attain the mechanical properties of the whole cell or a locality of the cell.

The mechanotransduction of chondrocytes in response to shear stress has mostly been studied through fluid-induced shear. These studies have shown an array of effects in response to shear, some regenerative and some degenerative. Several investigations explore the changes in morphology and gene expression of chondrocytes under shear by applying a uniform average shear or simple shear to cartilage explants, while others explore cartilage tissue constructs in bioreactors. Many bioreactor studies compare static versus perfused culture\textsuperscript{29-35} or attempt to tissue engineer cartilage constructs in a low shear or simulated microgravity environment.\textsuperscript{36-39} Static versus perfused versus rotating-wall bioreactor comparisons allow conjecture as to the relative shear levels experienced by the cells and their phenotypic and genotypic response.

Consequently, biomechanical shear forces are important and vitally integral in OA and tissue engineering of cartilage. While compressive, cyclical forces of a certain level are beneficial to chondrocytes, the effects of shear stress are largely unknown. One particularly difficult task is separating out the effects of shear stress from mass transit effects, while another intricacy entails knowledge of the forces transduced to the cell
from the extracellular or pericellular matrix. Therefore, shear stress plays a vital and as yet not fully recognized function for chondrocytes.

**CHONDROCYTES AND CELL ADHESION**

Cell adhesion, which is important to tissue formation, growth, and maturation, involves straightforward attempts to understand how well chondrocytes adhere to a material, which can indicate the level of biocompatibility or the receptors present in the membrane. Generally, a method is devised to remove cells from a substrate and measure the force or pressure of removal. The substrate may be coated with an extracellular matrix or basement membrane protein, such as fibronectin or laminin. Many studies were performed demonstrating how certain cells adhered better to certain substrates. The methods utilized included micropipette aspiration,\textsuperscript{40-43} cytodetachment,\textsuperscript{13,14} fluid-induced shear and cone viscometry,\textsuperscript{15,44,46} and could include microplates.\textsuperscript{47} The techniques used for cell detachment are not only important for adhesion studies, but they can also be modified to examine the effect of shear on genetic response.

**Micropipette Aspiration**

Micropipette aspiration uses a glass pipette with a micron scale tip combined with controlled negative pressure to pluck cells off of a surface (Fig. 2a).\textsuperscript{17,41,42,48,49} The pressure is increased in small steps until the cell is detached from the substrate. Moussy et al.\textsuperscript{41} investigated the adhesion strength of endothelial cells on four different surfaces in either phosphate buffer solution (PBS) or culture medium, finding that the force of
adhesion on both surfaces increased with time. In PBS, the force of detachment increased with surface tension, while in culture medium the opposite occurred.

Other experiments utilized chondrocytes or connective tissue cells. Lee and associates\textsuperscript{17} performed experiments that measured the pressure necessary to pull chondrocytes off of the surface of cut cartilage that was treated with different amounts of chondroitinase ABC. Chondrocytes were seeded onto different zones of the transversely cut cartilage. Adhesion pressure increased with the duration of seeding four to six times and with chondroitinase treatment, though to a lesser extent. Sung and associates,\textsuperscript{42,48,49} in three different studies, used micropipette aspiration to explore differences in adhesion of fibroblasts from two ligaments of the knee, the anterior cruciate ligament (ACL) and the posterior cruciate ligament (PCL). These studies are analogous to using chondrocytes from different zones, as the fibroblasts are two populations of the same cell type. The ACL fibroblasts adhered better to laminin,\textsuperscript{42} while the MCL fibroblasts adhered better to fibronectin.\textsuperscript{48} These results are thought to be due to the different receptor types present in different densities on the fibroblasts.

The micropipette studies demonstrate how cell adhesion studies extract detailed information about cell behavior and allow inferences as to cell membrane composition and organization. Using micropipette aspiration for cell adhesion studies does not measure shear, as the pipette pulls the cell perpendicular to the surface. The complex mechanical environment complicates attempts to compare this technique to others.
Cytodetachment

Cytodetachment is a method that measures the adhesion force by applying shear through a thin cantilever probe under microscopy. A similar method also allows measuring cell adhesion. The force is measured through the deflection of the probe by a fiber optic sensor or a photodiode (Fig. 2b). Athanasiou and associates found that chondrocytes adhered to fibronectin-coated glass better than plain glass and bovine serum albumin-coated glass. This study also examined the cytoskeletal organization of chondrocytes on the different substrata, finding that the cytoskeleton was more organized on fibronectin than glass or bovine serum albumin. Hoben and associates used cytodetachment and found fixed cells had more adhesiveness than living cells and that seeding time increased the adhesiveness. Using the cytodetacher, a study by Huang et al. showed adhesion forces increased and cell height decreased with seeding time. Yamamoto et al. used fibroblasts and calculated the shear strength and adhesive energy by using the force to detach the cell and the projected area of the cell. The adhesion shear strength and detachment surface energy found necessary to detach the fibroblasts were 1.5kPa and 29pJ on collagen-coated polystyrene and 1kPa and 16pJ on fibronectin polystyrene, while these values were approximately 420-670kPa and 7-11pJ for both glass and uncoated polystyrene. These studies were able to find the shearing force of detachment, which allows inference as to the surface composition and binding site density. These methods also lend themselves to mechanical modeling. Further, cytodetachment could be used to explore the mechanical properties of single chondrocytes and combined with reverse transcriptase polymerase chain reaction (RT-PCR) to investigate the genetic and phylogenetic response to shear stress.
**Microplates**

Microplates are glass beams of micron-scale thickness pulled from rectangular glass that are used to apply forces to single cells. The microplates are coated with fibronectin or laminin to allow the cells to adhere more firmly and specifically. One of the plates is rigid, while the other plate is flexible and of known elastic moduli (Fig. 2c). A cell is attached to the rigid plate, and then to the flexible plate. For testing cell adhesion, the rigid plate applies a force pulling the cell away from the flexible plate. The flexible plate’s deflection allows the calculation of the force, from 1-1000nN, exerted upon the cell beam theory since its modulus and thickness is known.

Thoumine and Ott tested chick embryonic cardiac fibroblasts in tension and compression, defining three regimens of mechanical behavior delineated by time scale. During the initial seconds of deformation, the cells behaved elastically, while on the scale of several minutes, the cells behaved viscoelastically. After ten minutes, the cells were found to have contractile forces dependent upon the actin cytoskeleton. The behavior of the cell was modeled using a three element standard linear solid model. The cells’ two elastic moduli were found to be from 0.6 – 1.0 kPa with an apparent viscosity of 10^4 Pa-s.

**Fluid-Induced Shear**

Fluid-induced shear was one of the first methods used to study cellular adhesion. Most fluid-induced shear devices were created to test endothelial cells, but have also been used for connective tissue cells, including chondrocytes. Several types of devices have been created for inducing shear stress with fluid flow; including parallel plate flow...
chambers,\textsuperscript{15} coaxial parallel plate rotation,\textsuperscript{54} cone and plate viscometers,\textsuperscript{45} and jet impingement (Fig. 3).\textsuperscript{55} These methods attempt to create a constant shear force across a monolayer culture of cells. To test adhesion, the percent of cells that detach can be noted, a standard number being the shear stress at which half of the cells detach.\textsuperscript{15} Some studies indirectly study cell adhesion through bioreactors. For instance, Vunjak-Novakovic and colleagues\textsuperscript{56} found that seeding thick scaffolds uniformly requires fluid-flow in mixed flasks versus static culture. The seeding kinetics were modeled with fluid flow, though the role of the resulting shear stress in chondrocyte adhesion to the scaffold is unclear.

Bussolari and Dewey\textsuperscript{45} described a cone and plate viscometer that applies a constant shear stress over a surface, including modeling the flow, which must be maintained at a very low Reynolds number (Fig. 3a). Blackman and colleagues\textsuperscript{44} described an advanced design of a cone and plate viscometer that includes a microstepper motor to allow close control of the rotation of the cone and the ensuing shear stress. The modeling of this device includes a nonsteady state startup three-dimensional flow solution, as well as the steady state solution. Wendl et al.\textsuperscript{54} described a model of a parallel coaxial disk device that simulates shear loading, solving for oscillatory flow conditions in addition to steady state (Fig. 3b). The models of these devices have increased in complexity over time, demonstrating the complexity of achieving a steady shear stress over groups of cells. Schnittler et al.\textsuperscript{46} developed a cone plate viscometer for specifically measuring shear detachment force and showed endothelial cell adhesion increased with laminin and that stress fiber formation was induced under fluid-induced shear.
Schinagl and associates\textsuperscript{15} used a parallel plate shear flow chamber, seeding chondrocytes onto a cartilage surface for between 5 and 40 minutes, inverted the chamber to count the percentage of cells still adherent, and then applied between 6 and 90 Pa of shear stress through fluid flow (Fig. 3c). The fraction of detached cells was then measured. At nine minutes of seeding time, half of the cells were detached due to the inversion and gravity. At 40 minutes of seeding time, half of the cells were detached by 26 Pa of shear stress. The study also found increased seeding time increased adhesion.

Bundy and colleagues\textsuperscript{55} used jet impingement to measure adhesion forces of fibroblasts and the bacteria, \textit{Staphylococcus aureus} (Fig. 3d). This technique uses a submerged jet of fluid to apply shear to a surface. The jet of fluid causes peak shear around its diameter and a gradient of less shear farther away from the stream. The jet detaches cells within a certain area on a plate, the diameter of which is fit with a computer. By modeling the shear stress as a function of radius and noting the greatest average diameter at which cells are detached, the approximate detachment force is calculated. This study found fibroblasts tend to detach due to shear, while the bacteria detached more due to pressure. Further, fibroblasts adhered to titanium and tissue culture plastic approximately the same, likely due to adsorbed proteins. The adhesion strength of bacteria was not as dependent on seeding time as fibroblasts, possibly due to fibroblasts' more complex cytoskeleton organization.

All of the fluid-induced shear studies of cell adhesion test groups of cells. The fluid-induced shear stress is an overall calculated average. While these studies are important to understanding how cells behave under shear, applying a uniform mechanical environment
to each cell is not possible, due to small differences in adhesion, extracellular matrix composition, and cell geometry. The genetic response of the cells gaged by these studies is also of the whole population of cells and does not account for possible differences between cells of the same type, such as fibroblasts from different tendons or chondrocytes from different zones.

**MECHANICAL PROPERTY MEASUREMENT OF SINGLE CELLS**

Cell mechanical properties must be obtained to understand the stress environment of the cell. Cells exhibit a wide array of mechanical properties. For example, while neutrophils tend to behave as a fluid, the chondrocyte exhibits viscoelastic behavior. Koay and colleagues demonstrated viscoelastic chondrocyte behavior in response to a step load using cytoindentation. The material properties of the chondrocyte were obtained using a standard linear solid model. As the chondrocyte and its cytoskeleton are complex, different models are used depending on the type of loading and properties desired. Thus, different cells and different loading regimes require different models. Many of the same techniques used for cell adhesion strength are the same for calculating mechanical properties. Once cell mechanical properties are known, the stress and strain profile of a cell undergoing a specific deformation can be interpreted and compared to other mechanical, genetic, and biochemical environmental factors.

*Micropipette Aspiration*

Micropipette aspiration to obtain mechanical, viscoelastic properties of chondrocytes has used a homogeneous elastic solid model or a standard linear solid model.
which assumes an axisymmetric half-space and incompressibility (Fig. 4a). The differences between using this technique for detachment and using it for measuring cell properties include a pipette tip diameter that is smaller than the cell and recording time displacement curves for the displacement of the cell membrane in the center of the pipette, L. Using an elastic solid model, the chondrocyte was found to behave as a viscoelastic solid; the Young’s modulus of both osteoarthritic and non-osteoarthritic chondrocytes was calculated to be 0.67 and 0.65 kPa, respectively, and not significantly different. The volume change after aspiration was greater in osteoarthritic chondrocytes. Trickey and colleagues employed a more complex standard linear solid model with three parameters to characterize normal and osteoarthritic chondrocytes, finding that osteoarthritic chondrocytes displayed an increased instantaneous and equilibrium modulus, as well as a higher apparent viscosity. This model employs a Kelvin Body to model the chondrocytes’ viscoelasticity phenomenologically (Fig. 5a). Another study found decreasing osmolarity led to decreased moduli and apparent viscosity in association with dissociation of actin, though no change to hyperosmotic stress. A study of the pericellular matrix with a chondrocyte found that the pericellular matrix was two times as stiff as the chondrocyte. These models demonstrate viscoelastic solid behavior by the chondrocyte and alterations in mechanical properties that were due to loading, osmotic environment, and disease states. Further, the surrounding pericellular matrix was found to be stiffer than the cell and thought to be less stiff than the extracellular matrix. These results have implications for the transduction of force from the tissue to the cell and may be used in future modeling.
Single-Cell Cantilever Techniques

Another method of obtaining the material properties of a cell involves applying a force to the cell via a cantilever beam. Cytoindentation uses a thin probe to compress the cell (Fig. 4b). Modeling the cell with two continuum mechanics models, including a mixed-boundary value, linear elastic solid with Bousinesq-Papkovitch potential functions and a linear biphasic model, the compressive modulus of a cell line was found. This apparatus allows a prescribed displacement and force profile on a single cell. Another indentation technique used a glass probe to indent the surface of mouse embryonic carcinoma cells, looking at differences between cells with and without vinculin. Vinculin-deficient cells were less resistant to indentation, indicating vinculin as an important part of the cytoskeleton. Similar cantilever techniques, such as microplates, have also been used to obtain cell mechanical properties, while other cantilever techniques could be modeled to obtain properties. Cytodetachment, discussed earlier, could be modeled to further understand how a single cell behaves under shear.

Atomic Force Microscopy

Atomic force microscopy has been used to investigate the differences in mechanical properties of various cells, including endothelial, cardiac and skeletal muscle, liver endothelial cells and fibroblasts, aortic endothelial cells, osteoblasts, and fibroblasts cell line cells (Fig. 4c). The Hertz model is usually used and assumes a pointed or spherical tip indentation probe and an elastic, homogenous material. The deflection of the probe is measured, and the applied force can be calculated using beam theory. The deformations are infinitesimal. Sato and colleagues looked at the changes in local cell
properties in endothelial cells exposed to fluid-induced shear stress, similar to studies of chondrocyte metabolism. Comparison of cardiac and skeletal muscle viscoelastic properties is similar to ascertaining the differences between zonal chondrocytes or between fibroblasts from two similar ligaments.\textsuperscript{66}

\textit{Laser Tracking Microrheology}

The procedure of using laser tracking microrheology involves implanting small spheres into the cytoskeleton and recording their micromotion.\textsuperscript{69} Yamada and colleagues tracked polystyrene particles in polymerized actin and COS-7 cell cytoplasm without applying any forces. The Brownian motion of the particles is indicative of the local mechanical properties. The technique demonstrated the viscoelastic nature of cytoplasm and measured local differences in the cytoplasm.

\textit{Magnetic Beads}

One method that can test local membrane mechanical properties is called magnetic bead microrheometry.\textsuperscript{26,70} A 4.5 \(\mu\)m magnetic bead is coated with a molecule that will bind a specific receptor and a magnetic field is applied to the bead, which applies a force to the membrane (Fig. 4d). The beads that are transmitting force onto the cells are tracked versus time, allowing force response curves to be generated. The viscoelastic behavior is modeled as a dashpot in series with a Kelvin body (Fig. 5). The data is curve fit and an effective modulus, relaxation time, and a viscosity is calculated. A similar study utilized the same size beads and applied tension on part of the cell membrane, but did not include
modeling. Force versus displacement curves were compared between different cells with and without vinculin.

**EFFECT OF SHEAR STRESS ON CHONDROCYTES**

While compressive or hydrostatic forces have been studied and shown to be beneficial at specific frequencies and levels, the effect of shear stress on chondrocytes remains controversial. While shear has shown to increase proliferation, glycosaminoglycan (GAG) size and amount, proteoglycan size, and protein amount, other effects of shear appear injurious to chondrocytes. Fluid-induced shear has been shown to increase pro-inflammatory mediators such as interleukin-6, nitric oxide, and prostaglandin E2. Protective molecules such as tissue inhibitor of metalloproteinase (TIMP-1) and interleukin-4 are also induced by shear, possibly indictating activation of protective mechanisms. The effects of shear on metabolism and proliferation are summarized in Table 1. Further adding to confusion, many studies compare static to perfused to rotating wall bioreactors. Rotating wall constructs attain the best results, yet the level of shear stress in these reactors is an average over a population and not always characterized. Furthermore, mass transport effects cannot be separated from the mechanical effects of the shear using fluid-induced shear in a viscometer or bioreactor.

**Metabolism and Proliferation**

The metabolism of chondrocytes in response to shear appears increased and reflective of an injurious state. Smith and colleagues found 1.6 Pa of fluid-induced shear in a cone viscometer caused bovine chondrocytes to align along the axis of flow and doubled GAG
synthesis. Proteoglycan molecule size was increased, while prostaglandin E2 (PGE2) increased by a factor of nine. Other pro-inflammatory mediators were not increased, including matrix metalloproteinase-3 (MMP-3), collagenase, and stromelysin, but an inhibitor of MMP-3, tissue inhibitor of metalloproteinase 1 (TIMP-1) was increased by a factor of 10. Archambault et al.\textsuperscript{78} found that rabbit fibroblasts produce MMP-1, MMP-3, and cyclooxygenase-2 under fluid-induced shear. Das and colleagues\textsuperscript{75} found that GAG and nitric oxide synthesis was increased in response to fluid-induced shear. Nitric oxide increased in proportion to the magnitude and duration of the shear stress. Mohtai et al.\textsuperscript{74} explored the expression of the pro-inflammatory cytokine interleukin 6 (II-6) in chondrocytes. Osteoarthritic chondrocytes produce II-6, while normal chondrocytes do not. When fluid-induced shear was applied at 1.6 Pa for 48 hours, II-6 mRNA and protein increased approximately 10-fold. Signals for mRNA from interferon-1\(\alpha\) and 1\(\beta\), tumor necrosis factor-\(\alpha\), and transforming growth factor-\(\beta\) were not affected by shear. II-6 mRNA was increased within one hour of applied shear, indicating a possible role in mechanotransduction. Under 1.64 Pa of fluid-induced shear stress, chondrocytes augmented the production of nitric oxide synthase gene and increased nitric oxide delivery by the cells up to 3.5-fold at 24 hours.\textsuperscript{79} Collagen type II mRNA and aggrecan mRNA were both inhibited by shear stress, but inhibiting nitric oxide production increased both mRNA levels. Based on these studies and others, Smith and colleagues\textsuperscript{77} put forth a model where shear stress causes chondrocytes to convert to an injurious state characterized by certain metabolic products, such as II-6, PGE-2, and TIMP-1.

Other studies confirmed the increase in GAG synthesis and showed other beneficial effects of shear. Gooch et al.\textsuperscript{32} explored static versus mixed flasks and rotating
bioreactors with and without IGF-I. The mixing in bioreactors causes fluid-induced shear; the rotating bioreactor likely has less shear stress than the mixed bioreactors. The mixed bioreactor constructs proliferated more than the static, though not significantly different from the rotating bioreactor. IGF-I caused an increase in cell count in all bioreactors and an increased amount of GAGs in the rotating bioreactor. Interestingly, a decrease in GAG content was seen in the mixed flask versus static if IGF-I was not present. Collagen was increased in the mixed and rotating bioreactors, while GAG was increased the most in the rotating bioreactors with added IGF-I. This study found a non-linear correlation between GAG content and equilibrium compressive modulus. Although these results were rigorously obtained, comparison of these results with other studies on chondrocyte metabolism is confounded due to the uncharacterized nature of the flow and stress fields within each bioreactor. Millward-Sadler et al.76 studied the effect of II-4 on chondrocytes, which induces hyperpolarization of the membrane under hydrostatic pressure. Antibodies to II-4 blocked the mechanical-induced hyperpolarization, and II-4 knockout mice did not exhibit hyperpolarization. These results indicate a role in mechanotransduction for II-4, but do not indicate a mechanism. In another study, normal, but not osteoarthritic chondrocytes under intermittent hydrostatic pressure decreased the amount of MMP-3 and increased the amount of aggrecan.80 These changes in metabolism for normal chondrocytes were found to be coupled to mechanotransduction through II-4. Malaviya and Nerem71 found an increase in proliferation of chondrocytes under shear of 3.5 Pa (35 dynes/cm²) after four days. Jin et al.73 applied 1-3% simple shear strain at frequencies of 0.01 to 1.0 Hz to cartilage explant disks, illustrating an increase of protein and proteoglycans of approximately 50 and 25%, respectively. The only significant difference
was between the static culture and shear; no significant differences were found based on the level of shear stress or frequency. These increases only occurred with fetal bovine serum, not in serum-free media. Jin and colleagues\textsuperscript{73} concluded that the stimulatory effect of shear is mostly due to deformation, not fluid flow.

These studies demonstrate that shear stress, fluid-induced or not, have a dramatic effect on the metabolism and gene expression of chondrocytes. Somewhat contradictory data are also present in these studies, for example one found MMP-3 mRNA levels change with intermittent hydrostatic pressure and another found no MMP-3.\textsuperscript{72,76} The experiments involving fluid flow pose the problem of delineating the effects of mass transport and mechanical shear. Though mechanically-induced shear stress did not exhibit a dose-dependent effect, the range of shear stresses was small, and the force transmission to the cells in matrix is not wholly known. So, a dose-dependence of cartilage gene expression and shear stress is possible. The pro-inflammatory and cytoprotective cytokines produced by chondrocytes in response to shear stress can be interpreted as a response to damage.\textsuperscript{77} The increase in ECM production, likewise may be a consequence of attempts at repair. However, the differential effects of between fluid-induced shear, mechanical shear, and mass transport remain unidentified.

**Bioreactors**

A major thrust of tissue engineering involves developing cartilage constructs in bioreactors. Cartilage develops better in bioreactors that have some perfusion or flow versus static conditions, and fluid flow induces shear stress. These observations can indirectly aid in understanding the effect of shear stress on chondrocytes, though the
exact levels of shear are unknown for each cell and mass transport effects are again inseparable. Two major classes of bioreactor studies involve comparing static to perfused to rotating wall bioreactors\textsuperscript{29-35} and simulated microgravity bioreactors\textsuperscript{36-39}

\textit{Static versus Mixed versus Rotating Bioreactors}

Comparative studies between static culture, mixed flasks, and rotating bioreactors have shown that rotating bioreactors tend to produce the best constructs, followed by mixed flasks. Pazzano et al.\textsuperscript{35,81} compared static to perfused bioreactors, finding that the perfusion of 1 μm/s of flow increased DNA content, GAGs, and hydroxyproline by 118%, 184%, and 155%, respectively. Dunkelman et al.\textsuperscript{82} built a bioreactor with a continuous flow rate of 50μm/min (0.83 μm/s), achieving a 15% and 25% dry weight of collagen and GAG, respectively. Freed and colleagues\textsuperscript{31} demonstrated that doubling time decreases approximately 60% with mixing versus static, which was attributed to increased mass transport. Freed and colleagues\textsuperscript{30} attempted to recapitulate native cartilage in a rotating wall bioreactor using bovine chondrocytes on PGA for 40 days, finding comparable cellularity, 68% GAG, and 33% collagen of native cartilage. Gooch et al.\textsuperscript{32} also using PGA and bovine chondrocytes, compared static culture to mixed Petri dishes and flasks and a rotating wall bioreactor with and without IGF-I. In all cases IGF-I increased wet weight. IGF-I also increased GAG content 1.7-fold in mixed flask and 2.9-fold in the rotating bioreactor and increased collagen 1.6-fold in the rotating bioreactor, while decreasing collagen in static culture. GAG content was found to be correlated to the equilibrium modulus. Mizuno et al.\textsuperscript{34} used bovine chondrocytes on collagen sponges with and without perfusion. In contrast to other studies, the static cultures were found to
have 3.5-fold and 2.4-fold increases in aggregan and collagen type II compared to perfused cultures, respectively. Martin et al.\textsuperscript{33} compared the mechanical properties of PGA-ovine chondrocyte constructs cultured in static versus mixed flask versus rotating bioreactors. Grossly, the rotating bioreactor construct was the most like native cartilage with 75% of the GAG, 39% of the collagen, and 20% of the equilibrium modulus. The study also included up to 7 months of culture in a rotating bioreactor and achieved the equilibrium modulus and GAG content of native cartilage, though not the stiffness or collagen content. While these studies have some conflicting results, rotating bioreactors seem to produce the best constructs. However, what combination of increasing mass transport and adding mechanical stimulation will optimize the cartilage tissue engineering is not fully understood.

One other study that may shed light upon the issue of shear’s effect on chondrocytes combines different bioreactors for the same culture. Carver and Heath\textsuperscript{39} used five experimental groups with different combinations of spinner flasks (s), intermittent compression/perfusion system (p), and a control without the intermittent compression (c). The fluid flow in the spinner flasks was turbulent, while the rotating bioreactor likely had less fluid-induced shear, though neither was modeled. The five groups were noted by 1s5c, 1s5p, 2s4p, 4s2p, and 6s. The 2s4p experimental group outperformed the others in collagen and GAG content, though the 4s2p group was next best. This study, though the mechanics and mass transport are uncharacterized, put the chondrocytes in a higher-shear environment initially and switched to a lower shear environment, producing better constructs.
Bioreactors and Microgravity

Mathematical models of bioreactors attempt to characterize the mechanical and mass transport environment due to fluid flow, though this characterization is not always included in bioreactor studies. Much of the mathematical modeling of bioreactors explores simulated microgravity. The rotating wall bioreactor was designed to minimize shear stress and still provide mass transport. While several bioreactors have been designed to simulate microgravity for cells or aggregates of cells, some mechanical stresses are present.

The simplest model of the rotating wall bioreactor involves a force balance on a PGA construct.\textsuperscript{38} The forces balanced were the gravitational, hydrodynamic, and centrifugal, resulting in a hydrodynamic force due to drag of 0.15 Pa (1.5 dyne/cm\textsuperscript{2}). This study assumed two-dimensional flow and that the constructs did not interrupt the fluid flow. The model also assumes a consistent construct size. Another early model of the rotating wall bioreactor by Tsao and colleagues\textsuperscript{83} assumed steady, Couette flow (flow between two parallel plates), modeled with Navier-Stokes equations. This model showed a shear stress component in unit gravity up to approximately 0.1 Pa (1 dyne/cm\textsuperscript{2}), especially as the particle size increased. In the model, space-based operation of the bioreactor substantially decreased the shear stress component. Begley and Kleis\textsuperscript{36} modeled the rotating wall bioreactor only assuming axisymmetric flow and validated the model with laser velocimetry. Their model also found large differences between space-based operation and simulated microgravity and used the inner and outer rotation regimes normal for space (inner rotation slower) or earth-based (inner and outer rotation equal).
operation. Mean shear was found to be increased two to three-fold in earth-based operation. Though the shear levels were still low, the mass transport effects cannot be separated from the hydrodynamically-imposed shear effects.

A new design for a bioreactor, the hydrodynamic focusing bioreactor designed for NASA, was modeled with results showing even less shear stress on constructs than the rotating wall bioreactor. This model assumed that mean shear stress is the same in space and earth-based applications, which was shown not to be the case for the rotating wall bioreactor. Using FLUENT, software to model fluid-mechanics, the model of the dome-shaped bioreactor was calculated to have a maximum shear of 0.001 Pa (0.01 dyne/cm²).

The bioreactor studies show promising results for tissue culture of cartilage, but the mass transport and mechanical environment, even if well-characterized, are only averages over a population of cells. The genetic and phenotypic response of cell subpopulations, for example different zones of chondrocytes, would be masked in these experiments. In both the bioreactor and metabolism studies, the reaction of a single chondrocyte to a particular level of shear is unknown and inseparable from the different levels of mass transport.

**Mechanotransduction**

The surface and cytoskeleton of the chondrocyte interact with each other and their environment to transduce mechanical signals into genetic expression and biochemical states. Schmidt et al. found that stressing β1 or α2 integrins on osteoblast membranes
caused an increase in tyrosine phosphorylation, as well as increased phosphorylation of mitogen-activated kinases. The stresses were induced magnetically and nonspecifically. Das et al.\textsuperscript{75} established that nitric oxide increases in response to shear 18-fold in two hours. By utilizing inhibitors of cellular cascade pathways, the investigators discovered that G-proteins, nitric oxide, and phospholipase C are involved in the transduction of GAGs. Calcium and potassium channel blockers did not inhibit GAG synthesis.

While studies found ion channel blockers did not inhibit GAG synthesis, several studies do implicate ion transients as a method of mechanotransduction.\textsuperscript{61,85,86} Guilak and colleagues\textsuperscript{61} found changes in the viscoelastic moduli with osmotic stress. Hypo-osmotic stress generated decreased instantaneous and equilibrium moduli, as well as apparent viscosity. Hypo-osmotic stress also caused actin cytoskeleton dissociation. Erikson et al.\textsuperscript{85} illustrated hyper-osmotic stress increased calcium in the cell and decreased cell volume.

Mobasheri et al.\textsuperscript{86} put forth a mechanoreceptor model involving integrins, ion channels, extracellular matrix (ECM), and cytoskeleton based on the co-localization of the ion channels and integrins. These mechanoreceptor complexes are thought to respond to deformation, changes in ion concentration, and possibly streaming potentials. The specific configuration or operation of these complexes is still unknown. Much of the chondrocyte mechanotransduction apparatus remains to be deciphered.
CONCLUSION

The relationship between shear and chondrocytes is not fully elucidated, though some aspects are more explicitly grasped than others. Cell adhesion studies result in information about chondrocyte mechanotransduction. The same techniques, accompanied by appropriate continuum mechanics models, furnish viscoelastic cell mechanical properties. Knowledge of these properties permits acquisition of the amount of shear stress the cell experiences when loaded in a certain way. Understanding the mechanical properties and the mechanical environment of the chondrocytes allows detailed study of how shear affects chondrocytes.

The metabolism, gene expression, proliferation, and phenotype of the chondrocyte in response to shear loading has been demonstrated to have various results. While pro-inflammatory cytokines increase in response to fluid-induced shear stress, proliferation and extracellular matrix products have also been found to increase.\textsuperscript{72,74,76,79,86} From the perspective of tissue engineering, increasing proliferation and extracellular matrix is beneficial, possibly decreasing the currently lengthy culture times for chondrocytes. However, the increase in pro-inflammatory cytokines due to shear stress is likely unsuitable for chondrocyte culture. Bioreactor studies have also had mixed results, showing static culture is not as suitable as perfusion or rotating wall bioreactor cultures, but the studies are unable to differentiate mechanical and mass transport effects on the culture. Simulated microgravity bioreactor studies have had success in improving tissue constructs, but have not reached native cartilage levels and cannot separate out effects of mass transport and mechanical environment. Further, earth- and space-based operation of
the same bioreactor results in different mechanical and flow environments, which also depend on the particle or construct size. While shear stress has both regenerative and degenerative effects on chondrocytes, the amount of stress and the effects of mass transport on the cells to achieve these effects remain unknown.

Future work in the form of a single cell approach\textsuperscript{19} to shear stress and chondrocytes is needed to be able to fully understand effects of shear in regenerative and degenerative processes. The single cell approach involves applying a known shear stress to a single chondrocyte with known cell material properties and measuring its genetic and phenotypic response. Using reverse transcriptase polymerase chain reaction (RT-PCR), the mRNA levels of degenerative and regenerative genes can be attained in chondrocytes that experience different shear stress regimes. By understanding what levels of shear induce particular cytokines and at what level, inhibitors of injurious cytokines at specific concentrations can be used to counteract negative effects. Knowledge of the intensity and duration of shear stress that induces proliferation and extracellular matrix permits shorter culture time and improved constructs. Combining current studies with a single cell approach will result in more comprehensive strategies for tissue engineering cartilage.
ACKNOWLEDGMENTS

We gratefully acknowledge the support of the Whitaker Foundation and The Arthritis Foundation.
Table 1. Effect of shear stress on metabolism and proliferation of chondrocytes.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Parameter</th>
<th>Change from baseline</th>
<th>Shear Stress</th>
<th>Time</th>
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<td>Smith et al., 1995</td>
<td>Cell orientation, peak angle 35-45° and 125-135°</td>
<td>increased 2 fold</td>
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<td></td>
<td>Glycosaminoglycans</td>
<td>increased</td>
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<td>24, 48 hrs</td>
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<td></td>
<td>Glycosaminoglycan size</td>
<td>increased</td>
<td>1.6 Pa</td>
<td>48 hrs</td>
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<tr>
<td></td>
<td>Proteoglycan size</td>
<td>increased</td>
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</tr>
<tr>
<td></td>
<td>TIMP-1</td>
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Figure 2. Techniques for cell adhesion experiments.

a.) Micropipette aspiration – the pressure is recorded at the time the cell detaches. b.) Cytodetachment – the deflection of the probe is recorded and calculated into the applied force at the time of detachment. c.) Microplate adhesion rupture – the deflection of the flexible plate can be recorded and calculated into the applied force at the time of detachment.
Figure 3. Techniques to apply fluid-induced shear stress over a population of cells.

a.) Cone and plate viscometer. b.) Coaxial plate viscometer. c.) Parallel plate flow chamber. d.) Jet impingement and lesion size.
Figure 4. Techniques to obtain mechanical properties.

a.) Micropipette aspiration. b.) Cytoindentation. c.) Atomic force microscopy. d.) Magnetic bead microrheometry.
Figure 5. Phenomenological models of viscoelastic behavior.

a.) Kelvin body with a spring in parallel with a series of a dashpot and a spring. b.) Kelvin body in series with a dashpot.
CHAPTER 2: DEVELOPMENT AND VALIDATION
OF VERTICAL SCANNING INTERFEROMETRY AS
A NOVEL METHOD FOR ACQUIRING
CHONDROCYTE GEOMETRY*

ABSTRACT

Chondrocytes are sensitive to changes in shape which depend on the type of substrate, mechanical factors, or biochemical stimuli. Shape changes can cause metabolic and phenotypic alterations. Cell geometry is also important for mechanical models, determination of mechanical properties, and the study of cell attachment and spreading. In this study, a novel method called vertical scanning interferometry (VSI) was developed to allow rapid and straightforward determination of the height, diameter, surface area, volume, and curvature of single chondrocytes. The dimensions of single chondrocytes at 4 h and 18 h were obtained and validated. Differences in the heights of zonal chondrocytes were found to be statistically significant. This method was also used to capture the geometry of a cell dehydrating as it was exposed to air. VSI has advantages over confocal microscopy and atomic force microscopy in terms of speed,

ease of use, field of view, and precision. VSI quickly obtains and graphically represents the three-dimensional geometry of chondrocytes in a simple format. These methods could be expanded to image many types of cells on various biomaterials to assess biocompatibility and attachment and cell spreading characteristics.
INTRODUCTION

Obtaining three-dimensional cell geometry is beneficial for several fields of study, including tissue regeneration, cytomechanics, and cell biology. Many types of cells are in a dynamic environment with either mechanical or chemical stresses constantly changing.\(^1\) Chondrocytes in particular have been shown to change their metabolism and even phenotype in response to cell shape.\(^87-89\) Three-dimensional geometric representation of the attachment and spreading of chondrocytes has important implications for tissue engineering, as well as for understanding basic cellular functions.\(^14,50,59,90\) One method used to explore the response of chondrocytes to mechanical forces involves a single cell approach, which can allow measurement of genetic responses to a known stress field experienced by a single cell.\(^91\) Using this single cell approach, the three-dimensional shape of a cell seeded onto a surface could be input into a biomechanical model for more accurate elucidation of its mechanical properties.\(^13,21\) For example, the curvature of a chondrocyte could be utilized by analytical models of cytomechanics that assume radial symmetry, while the cell's other dimensions can be used to generate finite element models. Thus, the three-dimensional geometry of the single cell is vital for accurate acquisition of its mechanical properties, knowledge of its mechanical environment, and characterization of attachment and spreading.

Several methods exist for obtaining the geometry of a single cell. Confocal microscopy,\(^92-94\) atomic force microscopy (AFM),\(^95,96\) and idealization of the shape of a cell\(^50\) are all utilized to acquire or estimate the shape of a cell. Confocal microscopy uses
lasers to scan different planes of the cell and reconstructs those planes mathematically into a solid.\textsuperscript{50,97,98} The surface area and volume can then be obtained from computational, stereological geometric reconstruction or other calculations.\textsuperscript{92,94,97} AFM records the deflections of a very small probe over surfaces and is able to reconstruct these lines of data to attain a three-dimensional geometry.\textsuperscript{95} AFM is more often used to visualize structures smaller than complete cells, such as membrane surface features or chromosomes.\textsuperscript{99,100} Both AFM and confocal microscopy are valuable tools for describing the shape of single cells.

A novel method that may work for imaging cells is vertical scanning interferometry (VSI), which can produce the three-dimensional geometry of a surface in the form of a height map. VSI uses optical interference patterns to construct the three-dimensional geometry of a surface. VSI has been used to study dissolution rates of minerals under different environmental conditions.\textsuperscript{101} VSI is also used by the semiconductor industry to image circuit boards for quality assurance. Since cells seeded onto a substrate can be geometrically described as a surface, VSI was applied to attempt to acquire detailed chondrocyte geometry. The objective of this investigation is to develop and validate VSI as a method to acquire salient geometric features of chondrocytes seeded onto a substrate. This study validates the diameter measurement and demonstrates the feasibility of obtaining the height, surface area, volume, and curvature of chondrocytes seeded on glass coverslips using VSI. A feasibility of performing a live cell air dehydration study as a function of time is also performed. Differences in the height between superficial and middle/deep chondrocytes as a function
of seeding time are demonstrated. This experiment also exhibits the proficient visualization capabilities of VSI for viewing single cells.

**MATERIALS AND METHODS**

*Chondrocyte Harvest*

Articular chondrocytes were extracted from the distal metacarpal of the metacarpo-phalangeal joint of adult male bovine cartilage using sterile technique within 24 h of slaughter. The cartilage was zonally harvested using an abradement method. Briefly, the abradement technique consisted of scraping the surface three times with a #10 scalpel blade at an acute angle to the cartilage surface to remove the superficial zone, and then removing the middle/deep zone. Histology illustrated the superficial layer was completely removed using this technique. The zonal cartilage was digested in 2 mg/ml of collagenase (Biowhittaker, Maryland) overnight in Dulbecco’s Modified Eagle Medium (DMEM) (Biowhittaker) supplemented with 100 units/ml Penicillin (Biowhittaker), 100 mcg/ml Streptomycin (Biowhittaker), 10% Fetal Bovine Serum (FBS) (Biowhittaker), 2.5 mcg/ml Fungizone (Biowhittaker), 2 mM L-Glutamine (Biowhittaker), 0.1 mM non-essential amino acids (NEAA) (Invitrogen, Grand Island, NY), and ascorbic acid (50 mg/L).

*Chondrocyte Seeding*

Primary chondrocytes were seeded onto glass coverslips (Fisher Scientific, Pennsylvania). At 4 and 18 h after seeding, the chondrocytes were fixed using 0.3%
solution of glutaraldehyde (Sigma, Missouri) with 80mM Kpipes (Sigma), 5mM EGTA (Sigma), and 2mM MgCl2 (Fisher, New Jersey) for 20 min. Glutaraldehyde is a commonly used fixative for electron microscopy since it has been shown to preserve subcellular structures well and cross-links proteins quickly. The chondrocytes were dehydrated in serial ethanol dilutions. Ethanol solutions of phosphate-buffered saline were made and the coverslips were serially immersed for 5 min in the following dilutions: 10%, 25%, 50%, 75%, 85%, 95%, 100%, and 100%. The ethanol was allowed to evaporate right before the data were acquired.

Vertical Scanning Interferometry (VSI)

An optical vertical scanning interferometry (VSI) system was used to obtain the height maps of the cells, from which the height, surface area, volume, and curvature were determined. A scanning white light phase shift MicroXAM interferometer (ADE Phase Shift, Tucson, Arizona) was used with a Mirau objective. VSI consists of a light microscope with a Mirau interferometer objective, a motorized stage, CCD camera, and white light source. This technique uses the interference of two beams of light, a reference beam and a sample beam. The sample beam is reflected off the surface of the chondrocytes and their substrate, while the reference beam is reflected off of a mirror inside the Mirau objective (Fig. 6). Once these two beams have been reflected, they are focused back together and form an interference pattern of light and dark fringes, known as an interferogram (Fig. 7). The fringes are horizontal on the main part of the Fig. 7, while they form more of a target pattern on the two inset cells. The light and dark fringes are used in combination with the wavelength of the light to determine height differences
between each of the fringes. A piezoelectric (PZT) stage moves the sample vertically with nanometer-precision, which produces phase shifts in the interferograms. All of the interferograms are digitized with the CCD camera and used to calculate a topographic surface map. The vertical resolution of VSI is about 2 nm, while the horizontal resolution is currently 0.5 μm.

**Chondrocyte Diameter Validation**

Following implementation of interferometry to visualize single chondrocytes a validation study was performed to insure the fixation process did not shrink the cell, as evidenced by a decrease in diameter. Using a reticle (Nikon Instruments, Japan), cell diameters were measured on ten live cells, ten fixed cells, and ten dehydrated cells on the same coverslip. Interferometry was then performed on ten cells on the same coverslip and the diameters were measured from the height maps and compared.

**Height, Surface Area, Volume, and Curvature**

Height data of 15 chondrocytes were taken at 4 and 18 h for superficial and middle/deep cells. The volume and surface area of ten middle/deep and ten superficial cells at 4 and 18 h were acquired using MapVUE software (ADE-Phase Shift, Tucson). The data is in the format of a height map, much like a topographic map. To acquire the curvature a row of height values through the center of the cell with their corresponding spatial location were taken and graphed in two dimensions. A second-order polynomial curve-fit was then performed on each curve. The height was taken as the largest vertical dimension in the height map of the cell minus the substrate. The surface area and volume
measurements were acquired using the MapVUE measurement tools. In short, the surface area and volume were calculated by first subtracting out the plane of the substrate or surface of attachment, which was like a half-space, and taking the topography of the cell above the substrate. Then, the surface area was considered to be the calculated area encompassed by the parts of the height map above the substrate, while the volume of the cell was calculated from the volume of the height map above the substrate. Since the steepest parts of the cell did not reflect well, they were interpolated with a lower order polynomial using the MapVUE software, but only for the surface area and volume calculation. Interpolation was only needed for a small area of the cell, and was unnecessary to acquire the height, curvature, or diameter.

*Chondrocyte Air Dehydration*

To explore the feasibility of a cell air dehydration study, live, unfixed chondrocytes were exposed to air while imaging the geometry with VSI. The cell geometry was taken at 20 min, 45 min, and 3 h.

*Statistics*

The diameter, height, surface area, and volume data were statistically analyzed by analysis of variance (ANOVA). A p-value less than 0.05 was deemed significant. When significant, a Fisher's PLSD post-hoc was performed.
RESULTS

Height Map Visualization

To demonstrate the visualization abilities, chondrocytes were seeded upon glass coverslips, and a height map of single cells was obtained. Figure 8 illustrates the height map of a chondrocyte from the middle/deep zone, seeded for 4h, from nine different perspectives. The different colors correspond to different height ranges, and the scaling of the geometry can be customized. The height map in the center is viewed from the side, while the height map above it is being viewed from above and so forth. At the base of the cell, a faint ring of raised substance is also visible. A diagram of a house to the lower right of each image illustrates the corresponding perspective. The bottom row reveals the ability to peer at the geometry from the inside. The same height map was visualized without color-coded height ranges, as well.

Diameter Validation

The diameters of the chondrocytes measured using an inverted Eclipse TS-100 microscope (Nikon USA, Melville, NY) with a reticle (Nikon USA, Melville, NY) under 400X showed no statistical difference (p < 0.05) among the different conditions of live cells (14.21 ± 0.86 μm), fixed cells (13.84 ± 1.01 μm), or cells serially dehydrated with ethanol (13.84 ± 1.16 μm). The diameters of the same population of cells that were dehydrated with ethanol measured using VSI (13.24 ± 0.81 μm) were not statistically different from the other conditions measured with a reticle. All measurements were taken on the same population of cells. The reticle measurements had a slightly higher standard
deviation owing to decreased precision. The reticle measurements were ± 0.5 μm. Since no statistical difference existed between the diameters of the live cells, the fixed cells, or the ethanol dehydrated cells, the fixation process does not seem to significantly affect the diameter of chondrocytes. The VSI measurements were also not statistically different from any of the reticle measurements, so the diameter measurement of VSI is valid.

**Zonal Chondrocyte Heights**

The heights of the zonal chondrocytes measured with VSI were statistically different (p < 0.05) at the different time points, as well as between superficial and middle/deep cells (Fig. 9a). At 4 h the superficial chondrocytes (5.56 ± 0.34 μm) were statistically shorter than the middle/deep chondrocytes (6.36 ± 0.44 μm). At 18 h, the superficial cells were 4.41 ± 0.46 μm and the middle/deep cells were 5.12 ± 0.26 μm. Between 4 and 18 h, the middle/deep and superficial cells became 19% and 21% shorter, respectively. The morphology of the cells at both time points was round as opposed to oval- or spindle-shaped.

The 4 h time point was in reasonable agreement with a previous study by Huang et al.\(^{50}\) which did not separate superficial from middle/deep or utilize bull chondrocytes. The data from Huang et al.'s\(^{50}\) study (7.6 ± 1.3 μm) were obtained using confocal microscopy and are shown in Fig. 9a. The standard deviation of the confocal microscopy study is substantially larger than that of the VSI measurements.
Surface Area and Volume

The surface area of the cells was only significantly different (p < 0.05) between the 4 and 18 h time points of the superficial chondrocytes (Fig. 9b). The surface area did not significantly decrease between the 4 h middle/deep (293 ± 121 \( \mu \text{m}^2 \)) and the 18 h middle/deep (280 ± 65 \( \mu \text{m}^2 \)). The 4 h superficial chondrocytes were 407 ± 175 \( \mu \text{m}^2 \), while the 18 h superficial chondrocytes were 186 ± 133 \( \mu \text{m}^2 \). The standard deviations for the surface area were fairly large.

The volume of the cells mirrored the changes that occurred in the surface area. The volume decrease from 4 to 18 h was larger than the decrease in surface area (Fig. 9c). The volume of the superficial cells at 4 h was 241 ± 23 \( \mu \text{m}^3 \) and decreased to 138 ± 40 \( \mu \text{m}^3 \) at 18 h. The volume of the middle/deep cells decreased from 227 ± 35 \( \mu \text{m}^3 \) at 4 h to 172 ± 32 \( \mu \text{m}^3 \) at 18 h. The 4 h and 18 h superficial cells were significantly different (p < 0.05), but the middle/deep cells were not significantly different between 4 h and 18 h. Other significant differences include the 4 h superficial and the 18 h middle/deep and the 4 h middle/deep, and the 18 h superficial (p < 0.05).

Curvature

The curvature was obtained by taking a line of height values through the middle of the cell. The curvature fits of the 4 h and 18 h chondrocytes were high-quality with \( R^2 \) values both above 0.94 (Fig. 10). The solid line is a polynomial fit. In the examples of Fig. 10, the equation of fit for the 4 h chondrocyte is \( y = -287.27x^2 + 6060.8x - 26116 \), while the equation of fit for the 18 h chondrocyte is \( y = -170.36x^2 + 3203.6x - 10146 \).
The cell curvatures at both time points shows chondrocytes to be somewhat flattened on the top.

*Air Dehydration of Live Cells*

Figure 11 illustrates the results of a live chondrocyte exposed to air at 20 min, 45 min, and 3 h. The cell becomes noticeably flatter and shrinks unevenly. Even at 20 min, the cell has very noticeably flattened into a coin shape and has an irregular, plateau-like surface with protrusions. The 20 min image of the cell was the first available after the media had evaporated. At 45 min and 3 h, the cell has become even flatter and done so asymmetrically. The image at 45 min shows a fairly flat cell with some small peaks on the plateau and a small rim around part of the cell. The 3 h image is more asymmetric and shows a wedge of the cell flatter without any raised edge.

**DISCUSSION**

Overall interferometry is a promising technique for nanometer-precision cell geometry visualization. The results show that the geometry of the cell can be ascertained with relative ease and high precision. The validation study illustrated that the diameter of the single chondrocyte does not change due to fixation with 0.3% glutaraldehyde, serial dehydration with ethanol, or air drying. While some shrinkage may occur of the cell height, previous data using goat chondrocytes is within a micron and a half. Further, glutaraldehyde or any aldehyde fixation can cause blebs in the cells, which is a potential source of artifact. Any contraction of the cell in the vertical direction is likely to be mostly uniform, still producing the essential geometry. Further validation in terms of the
height measurement of single cells may be beneficial, though VSI vertical measurement capabilities have been established. One other difficulty is the steepest parts of the cell do not reflect very well, thus some of the data must be interpolated using a lower order polynomial fit for surface area and volume calculation, but the interpolation is unnecessary for height, diameter, and curvature.

VSI is a fast method for obtaining the height maps of cells seeded onto a substrate. The vertical scan rate is 2 μm/s, which allows all cells in a field of view at 400X to be scanned in under 10 s. An advanced software package (MapVUE, ADE-Phase Shift, Tucson) allows fast calculation of the maximum height, surface area, and volume. The software also allows three-dimensional visualization of the cells from any perspective. Cells with complex geometry can be visualized and analyzed relatively easily without making large assumptions or idealizing the shape. It is also noteworthy that VSI allows for visualization of other objects around the cell, such as phosphate buffered-saline crystals (not shown). Another example of the versatility of the VSI approach is illustrated in Fig. 11, which shows the effects of air drying on a live chondrocyte as a function of time. These examples are indicative of the plethora of studies that can be performed to elucidate the phenotypic, morphological, and structure-function characteristics of single chondrocytes.

In our hands, the use of VSI allowed acquisition of all salient geometric data of a single chondrocyte in approximately 12 s. The heights of the cells at the different time points were all statistically different (p < 0.05). These results clearly show that the superficial cells are shorter than the middle/deep cells at every time point. In cartilage,
the superficial chondrocytes are more flattened, while the middle/deep chondrocytes are more rounded. The rate of attachment and spreading could be utilized in ascertaining the biocompatibility of a biomaterial. For instance, imaging osteoblasts attaching to an implant would give an indication of how fast the cells attach and spread, which is one possible metric of biocompatibility. Also, since chondrocytes tend to maintain their phenotype when rounded, the speed of flattening or elongating might be a marker for chondrocyte dedifferentiation into a fibroblast.

To help further characterize the geometry of the cells, the surface area, volume, and curvature of the cells were also acquired. The surface area of the superficial chondrocytes at 4 h and 18 h was the only significant difference. The 4 h superficial chondrocytes had a large standard deviation of the surface area compared to the other conditions, which may have been the result of a small number of middle/deep chondrocytes having been abraded off with the superficial zone. The volume significantly decreased from 4 h to 18 h for both the superficial and the middle/deep chondrocytes. This volume change is likely the result of the process of cell attachment and cytoskeleton reorganization. The curvature was obtained by taking a two-dimensional slice through the center of the cells at each time point. The curvature could also feasibly be compared between cells under different conditions, adding a layer of understanding to how cells spread. These curvature results suggest that differences in curvature may exist between the 4 and 18 h time points and can be quantified using VSI. While chondrocyte geometry is not very complex, cells with more difficult geometry, such as fibroblasts or endothelial cells could also have their surface area and volume tabulated with relative ease.
These descriptive parameters of the cell can be input into continuum or computational mechanical models of single cells to allow more accurate mechanical property measurement and stress field calculation. Additionally, the actual geometry of a cell as it spreads on a substrate can directly be inserted into computational models or used to build an adaptive model of cell spreading. To attain the actual stress environment of a cell to understand how it reacts to certain forces, quantification of its intrinsic mechanical properties is imperative. Mechanical models are necessary to calculate the mechanical properties, and an accurate geometry is an essential element of any mechanical model. Knowing the geometry of a single cell as it spreads can help elucidate the stress environment and allow more detailed manipulation of the cells’ overall environment via substrate design and mechanical loading regimes for tissue regeneration or for further understanding cell biology and cytomechanics. Even just tracking the metrics of cell attachment and spreading has the potential to yield detailed knowledge of the behavior of single cells.

Confocal microscopy and atomic force microscopy can also be used to visualize cells. AFM shows submembranous structures well, and can also be used to mechanically stimulate cells. AFM is not as often used to image whole cells as membranes and has more limitations as to the depth it can visualize. Confocal microscopy is used to obtain the geometry of whole cells, but requires substantial contour or edge identification and calculation to estimate the surface area or volume. A recent study also reported that using confocal microscopy induces calcium transients and even cell death. Cell death was substantially increased for cells that were fluorescently stained. Calcium transients were decreased with decreased laser power, but light-sensitive calcium signaling still
occurred. One disadvantage to VSI compared to AFM and confocal microscopy is that
the current technology requires cells to be fixed, which, as mentioned before, can cause
artifact. VSI is also not able to obtain the geometry of cells inside a biomaterial or
matrix, but the light source is not a laser and should not cause the problems with calcium
transients or cell viability. While VSI is not able to visualize submembranous structures
like AFM, it can scan the depth of multiple cell diameters. With vertical scans of 50 μm,
VSI has more than three times the vertical range of AFM.107

Due to technological advances, future applications of this technique may include
observing immersed, live cells as they spread. The possibility of creating an in situ
interferometer101 would allow obtaining the height map of live cells in phosphate-
buffered saline or media. VSI could allow visualization and calculation of the three-
dimensional details of cells spreading on different biomaterials with different peptides
attached, as well as migration of a cell with the geometry and velocity of the individual
cell membrane extensions or, in some cases, pseudopodia. With the short time period
necessary for acquiring data, movies of the cells would even be feasible. Thus, VSI
would allow much greater description and comprehension of how cells attach and spread,
which has wide-ranging implications.

Compared to many techniques to obtain three-dimensional cell geometry, the
method described here is fast and precise with a wide field of view. The vertical 2 nm
resolution can quickly show very small details almost on the molecular level and could
allow visualization of a cell as it makes and exports extracellular matrix. The scan rate of
2 µm/s allows quick scans over a large vertical space, and the field of measurement includes the entire field of view of the microscope.

VSI advances the ability to visualize and measure single cells attaching and spreading on a substrate in terms of detail and speed. The results show that surface area, volume, height, and even curvature can be determined in single cell experiments, allowing comprehensive cytomechanical models to determine the mechanical properties and the stress-strain environment of the cell. By acquiring all of the salient geometric characteristics of an anchorage-dependent cell, this technique allows cytomechanics, cell-substrate interactions, and other cellular processes to be studied more accurately and in more depth.
ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support of the Whitaker Foundation, the expertise of Tom Fewless, Darlene Weist and LadPak of Needville, TX, and the MSTP of the Baylor College of Medicine.
Figure 6. Diagram of vertical scanning interferometry (VSI).

The diagram illustrates the process and components of VSI. The components include a white light source, CCD camera, beam splitter, piezoelectric stage (PZT), and a Mirau objective.
Figure 7. Sample interferograms.

The light and dark fringes are the interference pattern known as an interferogram. Each fringe is a fixed difference in height from the adjacent fringes. By varying the height of the sample and taking many interferograms, the height map of the sample is calculated.
Figure 8. Height map visualization.

Nine perspectives of a single cell illustrate some of the different points of view possible with VSI. The houses below and to the right of each image demonstrates the relative point of view, and the different colors represent height ranges.
Figure 9. Height, volume and surface area of zonal chondrocytes at 4 and 18 h.

a) Mean height and standard deviation of the zonal chondrocytes at 4 and 18 h with n = 15. Data from Huang et al. at 4 h are also included for comparison. b) Mean volume and standard deviation of the zonal chondrocytes at 4 and 18 h with n = 10. The symbols (#*~) indicate pairs of conditions that are significantly different from each other (p < 0.05). c) Mean surface area and standard deviation of the zonal chondrocytes at 4 and 18 h with n = 10. The symbol (*) shows the pair of conditions with a significant difference.
**Figure 10.** Curvature of chondrocytes at 4 and 18 h.

Curve fit of a two-dimensional slice through the center of a cell at 4 and 18 h.
Figure 11. Live cell dehydration.

The height map of a live cell exposed to air as it dehydrates for 20 min, 45 min, and 3 h.
CHAPTER 3: CHARACTERIZATION OF FIBROBLAST MORPHOLOGY ON BIOACTIVE SURFACES USING VERTICAL SCANNING INTERFEROMETRY

ABSTRACT

Tissue donor scarcity is a major hindrance to articular cartilage tissue engineering. Previous research shows that dermal fibroblasts express chondrocytic markers after seeded on aggrecan-coated surfaces. Since cell roundness appears to correlate with chondrocytic behavior of dermal fibroblasts, this study quantified roundness by measuring cell height and surface area-volume ratio. In addition to aggrecan as a surface coating, collagen type II and decorin, two other major extracellular matrix components of articular cartilage, were examined. Aggrecan, collagen type II, and decorin were coated onto a glass substrate using three application techniques: static drying, airbrush, and painting. Vertical scanning interferometry (VSI) is a novel technique that allows for the expedient morphological determination of single cells. Interferometry was used for the characterization of protein-coated surfaces in addition to characterizing the morphology of single dermal fibroblasts after 24 h of seeding. Fibroblast height was found to vary

from 1.0 to 4.0 μm and protein coating, application technique, and seeding position were significant factors ($p < 0.002$). The largest cell heights were observed on aggregan and collagen type II coated surfaces using the air brush and static applications. Additionally, variations were observed for surface area-volume ratio, ranging from 1.75 to 11.94 μm$^{-1}$ with decorin resulting in the lowest ratio, followed by collagen type II and aggregan. This study identifies optimal coating conditions for stimulating morphology in dermal fibroblasts that is characteristic of the chondrocytic phenotype. These conditions can be employed to attempt articular cartilage regeneration and bypass difficulties due to a paucity of donor tissue.
INTRODUCTION

Shortage of tissue for clinical application of tissue engineering of articular cartilage presents a serious problem, as chondrocytes have a low capacity for proliferation and long-term culture causes a loss of functionality.\textsuperscript{108} One approach in the field of cartilage regeneration is focused on initiating phenotypic changes in cells from non-traditional tissues using protein-coated surfaces. Modification of surfaces is a widely employed technique for controlling cell attachment, proliferation, and differentiation.\textsuperscript{109-111} One method utilizes protein coatings to modify the surface and investigate the ability of dermal fibroblasts to effectively serve as an alternate cell source to chondrocytes.\textsuperscript{112}

Dermal fibroblasts seeded on surfaces coated with various proteoglycans native to articular cartilage have been examined for their ability to differentiate into a chondrocytic phenotype.\textsuperscript{112,113} Briefly, perlecan and aggrecan (which are proteoglycans present in articular cartilage) have been used to modify the surface of tissue culture treated plastic to grow both adult rabbit dermal fibroblasts and mouse embryonic fibroblasts. When seeded on the coated surfaces, fibroblasts become round, aggregate into nodules, and stain positively with Alcian Blue, indicating the presence of glycosaminoglycans. In contrast, fibroblasts cultured on uncoated tissue culture plastic stain negatively and maintain a fibroblastic phenotype.\textsuperscript{113} Additionally, cells on protein-coated surfaces demonstrate higher amounts of collagen type II and aggrecan gene expression, both phenotypic markers of chondrocytes.\textsuperscript{112} Though these studies were performed using perlecan and aggrecan as substrates, the results suggest that additional proteins found in cartilage extracellular matrix may be able to promote differentiation.
A common and simple method to coat surfaces with proteins is protein adsorption.\textsuperscript{109} Protein adsorption occurs through the application of an aqueous solution of proteins to a surface and allowing the proteins to adhere to the substrate over several hours. Protein adsorption for cellular studies and applications depends on many variables, including protein surface density, differences in adsorption between material surfaces, protein conformation on the surface, surface roughness, and retention of biological activity post-adsorption.\textsuperscript{109,114-117} The characteristics of the surface coating can potentially be varied by altering the method of protein application.

Several techniques are currently used for the characterization of protein-coated surfaces as well as characterization of the geometry of a single cell. Previous studies have used atomic force microscopy,\textsuperscript{109,118-121} X-ray photoelectron spectroscopy,\textsuperscript{119,120,122} attenuated total reflectance-infrared,\textsuperscript{109,118,119} total internal reflection microscopy,\textsuperscript{123} and scanning electron microscopy\textsuperscript{109,118,122} to characterize protein-coated surfaces. These techniques have been used to gain information about surface roughness, surface density, cell adhesion, and average protein height.\textsuperscript{114,120,124} These same techniques, along with confocal microscopy, have been used to gather information about cell surface area, volume, and the overall shape of a cell.\textsuperscript{92,93,95,125} Some results suggest a model where collagen coating density induces a qualitative transition in the fundamental way that fibroblasts interact with the coated substrate.\textsuperscript{114} These findings suggest the applied concentration of a surface coating may alter the morphology of fibroblasts once seeded. Additionally, the spread area of fibroblasts seeded on fibronectin and RGD- modified substrata is investigated and the data reveal that cellular interaction with modified surfaces can greatly change a cell’s ability to spread, migration speed, and generated
traction force.\textsuperscript{125} It is clear that seeding cells on modified surfaces can change many characteristics, including morphology and adhesive properties.

Recently, VSI has been introduced as an alternative technique for imaging cells and calculating their respective height, surface area, volume, and curvature.\textsuperscript{126} Essentially, interferometry relies upon the interference patterns created between light reflected off the surface and a reference beam. The height of the surface is changed with nanometer precision during scanning, and analyzing the interference patterns creates a surface topography map.\textsuperscript{127} Previously, it has been demonstrated that VSI can be used to visualize the three-dimensional geometries of individual cells.\textsuperscript{126} Interferometry results are a mathematical matrix of heights, which allows flexible processing of the data to obtain metrics of single cells or to accurately characterize a surface.

This study utilizes VSI as a tool for analyzing mouse embryonic fibroblasts seeded on protein-coated surfaces to deduce the degree of chondrocytic morphology imparted by the surface coating. To our knowledge, this technique has not yet been developed for the characterization of protein-coated surfaces and has only recently been used to image cells. The purpose of this study is to analyze protein-coated surfaces both prior to and following seeding of fibroblasts on glass substrates and to determine optimal surface coating conditions based on the induction of chondrocytic morphology. The proteins applied to the substrate in this study include aggrecan, decorin, and collagen type II. Several application techniques were used to administer the proteins onto the surfaces, including air brushing, painting, and static drying. All proteins were applied to glass coverslips. Morphological data of individual cells, including cell height, surface area,
and volume, were obtained to assess the induction of chondrocytic morphology upon seeding on the protein-coated surfaces.

RESULTS

Height Map

To illustrate the change in cell shape due to seeding on surfaces coated with various proteins, fibroblasts were seeded on coated glass coverslips and VSI was used to obtain height maps of single cells at various locations on the glass coverslip. Interferometry was performed to acquire images at the center of the seeded area (position 1) and the periphery of the seeded area (positions 2-5, with position #2 located above and numbered counterclockwise). Surface scans of each protein coating treatment group demonstrate the appearance of surfaces prior to seeding with fibroblasts and allow the calculation of the average coating thickness of each surface (Fig. 12). These images are all scaled identically, with a base of 500 μm × 500 μm and a height of 1.0 μm. Figure 13 depicts representative height maps for cells seeded on each of the 13 surface treatments 24 h post seeding. The height maps for the seeded images are scaled the same with a base of 35 μm × 35 μm and a height of 3.0 μm. The diagram of the house in the lower right-hand corner of each image depicts the three-dimensional perspective of the cell. It is worth noting that the vertical axis is exaggerated when compared to the x- and y-axes (plane of cell seeding) and amplifies the cell and surface coating geometry. These visualizations show that cells seeded on protein-coated surfaces appear taller and have a lower degree of cell spreading, indicating a potential change in morphology when
compared to cells seeded on surfaces coated with water and an uncoated glass substrate. To quantitatively determine this change in morphology, several aspects of cellular geometry are calculated, including peak height and surface area-volume ratio.

*Coating Thickness*

The average coating thickness of each surface was calculated 18 h after coating with aggregan, collagen type II, decorin, and distilled water with one of each of the application techniques using surface scans from each interferometry image. The data reveal that coating, application technique, and position are all significant factors ($p < 0.0001$) in the average protein coating thickness. Surfaces coated with aggregan, decorin, and collagen had significantly thicker coatings than those coated with water; aggregan having the thickest overall coating. Additionally, static application resulted in a significantly thicker coating than air brushing or painting. The center of the coverslip (position 1) resulted in the thickest coating, being significantly thicker than any other positions. The average coating thicknesses and standard deviations for the 13 treatment groups for position 1 are shown in Table 2. The static aggregan coating was 21 times thicker than a static water coating, six times thicker than aggregan applied by painting or air brushing, and three times thicker than the next largest surface coating, decorin applied statically.

*Fibroblast Heights*

The heights of the fibroblasts seeded on aggregan- and collagen-coated surfaces for 24 h were significantly different ($p < 0.0001$) than those seeded on coverslips with
either a decorin coating or only a water coating (Fig 14a). Additionally, peak heights of fibroblasts on decorin were significantly different ($p < 0.003$) than those only coated with water. Application technique was a significant factor, with air brushing and static application resulting in significantly higher peak heights ($p < 0.0001$) than when the proteins were coated using a paintbrush (Fig 14b). The position of the fibroblast on the coverslip was also significant, with cells at positions 2, 3, and 5 having significantly larger peak heights than at positions 1 ($p < 0.02$) and 4 not being significantly different than any other position. The cells seeded on aggrecan via air brushing, painting, and static application had peak heights of $2.33 \pm 0.58 \mu m$, $1.61 \pm 0.32 \mu m$, and $2.06 \pm 0.82 \mu m$, respectively. Collagen type II coating yielded cells that were $2.50 \pm 0.78 \mu m$, $1.47 \pm 0.21 \mu m$, and $2.43 \pm 0.61 \mu m$ in height for the three application techniques. The cells on decorin-coated glass possessed heights of $1.61 \pm 0.35 \mu m$, $1.68 \pm 0.28 \mu m$, and $1.60 \pm 0.32 \mu m$ for air brushing, painting, and static application. Cells seeded on the water-coated surfaces and on the uncoated surface had heights of $1.25 \pm 0.15 \mu m$, $1.26 \pm 0.22 \mu m$, $1.56 \pm 0.32 \mu m$, and $1.62 \pm 0.30 \mu m$, respectively.

**Surface Area-Volume Ratio**

Coating type was found to be the only significant factor ($p < 0.0001$) for cell surface area-volume ratio. Application technique and position had $p$-values of 0.17 and 0.50, respectively. The surface area-volume ratio of the cells was significantly different ($p < 0.0001$) between the cells seeded on protein-coated surfaces and those seeded on water-coated surfaces (Fig 15a). Furthermore, cells seeded on decorin possessed significantly lower surface area-volume ratios than either the aggrecan or collagen
coating ($p < 0.025$). There was no significant difference of surface area-volume ratios between application techniques (Fig 15b) or position on the coverslip. Fibroblasts on the uncoated surfaces had a surface area-volume ratio of $7.62 \pm 1.70 \, \mu\text{m}^{-1}$. This control was not significantly different than cells seeded on water-coated surfaces, whose cells had ratios of $6.87 \pm 1.07 \, \mu\text{m}^{-1}$, $6.20 \pm 0.83 \, \mu\text{m}^{-1}$, and $7.31 \pm 1.73 \, \mu\text{m}^{-1}$ for air brushing, painting, and static applications, respectively. The other nine treatment groups were significantly different than these controls, with static coatings possessing the lowest surface area-volume ratio within each protein. For static application, the aggrecan, collagen, and decorin treatments yielded surface area-volume ratios of $3.62 \pm 1.47 \, \mu\text{m}^{-1}$, $3.62 \pm 1.14 \, \mu\text{m}^{-1}$, and $3.43 \pm 0.67 \, \mu\text{m}^{-1}$, respectively. Proteins coated using the air brushing technique resulted in cells with ratios of $4.28 \pm 1.72 \, \mu\text{m}^{-1}$, $4.27 \pm 1.02 \, \mu\text{m}^{-1}$, and $3.72 \pm 0.72 \, \mu\text{m}^{-1}$ for aggrecan, collagen, and decorin. Finally, coating by the painting technique resulted in cells with ratios of $4.96 \pm 1.15 \, \mu\text{m}^{-1}$, $4.52 \pm 0.80 \, \mu\text{m}^{-1}$, and $3.63 \pm 0.62 \, \mu\text{m}^{-1}$ for aggrecan, collagen, and decorin.

**DISCUSSION**

Interferometry was used for the first time to characterize protein-coated surfaces and to assess the induction of chondrocytic morphology of fibroblasts seeded on these surfaces. The results of the surface characterization show that the deposition of proteins onto glass substrata change the native environment cells are exposed to upon seeding. Previous work in our lab has utilized tissue culture plastic for protein coating and fibroblast seeding, but the initial data acquired that mapped tissue culture plastic illustrated the roughness of the surface when compared to glass (Fig. 16). At the
nanometer scale, the peaks and valleys of the tissue culture plastic cause difficulty when studying the morphology of the cell. Additionally, previous studies measuring the cell behavior of fibroblasts seeded on fibronectin-coated surfaces have used glass coverslips successfully.\textsuperscript{109} Varying thicknesses of coating occur and depend on the protein and application technique used. When mouse embryonic fibroblasts are seeded on these protein-coated surfaces, the cell morphology is altered from fibroblastic (spread) to chondrocytic (rounded). The degree to which the morphology changes, however, is again dependent on the protein and application technique. The data show that static application of aggrecan and decorin results in the most rounded (chondrocytic) morphology, which implies that decorin, like studies with aggrecan, \textsuperscript{112,113} may cause a phenotypic change from fibroblast to chondrocyte.

The average coating thickness was measured 18 h after application of the coatings to understand the protein surface geometry on which cells would be seeded. Linear surface scans were analyzed and used to calculate the average coating thickness for each surface treatment at each coating position of interest. The position on the glass coverslip is a significant factor, with the center of the coverslip (position 1) having the largest coating thickness compared to the periphery (positions 2-5). This was expected based upon unpublished data from our lab that have shown that fibroblasts, when seeded onto coated surfaces, aggregate more in the center of the coated area and produce more chondrocytic markers than cells seeded on the periphery. Additionally, application technique is a significant factor in coating thickness. As shown in data for position 1, coating aggrecan and decorin via static application resulted in significantly thicker coatings as compared to the 11 other treatment groups (Table 2). These findings were
also not surprising as the trend in average thickness follows the same trend of molecular weight, with aggregan having the largest molecular weight (~2,500 kDa) followed by decorin (~100 kDa)\textsuperscript{128} and finally collagen type II (~85 kDa)\textsuperscript{129}. Additionally, it appears that the static application provided the thickest overall coating within each protein type.

Several aspects of cellular geometry were obtained through the use of vertical scanning interferometry, including peak height and surface area-volume ratio, for the characterization of chondrocyte-like morphology of fibroblasts. The cell heights were obtained for each treatment group and all three factors (coating, application technique, and position) were significant. These results clearly show marked differences in that cells seeded on aggregan and collagen are taller than cells on decorin or water. The cells on decorin are also significantly larger than those on only a water coating. These differences in height are likely the result of cell attachment and cytoskeletal rearrangement due to the surface coating. The application technique is significant for cellular peak height, with static and air brush applications inducing a larger peak height when compared to painted surfaces. This is postulated to be an effect due to the differences in protein coating (Fig. 12). Also, some amount of protein may adsorb on the brush, reducing the density on the surface with the painting technique. As shown in the representative images of each coated surface, the static and air brushed surfaces have a higher coating density than the painted surfaces. The presence of the protein in higher abundance permits the cells to limit attachment, thus having an increase in cell peak height forming a more rounded cell.
Chondrocytes in their native environment exhibit a rounded morphology as opposed to fibroblasts, which are flattened cells with many pseudopodia-like extensions on the substrate. Through the use of vertical scanning interferometry, the surface area and volumes of cells were acquired. The surface area-volume ratio is indicative of the degree of spreading a cell has undergone, thus can be one marker for assessing amount of chondrocytic dedifferentiation,\textsuperscript{126} or induction of chondrocytic morphology of fibroblasts. For comparison, a smaller surface area-volume ratio is indicative of lower cell-substratum adhesion and of smaller amount of cell spreading (typical of chondrocytes) while a higher ratio is indicative of more attachments and more spreading (typical of fibroblasts). In this study, fibroblasts cultured on decorin possessed the smallest surface area-volume ratios and were significantly smaller than cells on both collagen- and aggrecan-coated surfaces, as well as the water-coated surfaces. The cells on collagen- and aggrecan-coated surface were also significantly smaller than those on the water-coated surfaces. This finding was expected as fibroblasts cultured on these substrates have been shown to have a more chondrocytic morphology and to express higher amounts of extracellular matrix components of cartilage (collagen type II and aggrecan) than fibroblasts on uncoated surfaces.\textsuperscript{112,113} The exposure of fibroblasts to protein-modified surfaces has a dramatic impact on the surface area-volume ratio 24 h after seeding, causing cells to become more round and chondrocyte-like as opposed fibroblast-like, characterized as flattened, spread cells.

Several unexpected results from the cellular study were observed, in particular a lack of significance in the surface area-volume ratios of seeded fibroblasts as a function of either the application technique ($p = 0.17$) or position ($p = 0.50$). Though there is a
trend in the application technique data that showed static application caused a smaller surface area-volume ratio than air brushing or painting, the difference is not significant. This trend agrees with the results found for peak height as well as coating thickness, with static application of protein producing the most chondrocytic morphology. As stated before, previous work with fibroblasts seeded on aggrecan-coated surfaces has indicated that Safranin-O staining is more intense in the middle of the seeded area (analogous to position 1) and less intense on the periphery (analogous to positions 2-5). Additionally, cells appeared more aggregated and rounder in the middle of the seeded area and more spread and less densely populated on the periphery when viewed macroscopically. Due to these results, our hypothesis was that position 1 would have the lowest surface area-volume ratio. However, the data suggest that position does not have an effect on the degree of induction of chondrocytic morphology in terms of surface area-volume ratio.

For comparison purposes, previous data using confocal microscopy report rabbit cartilage explants from the middle zone cultured for 30 min have a mean chondrocyte height of 12.2 μm and surface area-volume ratio of 0.60 μm⁻¹. Other data obtained using interferometry report middle/deep bovine chondrocytes seeded on glass have a peak height of 6.36 ± 0.44 μm after 4 h of seeding and 5.12 ± 0.26 μm after 18 h of seeding. Additionally, these chondrocytes possess a surface area-volume ratio of approximately 1.05 μm⁻¹ and 1.50 μm⁻¹, for 4 h and 18 h of seeding, respectively. These data, coupled with the results of the current study, suggest the coating of surfaces imparts some degree of chondrocytic morphology to the fibroblasts. The significance of the surface coatings can be observed for both cell peak height (increase) and surface area-volume ratio (decrease), with cells attaining a more chondrocytic morphology on these
surfaces. It has previously been shown that morphological changes occur that are potentially indicative of a phenotypic change, with a native fibroblastic cell being flat and spread while a cell undergoing chondrocytic differentiation exhibits a more rounded morphology. Moreover, decorin appears to have the most dramatic effect on the degree of spreading of fibroblasts with a 47% decrease in surface area-volume ratio while collagen and aggrecan have the largest effect on the overall cell’s peak height, increasing 55% and 47%, respectively, when compared to cells seeded on water-coated surfaces. The static application seems to be the most uniform of coatings and does the most efficient job of transforming the fibroblasts into more of chondrocytic morphology.

The data presented here correlate well with previous data assessing markers of chondrocytic differentiation of fibroblasts. Fibroblasts seeded onto protein-coated surfaces undergo a change in morphology, becoming more round as measured by a significant increase in peak height and decrease in surface area-volume ratio. Thus, cell shape can be used as one tool to quantify the level of chondrocytic phenotype induction. The ability to more effectively drive fibroblasts to become more chondrocytic in terms of morphology and phenotype increases the potential of future articular cartilage tissue engineering studies in addressing the problem of donor tissue scarcity.

**EXPERIMENTAL PROCEDURES**

*Protein Coatings*

Protein solutions were prepared using aggrecan and decorin from bovine articular cartilage, and collagen type II from chicken sternal cartilage (Sigma, St. Louis, MO).
Protein solutions were made with a stock concentration of 1 mg/ml in filtered, deionized, distilled water. The resulting solutions were passed through a 0.22-μm filter (Millipore, Billerica, MA). Surface coatings were applied using one of three methods: air brushing, painting, or static drying. In all cases, 2.6 μg/cm² of protein was coated on 12 mm diameter glass coverslips (Fisher, Pittsburgh, PA) and diluted to appropriate final volumes using filtered, deionized, distilled water. For air brushing, 200 μl of protein solution was applied to a dry coverslip using an air brush kit (Central Pneumatic, Camarillo, CA). Painting of the protein was performed by applying the appropriate volume of stock solution to the coverslip and distributed by brushing manually using a fine brush (Staedtler, Chatsworth, CA). All brush strokes were applied in a uniform direction, with 20 strokes used per coverslip. For static drying, 200 μl of protein solution was placed on the surface of a coverslip, creating a small liquid meniscus covering the entire surface of the coverslip. All coated coverslips were placed in individual 35 mm x 10 mm cell culture dishes (Corning, Corning, NY), face up, and dried for 18 h in a laminar flow hood. Following complete evaporation of the solution, the culture dishes containing the coated glass coverslips were covered until either imaging or being seeded with fibroblasts.

Fibroblast Seeding

Murine 10T1/2 cells (ATCC) were cultured in vitro in tissue culture flasks with Dulbecco's modified eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gemini, Woodland, CA), 1% penicillin/streptomycin/fungizone (Cambrex, Walkersville, MD), and 1% non-essential amino acids (Invitrogen). Passage
three 10T1/2 cells were seeded at 40,000 cells/coverslip in 200 μL medium. At 24 h after seeding, the fibroblasts were fixed using a 0.3% solution of glutaraldehyde (Sigma) containing 80 mM Kpipes (Sigma), 5 mM EGTA (Sigma), and 2 mM MgCl₂ (Fisher, Fairlawn, NJ) for 20 min. Following fixation with glutaraldehyde, the fibroblasts were dehydrated using serial ethanol dilutions. Phosphate-buffered saline solutions of ethanol were made in which the coverslips were fully immersed for 5 min and dilutions were applied in the following order: 10, 25, 50, 75, 85, 95, 100, and 100% ethanol. The ethanol was allowed to evaporate immediately prior to data acquisition.

Vertical Scanning Interferometry

Images were obtained using a scanning white light phase shift MicroXAM interferometer (ADE Phase Shift, Tucson, AZ). This optical vertical scanning interferometry system consisted of a light microscope, CCD camera, motorized stage, white light source, and Mirau interferometer. Previous studies have described the detailed analysis of surface using this technique in which a height map is obtained with a horizontal resolution of 500 nm and a vertical resolution of 2 nm. Briefly, the system uses the interference patterns created between a sample and reference beam of light. The sample beam is reflected off the surface while the reference beam is reflected off the Mirau objective. The resulting interference pattern, or interferogram, is used along with the wavelength of light to determine height differences between points. To produce different interference patterns, a piezoelectric stage is used to vertically adjust the sample to change the pathlength of the sample beam with nanometer precision. For each surface treatment, a total of five images were acquired with one image taken at the center of the
coverslip (position 1) and the remaining four images taken near the perimeter (positions 2-5). Scans were acquired with a 10X interference objective for surface scans without cells and a 50X interference objective for scans on seeded surfaces.

*Coated Surface Characterization*

Following 18 h of adsorption of the coating to the glass substrate, interferometry was performed to acquire images at the center of the coating area (position 1) and the periphery of the coating area (positions 2-5). From each image obtained, a topographical map, or height map, was analyzed and height data collected for a specified area on the surface. Based on the obtained data, an x-y plot was constructed illustrating a height map of the imaged surface. From these plots, representative linear scans were obtained that were used to calculate the average height of each surface. Each linear scan returned maximum height values at each 1-μm step across the surface and averaged to return an overall coating thickness for each linear scan. Specifically, the average height of each surface was calculated for comparison among the 13 treatment groups. These data were used to extract the coating thickness of each surface coating as well as the surface roughness and the efficiency of coating of each application technique.

*Cell Height, Surface Area, and Volume Measurement*

After 24 h of seeding, interferometry was performed to again acquire images at the center of the coating area (position 1) and the periphery of the coating area (positions 2-5). From the height map of each image, three to five cells were analyzed to obtain height as well as surface area-volume ratio data. This resulted in 20-25 cells analyzed for
each surface treatment. A cell’s height measurement was taken as the difference in the largest vertical distance between the height map of the cell and its adjacent surface. The MapVUE software (ADE-Phase Shift) was utilized to calculate the surface area and volume data of each imaged cell. The software first identified the plane of the surface on which the cell is attached. This plane is treated as a half-space and the topography above this plane is considered the cell of interest. The surface area was calculated as the area of the height map above this plane. Similarly, the volume of the cell was calculated from the volume above the surface plane. A normalized volume is computed by dividing the given volume by the surface area. Surface area-volume ratios were calculated by taking the inverse of the normalized volume.

Statistics

For each of the 13 treatment groups, 20-25 cells were analyzed at the five positions for calculation of peak height and surface area-volume ratio, resulting in a total of 304 cells that were analyzed by interferometry for morphological characterization. The coating thickness, cell height, surface area, and volume data were analyzed by analysis of variance (ANOVA), with $p < 0.05$ considered significant. When significance was obtained, a Tukey HSD post hoc analysis was performed.
Table 2. Coating thickness for each treatment at the center of the coverslip.

Height data for surfaces coated with each treatment at the center of the coverslip. Static application of each protein resulted in the largest average coating thickness. Levels not connected by the same letter are significantly different ($p < 0.05$). Data represent mean ± standard deviation.

<table>
<thead>
<tr>
<th>Treatment (Coating + Application)</th>
<th>Coating thickness (nm)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan + Static</td>
<td>111 ± 34</td>
<td>A</td>
</tr>
<tr>
<td>Decorin + Static</td>
<td>34 ± 8</td>
<td>B</td>
</tr>
<tr>
<td>Collagen + Static</td>
<td>31 ± 25</td>
<td>B, C</td>
</tr>
<tr>
<td>Collagen + Paint</td>
<td>30 ± 6</td>
<td>B, C</td>
</tr>
<tr>
<td>Collagen + Air brush</td>
<td>28 ± 9</td>
<td>B, C</td>
</tr>
<tr>
<td>Decorin + Paint</td>
<td>23 ± 7</td>
<td>B, C, D</td>
</tr>
<tr>
<td>Aggrecan + Paint</td>
<td>19 ± 13</td>
<td>C, D, E</td>
</tr>
<tr>
<td>Aggrecan + Air brush</td>
<td>19 ± 7</td>
<td>C, D, E</td>
</tr>
<tr>
<td>Water + Paint</td>
<td>12 ± 4</td>
<td>D, E</td>
</tr>
<tr>
<td>Decorin + Air brush</td>
<td>9 ± 3</td>
<td>D, E</td>
</tr>
<tr>
<td>No Coating</td>
<td>8 ± 3</td>
<td>D, E</td>
</tr>
<tr>
<td>Water + Air brush</td>
<td>8 ± 3</td>
<td>E</td>
</tr>
<tr>
<td>Water + Static</td>
<td>5 ± 2</td>
<td>E</td>
</tr>
</tbody>
</table>
Figure 12. Height map visualization.

Representative images of each of the 13 treatment groups of the protein coating on the glass coverslips illustrate the differences between protein and application technique. The scale of each image has a base of 500 μm x 500 μm, while the height is 1.0 μm. The height is exaggerated in these images to magnify surface detail.
Figure 13. Fibroblast visualization.

Representative height maps of fibroblasts seeded for each condition visually indicate differences in cell peak height as well as surface area-volume ratio due to different protein coatings and application techniques. Each image has a height of 3.0 μm while the x-axis is 35 μm.
Figure 14. Cell peak height data in terms of surface coating (a) and application technique (b).

Surface coating and application techniques have significant effects ($p < 0.05$) for the cell peak heights. Levels not connected by the same letter are significantly different. Data represent mean ± standard deviation.
Figure 15. Surface area-volume ratio data by surface coating (a) and application technique (b).

Surface coating has a significant effect ($p < 0.05$) whereas application does not ($p = 0.17$). Levels not connected by the same letter are significantly different. Data represent mean ± standard deviation.
**Figure 16.** Visualization of glass and tissue culture plastic (TCP).

Vertical scanning interferometry image shows the differences between a glass coverslip on the left and TCP on the right. The non-uniformity of the TCP surface dictated the use of glass as the surface to be modified for this study.
CHAPTER 4: MECHANICAL IMPACT AND ARTICULAR CARTILAGE*

ABSTRACT

Mechanical impact forces upon articular cartilage can cause substantial damage. Car accidents, falls, and sports injuries have a tremendous effect upon the U.S. and world populations, both in terms of economic and quality of life costs. While the effects of impact forces are known to be damaging, tolerance levels of cartilage to these forces and the mechanobiologic sequelae are still mostly unknown. Impact studies can be difficult to compare to each other due to the complex array of mechanical factors that are involved in a single impact. Previous work includes mathematical models, acute effects of impact, and in vivo and explant models of impact. These experiments have found that articular cartilage has a threshold above which impact forces are damaging, though this threshold is likely dependent upon many factors, both genetic and environmental. This type of damage has been shown to vary according to the severity of the impact, from leaving the articular cartilage surface intact to fracture of the subchondral bone. Some studies have initiated investigations into ways to ameliorate the injurious response to impact, which may allow some patients to avoid the ensuing cartilage degeneration and osteoarthritis. Much work remains to be performed in understanding the genetic and biochemical

response to impact. The goal of this research is to eventually decrease the incidence of post-traumatic arthritis and possibly even delay primary osteoarthritis, which can be achieved by using a robust testing design that includes morphological, biomechanical, quantitative biochemical, and genetic characterization of a model system for articular cartilage impact. This model system can then be used to test treatments to prevent degenerative changes in articular cartilage.
INTRODUCTION TO IMPACT AND ARTICULAR CARTILAGE

Impact loading of articular cartilage can have negative consequences for the protective abilities of the tissue, especially with respect to the daily regime of weight-bearing, load-distribution, and joint motion.\textsuperscript{1,131} Detrimental trauma to joints was linked to "osteoarthrosis" as early as 1743 when described by Dr. William Hunter.\textsuperscript{131} More modern science has also shown severe injury of articular cartilage can lead to post-traumatic osteoarthritis (OA), which may permanently alter the quality of life, by affecting the ability to work, lift children, or even walk pain free.\textsuperscript{132} Post-traumatic OA is a possible consequence of sports injuries, automobile accidents, and other falls or mishaps. Athletes with a history of joint injury develop osteoarthritis more often than their peers.\textsuperscript{133} Further knowledge of the effect of impact upon articular cartilage will enable prevention or even reversal of tissue damage in certain cases. If prevention of post-traumatic or other types of OA were possible, treatments would preclude much pain and suffering, as well as save substantial amounts of money and productivity. The discovery of interventions that decrease the chances of post-traumatic OA development may be expandable to primary and other forms of OA.

Knowledge of the biochemical and biomechanical characteristics of mechanically impacted and injuriously compressed articular cartilage expanded exponentially in recent decades to include mathematical models\textsuperscript{134-141}, studies of acute effects,\textsuperscript{142-147} in vivo animal studies,\textsuperscript{148-155} explant studies,\textsuperscript{156-162} and growth factor studies.\textsuperscript{163-168} These investigations illustrated patterns of cell death, morphological and histological characteristics, and biomechanical and biochemical changes. However, much of the
biochemical, biomechanical, and genetic sequelae of impact loading is still unknown. In fact, the definition of "impact loading" is often interpreted in different ways.\textsuperscript{169} Many studies explore injurious compression, which occurs over a longer time span than impact.\textsuperscript{163,170} Also, descriptions of the genetic regulation induced by impact and how it correlates to the biomechanical and biochemical attributes are sparse in the current literature.

\textit{Significance}

Osteoarthritis affects more than 20 million people in the U.S. alone,\textsuperscript{171} including a fifth of the population over age 45 and almost half over age 65.\textsuperscript{3} OA is the eighth cause of disability in the world and the second most common cause in the U.S. OA of the knee occurs in 6\% of Americans and in the hip in 3\%.\textsuperscript{4} From the years 1989 through 1991, the cost of OA in medical bills and lost productivity was calculated to be over $115 billion dollars a year, 2.1\% of the gross national product.\textsuperscript{172} This figure may increase substantially as the baby boomer generation retires since the predisposing factors for OA include previous injury, fracture, obesity, and age.\textsuperscript{171,173-176} Much of the trauma that leads to OA can occur in one of the millions of annual car accidents or in the workplace.\textsuperscript{177} The legal costs of disability suits are not included in the above figure, and since OA presents anywhere from 2-10 years post-injury and has genetic influence, the legal implications are complex and multifaceted.\textsuperscript{178} Yet, with all of these costs to society and individuals, currently no lifestyle changes nor treatments will change the course of the disease, and only symptomatic relief is available.\textsuperscript{171}
OA is classified as either primary (idiopathic) or secondary due to a variety of causes. Most cases of secondary OA are due to trauma or injury, but a good deal of primary OA may also be due to a past injury. The Framingham study illustrated a relative risk, where 1.00 is defined as average risk, of 3.46 in men and 2.18 in women for developing OA after a major knee injury. Hannan et al. showed increased physical activity was not associated with knee OA, leaving trauma or injury as one of the main suspects. Risk factors for OA include obesity, age, previous injury, bone density, and genetics. Age may cause a slight decrease in articular cartilage properties in conjunction with decreased muscle strength, predisposing individuals to higher impact loading of softer cartilage. Obesity causes higher impact loads for normal activity and much higher loading during accidents or falls. Many of these factors will result in higher risk for increased impact loading of the articular surface. Indeed, high strain rates, which are seen in impact loading, are thought to be a vital factor in articular cartilage damage. One explanation for this results from the viscoelastic behavior of articular cartilage, since tissue under high-strain rates is less deformable and more brittle. If even a fraction of post-traumatic OA could be decreased or delayed in its progression, billions of dollars could be saved and millions of people could preserve fundamental, painless function.

*Experimental Impact*

In mechanical terms, impact of two bodies is a complex event. Articular cartilage, which is a fluid-filled, almost impermeable, porous material, behaves elastically under impact with high loading rates, but less so as the loading rate decreases.
The behavior becomes elastic at high loading rates because the fluid phase does not have enough time to flow out of the tissue. An impact event can be described in many ways, including peak load, time to peak load, duration of load, and energy.\textsuperscript{188} The impact of joints occurs between two nontrivial geometric structures encased in soft tissue, which complicates further the calculation of the mechanical environment. Additionally, studies using different loading rates, energies, and peak loads yield results that are challenging to compare. \textit{In vivo} experiments that impact a closed joint are beneficial for long-term investigations, but make it difficult to correlate the specific sites of damage with specific impact characteristics, such as peak load or time to peak load. Also, animal studies introduce such environmental variability as activity, oxygen tension, and cytokine levels. Explant studies provide the advantage of being able to attain a highly controlled impact and post-impact environment, but are further from the physiologic reality.

Different studies use variable impact levels and describe the impacts differently. Physiologic levels of impact on the hip,\textsuperscript{189,190} during a fall,\textsuperscript{191} during aerobics,\textsuperscript{192} or even in a car wreck\textsuperscript{193} have been investigated. These studies show peak loads from 3 to 4 times body weight on the hip during aerobic activity,\textsuperscript{190,192} 2-3 kN during a fall onto outstretched hands,\textsuperscript{191} and up to 21 kN on the knee in a wreck,\textsuperscript{193} but they do not quantify the pressure distribution or the time to peak or duration of the loading for the articular surface. Aspden \textit{et al.}\textsuperscript{169} states that the times to peak load from 30-150 ms and stress rates of 1000 MPa/s are physiological. Thus, to attain times to peak load less than 30 ms, a drop tower is often necessary. Many studies will use the term “impact” for different loading rates, and also report the characteristics of impact in different manners. While many researchers use a drop tower, others use materials testing machines with times to
peak load from 8 ms to over 1.5 s. These data are valuable for understanding how articular cartilage reacts to differing compression loads. However, when comparing results of different studies, the specific method of impact or loading and the methods used to quantify them must be noted in an attempt to normalize the impact characteristics. Many studies only report peak force or the energy of the impact based on the height and mass of the impacting object, which can make comparisons difficult. For the purposes of this article, any load applied over more than 30 to 50 ms will be considered injurious compression. To obtain impacts which apply a load in less than 30 ms, three types of devices are used (Fig. 17). Figure 17a shows a free flight mass, which is usually guided by a string and accelerated to a particular velocity. A drop tower (Fig. 17b) has a weight that falls onto a sample, usually under the acceleration of gravity. The weight or impact mass is called a tup. Pendulum impactors are also used (Fig. 17c). The instrumentation for these impacts varies and can include accelerometers, force transducers, and displacement measurement devices, such as optical systems or linear variable differential transformers (LVDT).

**Purpose**

The overall goal of current research is to prevent or slow the progression of articular cartilage degeneration and pain through an understanding of the biomechanical, biochemical, and genetic consequences of impact. In 1926, Magnuson originally published joint debridement as a surgical option for OA. This provides some temporary relief to certain patients. Thirty years ago the treatment of OA consisted of aspirin or another non-steroidal anti-inflammatory drug (NSAID) with lifestyle changes
and periods of rest in addition to surgery. Currently, treatment of OA has expanded with many possibilities on the horizon. Cyclooxygenase-2 (COX-2) inhibitors and NSAIDs, opiates, nutrition supplements such as chondroitin sulfate and glucosamine, acupuncture, arthroscopy, and total knee arthroplasty are all potential therapies. Therapies currently studied and utilized for OA include osteochondral autografts or allografts, tissue engineered constructs, stem cells, and various cytokine interventions. However, none of these has yet been found to clinically slow the natural course of the disease, highlighting prevention as another avenue to decrease the incidence of OA.

The intensity of impact on articular cartilage can cause different degrees of damage. One classification system involves three levels: 1.) the impact does not cause surface disruption, 2.) the cartilage surface is disrupted, and 3.) the cartilage and the bone are disrupted. Figure 18 shows one example of healthy articular cartilage with no surface disruption (Fig. 18a), one example of a low energy impact causing a surface fissure (Fig. 18b), and one example of a higher energy impact resulting in a deep fissure with surface fibrillation. Figure 18b and 18c both are part of the second category of impact of the above classification system and illustrate the range of this level. Prevention of progression to OA without surgery might be possible in the first two categories if the genetic cascade leading to articular cartilage breakdown and replacement with fibrous tissue can be halted or altered. Particularly, in cases where impact loads do not cause surface disruption, a combination of growth factors or bioactive molecules may decrease the percentage of patients that progress to OA or increase the amount of time before the onset of OA. Building on the following research studies, an experimental model can be
developed to allow evaluation of pharmacologic, biomechanical, or more invasive treatments aimed at slowing or preventing post-traumatic arthritis based upon the amelioration of the biomechanical, biochemical, and genetic sequelae of articular cartilage impact.

**Mathematical Modeling of Articular Cartilage Impact**

Modeling articular cartilage impact is difficult due to its heterogeneous composition, and the complexity of impact and contact mechanics. Articular cartilage has intricate mechanical behavior, since it has several components. Type II collagen gives cartilage tensile strength, while proteoglycans are responsible for the high levels of hydration and concomitant behavior under compression. Articular cartilage is often modeled as having two phases, a solid, linearly elastic phase, and an incompressible, fluid phase.\(^{198}\) This model, known as the biphasic model, allows the determination of stress distribution in the tissue under known loading conditions. Other characteristics can also be ascertained from the application of this model.

*Analytical Models of Impact*

Analytical models are usually confined to contact problems, while finite element models can be used to study impact. One analysis illustrated that the fluid phase of articular cartilage shields the collagen and proteoglycans from stresses by almost an order of magnitude during the first 100-200 s of loading.\(^{136}\) When put under stress, one model showed fluid flow occurs within the tissue and is dependent upon the permeability and
modulus.\textsuperscript{135} During impact, Burgin and Aspden\textsuperscript{199} illustrated that the peak force may occur before the peak displacement. Other models of articular cartilage contact described the electrical signals depending upon the dominance of either streaming potentials in a stiffer tissue or diffusion in a softer tissue,\textsuperscript{139} explained how the variation in the modulus by layer changes stress-relaxation behavior,\textsuperscript{141} or used composite theory\textsuperscript{200} to look at various aspects of the tissue. Aspden \textit{et al.}\textsuperscript{200} modeled the interactions of different types of collagen, such that type II collagen is stiffened by interaction with type X collagen in the growth plate. Eberhardt \textit{et al.}\textsuperscript{186} modeled articular cartilage contact to further understand how different parameters affect cartilage failure. The parameters investigated in this model included cartilage thickness and modulus, bone modulus, and joint curvature. This particular model simplified cartilage to a linearly elastic instead of biphasic material and found bone softening not to significantly affect the stress field of articular cartilage. In a model by Kafka,\textsuperscript{201} the tensile stress on the collagen fibers depended on the rate of loading, which may contribute to surface fissures during impact loading. In an application of this model, Kafka\textsuperscript{202} found that thinning synovial fluid might decrease the threshold of surface fissure formation. These models are useful in describing the stresses and strains in articular cartilage for loading rates lower than those seen in impact. For more complex geometries, computational models of articular cartilage contact, such as finite element analysis, are employed.\textsuperscript{138}

\textit{Computational Models of Impact}

Mathematical modeling of articular cartilage impact is quite complex and simplifications or empirical correlates, in addition to computational methods like finite-
element analysis, are used to make the analysis more feasible. Atkinson et al.\textsuperscript{137,203} considered criteria for fissuring of articular cartilage. They used a finite element analysis and biphasic theory to predict the stress state where fissures occurred and also ran a logistic regression to find the predictive abilities of the different stresses in the solid matrix. The study found the maximum shear stress was the best predictor of a fissure in areas where the tensile stress was low, although other predictors included maximum strain and mean stress. Anderson et al.\textsuperscript{134} used a dynamic plane strain finite element model of an impact load to a rabbit knee, a common animal model for this type of study. This model showed that two impacts, with time to peak loads of 50 ms and 500 ms, and the same peak force, had strain rates that were quite different. The 50 ms impact had a strain rate six times higher than the 500 ms impact. These results agreed with experimental evidence showing the 50 ms impact caused articular cartilage damage, while the 500 ms impact did not. This study also illustrates the necessity for accurate description of the impact in terms of both the time to peak force and peak force. Li et al.\textsuperscript{140} created a model of articular cartilage impact on rabbit patello-femoral joints using a quasi-static, plane strain, and linear elastic finite element model. This model was used to predict contact pressures and to correlate them with experimentally-observed damage, as well as correlate high shear and tensile stress to areas of damage. Donzelli et al.\textsuperscript{204} attempted to closely correlate peak stresses with areas of failure using a transversely isotropic, biphasic model. This model provided good agreement with experimentation, and it established that a transversely isotropic assumption, as opposed to an isotropic assumption, for the solid phase of articular cartilage is applicable for damage prediction. Further, this model found that the pressure sensitive film used in many impact
experiments is not indicative of the stress on the solid matrix, but of the interstitial fluid pressure. Dunbar et al.\textsuperscript{138} used a finite element model that used penetration data of undeformed tissue to formulate boundary conditions and found that it predicted the stress and deformation well, although to some extent underestimated the contact area.

Additionally, shear stress and tensile strains were found to be predictors of articular cartilage surface damage. Garcia et al.\textsuperscript{205} also modeled the rabbit patello-femoral joint with finite elements. This study found that a transversely isotropic assumption was better at describing the mechanical environment at the site of articular cartilage damage in accordance with the previous analytical model of Donzelli et al.\textsuperscript{204} A later model by Garcia et al.\textsuperscript{206} applied a hypo-elastic, transversely isotropic finite element model to impact of the rabbit patello-femoral joint. The goal of this model was to use the same theory to explain rapid and creep indentation experiments since previous models showed excessive deformation when extrapolating from creep to impact experiments. Another study explored the effects of stress waves during an impact upon articular cartilage, finding that the reflection of the stress wave off of the subchondral bone is probably not large enough to matter in impact loading.\textsuperscript{207} Since most mechanical models assume quasi-static conditions, stress waves might increase the local stresses resulting in an underestimation the loading. This finite element model found that stress waves are, in fact, much smaller than the quasi-static stress estimations and likely do not cause appreciable error.

The above models describe the impact or contact of articular cartilage, including the stress distribution throughout the solid and fluid phases. The nature and amplitude of
the stresses on the solid matrix, especially the shear and tensile stress, were used to explain the pattern of fissures and cracks seen after impact. These models increase our understanding of the complex event of the impact of articular cartilage, as well as how much of the biphasic behavior of cartilage is necessary to sufficiently describe its impact. Though difficult to implement and only applicable in defined circumstances, these models provide powerful predictions of the dilatational and deviatoric stresses experienced by articular cartilage. These stresses can then be correlated to the biochemical, biomechanical, and genetic changes seen in response to cartilage impact in order to expand the knowledge of tissue mechanobiology and analyze treatments for the prevention of OA. Further, models may assist in reducing impact loads on automobile passengers in car crashes, other accident victims, and athletes, both professional and amateur.

**ACUTE EFFECTS OF IMPACT UPON ARTICULAR CARTILAGE**

Many studies explore the acute effects of impact upon articular cartilage, from solely measuring the stress-strain curves to staining for cell viability shortly after an impact. Such studies help determine a baseline for the effects of impact upon cartilage, and a summary of the analyses is in Table 3.

*Stress-Strain and Load Attenuation*

Several investigators explored the stress-strain response of articular cartilage and how it and other tissues attenuate impact loads. Radin *et al.*\textsuperscript{208} compared the stress-strain curves of articular cartilage and bone explants using two different strain rates. The low
strain rate was applied with a materials testing machine, while the high strain rate was applied with a drop tower called an “impacting sleeve.” Articular cartilage was much stiffer under higher strain rates, and impact loads were orders of magnitude greater than the weight impacting the cartilage. Radin and Paul\textsuperscript{145} found articular cartilage and synovial fluid do not attenuate force well compared to the joint capsule and the subchondral bone. They also point out that the integrity of the subchondral bone could be important in the function and resilience of the joint. In a different study, Radin and Paul\textsuperscript{209} recreated these experiments with more control over the testing environment and still only found an 8\% increase in peak force without the cartilage, which agreed with their previous results. Bone was found to attenuate the peak force to a much greater extent, mostly due to the comparatively larger quantity. They concluded that bone spares articular cartilage from impact. Schuurman and Kauer\textsuperscript{210} investigated the effect of the triangular fibrocartilage of the wrist on the attenuation of injurious compression. The fibrocartilage attenuated about half of the compressive load. Fukuda \textit{et al.}\textsuperscript{211} illustrated the areas in the subchondral bone that had the most pressure applied to them using small pressure transducers, and then looked at the increase in load when the meniscus and articular cartilage were removed. The loading was greatest at the medial tibial epiphysis and the lateral subchondral bone. Removal of the meniscus with the legs in varus caused the load on the subchondral bone to increase four to five times. Removal of the articular cartilage resulted in the load increasing 1.1 to 1.4 times medially and 1.2 to 1.6 times laterally. This study shows the meniscus attenuates the load more than articular cartilage, but cartilage still seems to play a role in load attenuation. In another acute study on articular cartilage, Radin and Paul\textsuperscript{212} tested how impact loading versus constant loading
caused wear while a bovine joint was articulated. They found that static loads up to 4448 N (1000 lbs) did not cause much wear or increase the coefficient of friction during 500 h of articulation. In contrast, adding an impact load of 2224 N (500 lbs) to a 2224 N (500 lbs) static load caused wear and an increase in friction in just 200 h, leading to the conclusion that impact loading could be a primary cause of articular cartilage wear.

**Articular Cartilage Damage, Autoradiography, and Impact Characterization**

The use of a drop tower with histology and autoradiography allowed several studies to determine the viability of cells after impact and gather biomechanical data. Repo and Finley\textsuperscript{14,213} used a drop tower to impact osteochondral plugs to examine chondrocyte survival. The design of the drop tower allowed for the application of impact loads found during falls or in car crashes. After impact, the cartilage was incubated with tritiated proline to assess viability. The threshold for the gross damage of articular cartilage upon impact was 25 MPa at strain rates between 500 and 1000 s\textsuperscript{-1}. This level was much higher than the previous study by Radin,\textsuperscript{208} possibly due to the attached subchondral bone. Cells adjacent to the large fissures caused by the impact did not uptake the proline and were likely dead. The unconfined compression in the study also likely underestimated the critical load compared to impact upon a cartilage that had not been removed from the joint, as shown in later studies. Using the same setup, another study discovered that, up to 6 MPa, normal stress during walking would result in a 10% strain in tibial cartilage.\textsuperscript{214} The chondrocytes in the deep zone tended to survive all impacts unless affected by a deep fissure. One study demonstrated that the thickness of
bone cement used did not interfere with the strain measurements and showed massive fissuring at 50% strain whether or not the articular cartilage had been removed from the bone before impact.\textsuperscript{215} The energy absorption in the knee calculated for normal walking with the same drop tower setup was between 0.13 and 3.65 Joules, but large variations were found that might be due to tissues such as the meniscus and other factors.\textsuperscript{216} Vener \textit{et al.}\textsuperscript{217} considered canine joints impacted at many different levels. The average pressure generated in the fractured specimens was greater than 40 MPa, unlike the previous studies, which found lower peak stress levels. The discrepancy could result from the use of intact joints, as well as the differences in the energy and load rates from other studies. This study found some isolated cracks in the calcified cartilage apart from the subchondral bone fractures and surmised that damage begins in the calcified region of cartilage. These studies provide evidence of a critical level of impact for cartilage before gross damage, infer patterns of articular cartilage failure and cell death, and begin to characterize impacts with energy levels and strains.

Other studies delve into the specific contact pressures during impact, the effects of subfracture impacts, and the effects of joint angles and impact characteristics on articular cartilage. Haut\textsuperscript{146} used a free flying impact mass and pressure sensitive film to record the contact pressures in a human knee flexed at 90\(^\circ\). Fracture of the bone occurred at 8.5 kN and the pressure in the joint for an 8 kN load was 25 MPa, the stress level seen in the previous studies to cause visible cartilage damage. The pressures in the patello-femoral (PF) joint were non-uniform and larger areas of the joint experienced pressures over 25 MPa with increasing loads, even when subchondral bone fracture did not occur. Subfracture impacts that were 45% of the fracture load of the contralateral PF joint were
shown to cause subchondral bone damage as well as substantial cartilage fibrillation.\textsuperscript{218} The splits in the subchondral bone were not seen grossly and occurred at 48\% less load than the criteria for automobile certification. Testing the influence of impact energy and impact mass on the injuries to the PF joint, Atkinson \textit{et al.}\textsuperscript{219} found injuries to be more frequent and worse in nature with higher mass at the same energy level. Loading rate increased with increasing energy, independent of mass. Increased contact area and deeper fissures correlated with increasing energy. This study illustrates the importance of recording the energy of the impact, as well as peak force, time to peak force, and duration for comparison to the literature. The pressure sensitive film used in the experiment may have recorded the hydrostatic pressure of the fluid, not the solid, phase of the articular cartilage during the impact, as mentioned previously.\textsuperscript{204} Atkinson and Haut\textsuperscript{220} also investigated impact of the human knee with various flexion angles of 60°, 90°, and 120°. Both the peak pressure and load increased with increasing flexion angle, while the time to peak load decreased. The contact area varied with flexion and predicted the area of fractures or microfractures, allowing better understanding of ways to minimize injury due to car accidents or potential falls. In a similar study, different flexion angles were tested with a rigid and a deformable impact interface.\textsuperscript{221} The rigid interface was used with a fracture impact level, and the deformable interface was used at the same level. The deformable impact interface resulted in fewer bone fractures, but no fewer microfractures of subchondral bone or articular cartilage fissures. A study on horse distal interphalangeal joints also showed varying contact area and pressures with different joint flexions.\textsuperscript{222} Silyn-Roberts and Broom\textsuperscript{223} explored crack initiation and propagation in articular cartilage explants separated from bone with a pendulum impactor. The cartilage
was separated from the bone and some specimens had the superficial layer shaved off. All fissures formed at 45° to the surface, which suggests shear stress as the failure mechanism. The fissures only went to the intermediate/deep zone boundary and did not occur in more compliant cartilage or cartilage with the superficial layer shaved off. These studies further characterized impacts with respect to energy levels, weight of impactor, and flexion angles. The subfracture impact levels which did not cause gross bone fracture were shown to cause microfractures in the subchondral bone and to cause substantial articular cartilage damage.

**Viability Staining and Cell Membrane Response**

Other investigations of acute changes in articular chondrocytes include staining for viability, while one study investigated the biochemistry of the cell membrane. After impact, the phospholipid pool from articular cartilage receiving a "standard trauma" was tested. The arachidonic acid content, a precursor to inflammatory cytokines that can upregulate proteases and lead to soft tissue degradation, was increased by a factor of four. The viability studies all have similar patterns. Duda et al.\textsuperscript{224} used a drop tower to injuriously compress porcine osteochondral plugs with low energy impacts at 0.06, 0.1, and 0.2 Joules with a loading time of about 200 ms. Viability staining was done with thiazole blue and propidium iodide. Similarly to other studies, cell death occurred mostly in the superficial and intermediate zone and increased with increasing energy. Even with no fissures or other visible damage, chondrocytes died at low impact levels, possibly leading to long-term articular cartilage degradation. Thus, arthroscopy may be inadequate to visualize damage that will eventually lead to OA. Clements et al.\textsuperscript{147} had
similar results in their investigation of repetitive loading of bovine osteochondral plugs. Although, the repetitive loading was 3600 cycles at 1 Hz, and thus the duration was five times longer, the results were complementary with the previously discussed study. The mechanism of cell death in this study was partially apoptosis, but the authors are uncertain whether this was true in the unloaded samples. Cell death was seen mostly in the superficial zone, but was also observed, although to a lesser extent, in the area adjacent to the loaded cartilage. Cell death began at the 6 MPa level of repetitive loading and increased to 40% when gross damage of the cartilage occurred at 14 MPa. Such lower level impacts resulting in chondrocyte death may explain much of the primary OA, but the pressure associated with these impacts was 6 MPa, which is near the physiological pressures seen with walking. This incongruity may be partially explained by animal model and explant location. Studies of human articular cartilage would be useful to confirm these results.

Studies of the acute changes occurring after impact illuminate many aspects of articular cartilage damage. The mechanical behavior differs from that in slower load rate experiments and can vary depending upon different attributes of the impact. Impact was also shown to be a complex mechanical event with multiple methods of description, making comparison of some studies challenging. The flexion of a joint and the stiffness of the soft tissues can cause variation in impact forces and load rates. The viability of the chondrocytes was shown to be lower in cases of cartilage surface disruption and high impact levels, but chondrocyte apoptosis also transpired with lower impact loads. If chondrocytes generally die from low impact loads that do not cause articular cartilage surface disruption or subchondral bone trauma, this could explain some cases of primary
OA. In contrast to these studies on acute changes of articular cartilage in response to impact, work has also been done with animal models to examine longer-term effects of impact.

**ANIMAL MODELS OF ARTICULAR CARTILAGE IMPACT**

*In vivo* models of the effect of mechanical impact on cartilage have produced a great deal of insight into many aspects of injury to the cartilage and subchondral bone (Table 4). While many models of OA exist, most involve chemical induction or ligament resection, neither of which works well for post-traumatic OA. The post-traumatic OA models that will be discussed are produced by many different methods, including intra-articular fracture models, increased joint compression models, ligament resection, articular surface disruption models, and, of course, impact models. Post-traumatic arthritis has been documented secondary to intra-articular fractures and impact injury, making these injuries good animal models of OA. Since small animal models have many advantages, including cost and ease of manipulation, rabbit, canine, and guinea pig have been used. Simon and Radin used a guinea pig model with multiple daily impacts equal to the weight of the animal. This model found the subchondral bone became stiffer initially and returned to normal as the articular cartilage degenerated. The following canine and rabbit models demonstrate many other aspects of the effect of impact on articular cartilage.
Canine Articular Cartilage Impact Models

Canine models of impact on articular cartilage have utilized histology, biochemistry, electron microscopy, and magnetic resonance imaging. Donohue et al.\textsuperscript{148} impacted canine patellas with two subfracture loads through a small incision in the skin using a drop tower. These impact levels also did not disrupt the articular cartilage surface. At 2 and 4 weeks, the cartilage swelled with an increase in hexuronic acid content and unchanged DNA levels. The swelling could have been a result of the matrix damage drawing more water into the matrix. The hexuronic acid level increase was likely due to increased proteoglycan synthesis, as safranin-O staining, representative of proteoglycan content, decreased. The decreased staining was indicative of loss of proteoglycan, which may stimulate more production. The force was calculated, but not measured in this experiment to be 25 and 16 N. Thompson et al.\textsuperscript{232} applied an impact force of 2170 N over 5 ms onto closed canine patello-femoral joints using a drop tower. This load initially produced surface fissures and fractures in the calcified cartilage. At six months, the cartilage was fibrillated, the subchondral bone had thickened, and proteoglycans were lost adjacent to gross cartilage damage. This animal model produced serious osteoarthritic changes at six months. Oegama et al.\textsuperscript{231} compared this model to an open joint impact model, which offered the added benefits of controlling the impact more precisely, visualizing of the joint immediately, and changing the location of the impact more easily. The initial results of the open joint model produced some of the same features of the closed joint model, which included the surface fissures and fractures of the calcified cartilage. This study also explored the expression of the pro-inflammatory cytokines TNF-\(\alpha\), II-1\(\beta\), and stromelysin (MMP-3), finding that all of them increased
shortly after impact. The drawback to an open joint impact model is the trauma of surgery occurring at the same time as the impact. Thompson et al.\textsuperscript{235} also used the scanning electron microscopy (SEM) and magnetic resonance imaging (MRI) with the closed joint model. The SEM showed at baseline that calcified cartilage fractures were present, while the articular cartilage surface looked normal. The MRI showed soft tissue swelling and a decreased marrow signal in the patella. The MRI one year after the impact looked normal with few changes detected, although there were still clefts in the articular cartilage. The subchondral bone appeared to have healed. While some repair processes did take place in this model, the continued cartilage surface disruption would likely lead to worsening conditions for the joint and eventually OA.

\textit{Rabbit Articular Cartilage Impact Models}

Experiments using rabbit models of articular cartilage impact are numerous. Radin et al.\textsuperscript{142} used impulsive loading on rabbit hind legs approximately equal to their body weight for 1 h a day. The histological and observational results showed subchondral bone was thickened, effusions occurred, and surface and deep mucopolysaccharide loss at 20 to 30 days. Gross cartilage changes did not occur until day 30. The articular cartilage degeneration following subchondral bone thickening may have been secondary to cartilage wear, and the cartilage degradation might not have occurred had the impulsive loading not been continued. Dekel and Weissman\textsuperscript{149} cycled rabbit hind legs from flexion to extension with an impulsive force applied at full extension. The impacts were not characterized with peak load, energy, or time to peak load in either of these two studies. The SEM showed exposed collagen fibers
immediately and 7 d after loading and prostaglandin E was increased four-fold in the synovial fluid after 60 min of loading. The levels of cyclic adenosine monophosphate (cAMP), a secondary messenger, were decreased in the subchondral bone. This study found that a threshold existed below which no acute changes occurred, although the pressures of the joint at this threshold were not characterized. Another study using this model also showed subchondral bone stiffening and metabolic changes. The stiffness used was a relative value and was found using a compression device. The loading increased the glycine and sulphate uptake, while the hexosamine content decreased and the hydroxyproline levels remained constant. Mazieres et al. developed a rabbit impact model of OA with comparisons based on histology and morphology. This model impacts a rabbit patella with a 1 kg mass dropped from 1 m. The scoring is a combination of histological and morphological characteristics rated from zero to three. No recording of the impact was done, thus differences between impacts may occur, especially if this model is used in multiple laboratories.

More recent animal studies calculated the energy of the impact and characterized the impact in other ways, such as pressure-sensitive film. Borrelli et al. created an in vivo rabbit model that impacts the femoral condyles directly. The pressure sensitive film that had been associated with softening of the cartilage in another study was used to measure the contact pressure. Therefore, control animals should also have the film placed on the articular surface in future studies. This model was able to consistently apply and record an impact load. Haut et al. used a drop tower to impact a rabbit knee with pressure-sensitive film inserted into the joint. The energy levels were 0.9, 4.2, and 6.3 Joules, and the contact pressures varied from 13 MPa for the low energy to 22 MPa
for the high energy impacts. The force was recorded using a load cell. One interesting result of this study was that the peak pressure did not correlate with injury to articular cartilage as well as the gradient of the contact pressures, inferring that shearing was responsible for some of the fissuring. Another notable finding was the amount of variability in the contact pressures and peak loads between impacts of the same level, which highlights the need for careful control and measurement of the impact characteristics. The control animals in this study also showed softening of the articular cartilage just from the opening of the joint and insertion of the pressure-sensitive film. Likely, the surgery itself caused the softening, either through inflammation or another cause secondary to the trauma of the surgery. Two moduli of the cartilage were measured, an instantaneous modulus and an 100 s modulus, called a relaxed modulus, based on the Hayes et al.\textsuperscript{239} indentation theory. These moduli were used to determine that the articular cartilage had softened.

Newberry et al.\textsuperscript{240} made use of the above animal model for long-term studies of the effect of impact on articular cartilage. The subchondral bone thickened at 12 months compared to control based on histological measurements, while the mechanical stiffness did not change. So, while the subchondral bone thickened consistently up to a year, the cartilage did not soften in response. The previous study by Haut et al.\textsuperscript{153} showed a softening of the articular cartilage, but also included surgery to insert pressure-sensitive film into the joint during the impact. Thus, the film might have changed the local mechanical environment of the surface of the cartilage during impact. This study observed articular cartilage damage in the form of fibrillation and fissuring. Adding physical activity post-impact to the same model, Newberry et al.\textsuperscript{241} found the cartilage
degenerated using a histological score. The score was based on a 14 point scale with points assigned to the structure (surface irregularity to clefts to no organization), cells (normal to hypocellular), safranin-O stain (normal to no staining), and the tidemark (intact or gone) of the articular cartilage. The impacted articular cartilage was softer than the control side at 3, 6, and 12 months, and its histological score increased as well. However, the histological scoring of the controls increased, illustrating the difficulty in using contralateral limbs as controls because the non-control limb is often favored in injury models. To test for the threshold of cartilage injury, Newberry et al.\textsuperscript{242} used two levels of impact and compared them using histology, stiffness, and subchondral bone thickness. The low level impacts of 0.9 Joules did not produce changes characteristic of OA, while the higher intensity impacts of 6.3 Joules caused a decrease in the instantaneous and relaxed (150 s) modulus, an increase in the histological score, and increased subchondral bone thickness. This investigation used a finite element model, which calculated significantly different stress levels between the two impact intensities. The higher intensity pressure was 25 MPa, which is the level used in other studies.

Other studies of the Haut et al.\textsuperscript{153} rabbit model looked at subchondral bone, impact orientation, rate of loading, and other features. Ewers et al.\textsuperscript{243} found that this model resulted in articular cartilage softening without subchondral bone thickening when using a padded interface. The time points were 0, 4.5, and 12 months, with a computational model used to further characterize the impact at baseline. The cartilage was significantly softer at both 4.5 and 12 months, while the subchondral bone was not thicker. This study disagrees with the theory of Radin et al.\textsuperscript{185} that subchondral bone thickening occurs before articular cartilage breakdown. Ewers et al.\textsuperscript{81} also performed a
study varying the impact orientation to the rabbit patello-femoral joint. The two impact loads were either administered centrally or with the leg slightly abducted, which would cause the impact to be off-center. The centrally applied impact load did not show any changes in articular cartilage stiffness, but the subchondral bone was thickened. Using pressure sensitive film, they found the centrally-oriented impact had much lower contact pressure gradients, although the peak pressures were comparable. The abducted limb impact showed two contact pressure peaks with larger and more pressure gradients. The abducted leg impact groups had significant articular cartilage softening, as well as the subchondral bone thickening seen in the centrally-oriented impacts. Load rate was altered in this animal model to understand how it affects cartilage and subchondral bone.\textsuperscript{155} The previous model had a time to peak load of 5 ms, so this rate was tested against an order of magnitude higher time to peak load, 50 ms. The high loading rate, 5 ms to peak load, and the low loading rate, 50 ms to peak load, softened articular cartilage an equal amount, but the high rate of loading resulted in more surface fissures and fibrillation. The subchondral bone thickened more for the high rate of loading. Ewers \textit{et al.}\textsuperscript{154} explored changes out to 3 years with the same animal model. The softening of the cartilage was about 30% at 4.5 months and did not change out to 36 months. The articular cartilage thickness initially increased at 4.5 months, but then shrank to less than half the original thickness at 36 months. Interestingly, the surface fissures did not worsen or extend in length over the 36 months. The subchondral bone started to thicken at 7.5 months, and the authors note that the thickening of bone and the softening of cartilage may or may not be directly related.
The *in vivo* studies have shown how a single or repetitive impact can lead to osteoarthritic changes. Biomechanical softening of articular cartilage, altered surface characteristics, and the loss of proteoglycan staining in response to impact have been well-established over long-term studies. However, there is a disagreement on whether subchondral bone thickening and cartilage softening are coupled to each other or not. Experimental evidence exists on both sides of the dispute. Some future testing might consist of quantitative biochemistry that correlates with the biomechanics, as well as more exploration of genetic regulation of these degenerative processes.

**EXPLANT STUDIES OF ARTICULAR CARTILAGE IMPACT**

Explant studies of the effect of impact upon articular cartilage provide a substantial amount of control over the tissue environment, from the impact to the end of the culture period, along with any experimental treatments (Table 5). However, a drawback to explant studies is the fact that the physiological environment is substantially altered and thus temporal changes cannot be directly correlated to the *in vivo* situation. Many studies with explants use injurious compression instead of impact, according to the definition of Aspden *et al.*\(^ {169} \) The first section deals with explant studies that do not meet this definition, but still shed light on different aspects of the articular cartilage response to lower and intermediate rates of loading. The second section discusses studies that meet the definition of impact previously described.
Injurious and Cyclic Compression Explant Studies

Several devices have the capability to apply dynamic compression on explants, and many studies investigate cyclical loading.\textsuperscript{244,245} Farquhar et al.\textsuperscript{156} loaded canine articular cartilage explants up to 50 MPa for 30 min at a strain rate of 100 MPa/s. This investigation found increased fibronectin and water content after 10 d, similar to changes observed in OA. Torzilli et al.\textsuperscript{245} applied stresses to canine explants from 0.5 to 24 MPa at 1 Hz for 2 to 24 h. This loading resulted in decreased proteoglycan biosynthesis for both static and dynamic compression, while retaining cell viability. Morel and Quinn\textsuperscript{246} found that applying 14 MPa with low strain rates to osteochondral bovine explants caused cell death in the superficial zone and near cartilage fissures. The area of loading was demarcated by cell death until day 4, but by day 11, cell death had spread to adjacent tissue. Chen et al.\textsuperscript{247} varied the duration of loading on cartilage explants at 5 MPa for 2 to 20 min. Two h after loading, about one-third of the cells had taken up propidium iodide, while only 1% of the cells were terminal deoxynucleotidal transferase-mediated dUTP nick-end labeling (TUNEL) positive. TUNEL is an assay for apoptotic cells. After two days in culture, almost three-fourths of the cells were TUNEL positive. Thus, cell necrosis was thought to have occurred first in the superficial and intermediate zones, followed by apoptosis in the adjacent region. This result is important when attempting to prevent apoptosis to preserve articular cartilage function. If necrosis occurs first, then preventing apoptosis will limit cell death and ensuing cartilage damage. Another study of apoptosis subjected human cartilage explants to a static stress of 14 MPa and found apoptosis evidence in one-third of the cells at 96 h, with increased glycosaminoglycan (GAG) release.\textsuperscript{159} Apoptosis increased from 6 h to 7 d after loading, suggesting a time
frame for therapy that will inhibit apoptosis. This investigation also examined caspase inhibitors to prevent apoptosis. A follow up experiment looked at apoptosis in several animal models using the TUNEL assay and found that static loading, a drill hole, and impact all cause appreciable apoptosis.\textsuperscript{160} The drill hole may have caused high short term shear stress around the edges, where apoptosis rates were greater than half. Patwari \textit{et al.}\textsuperscript{248} also looked at injurious compression with the TUNEL assay and electron microscopy. This study illustrated that electron microscopy determined twice the percentage of apoptotic cells, 62\%, than the TUNEL assay, 33\%. Levin \textit{et al.}\textsuperscript{249} cyclically loaded articular cartilage in the middle of an explant to 5 MPa for 20 to 120 min. This study cultured the explants either whole or with the loaded and unloaded portions separated. When isolated from the loaded section of articular cartilage, the unloaded portion did not become TUNEL positive for apoptosis. The unloaded portion of the explant still attached to the loaded portion had increased numbers of TUNEL positive cells, illustrating that some form of intercellular interaction was contributing to the cell death occurring after loading.

\textit{Quinn et al.}\textsuperscript{250} looked at sub-impact loading of bovine articular cartilage explants between 3.5 and 14 MPa at strain rates below 0.7 s\textsuperscript{-1} and cultured for 4 d. They ascertained that fissuring and cell death occurred in the superficial zone with more release of GAGs at the higher strain rates. Cell metabolic rate was measured with fluorescein diacetate staining. Cell deactivation occurred throughout the explant, but was most apparent in the center of the loaded site and decreased with distance from the loaded site. Redman \textit{et al.}\textsuperscript{251} used blunt and sharp instruments to make cuts of articular cartilage, discovering that sharper instruments cause less cell death at the edge of the cut.
This study demonstrates that removal of articular cartilage explants is traumatic, but the trauma can be minimized using sharper instruments. D'Lima et al.\textsuperscript{252} cultured bovine cartilage explants for up to 96 h after loading at 30% strain for half a second and found apoptosis increased at every time point, so more apoptosis may have occurred at later time points. Similar results were shown in human knee and ankle articular cartilage. Clements et al.\textsuperscript{147} explored the role of nitric oxide and caspases in chondrocyte death from cyclically compressed explants and found that neither seems to be mediating cell death. This study used inhibitors of nitric oxide and caspases to investigate their role in impact-related cell death.

\textit{Matrix and Cell Viability Impact Studies}

Many impact explant studies explore chondrocyte viability and matrix degradation. Krueger et al.\textsuperscript{253} examined impacted explants with and without subchondral bone. This study used a high loading rate (600 MPa/s) and a low loading rate (30 MPa/s) to test chondral and osteochondral explants. The high rate of loading is just below Aspden et al.\textsuperscript{169} suggested definition of loading based on the load rate and the 42 ms time to peak load. The presence of the subchondral bone decreased cell death and matrix damage. Low rate of loading in chondral explants showed a more diffuse pattern of cell death, while the high rate of loading had more concentrated cell death in the superficial zone, which agrees with studies by Quinn et al.\textsuperscript{250} and Torzilli et al.\textsuperscript{161} Torzilli et al.\textsuperscript{161} applied a subfracture, acute load of 0 to 65 MPa at a load rate of 35 MPa/s, which is well below impact levels from a drop tower. The pattern of cell death in this study was similar to Krueger et al.\textsuperscript{161}'s study, but did not find cell death in appreciable amounts outside of
the compressed area. This investigation found water content increased with load, while the proteoglycan synthesis decreased with load. They also observed the explants after 24 h, but did not find much difference compared to immediately after impact. If the study had been taken out to later time points, more chondrocyte death may have occurred. Ewers et al.\textsuperscript{254} surveyed the effect of the rate of loading on bovine articular cartilage explant death and matrix damage. They used two loading rates, one at 900 MPa/s and one at 40 MPa/s. This experiment found the same results as the previous two in that low rates of loading resulted in diffuse chondrocyte death, while high rates of loading caused more death around the fissures and fractures. The high rate of loading, as in the previously described studies, caused more matrix damage. Phillips et al.\textsuperscript{255} used a low rate of loading on chondral explants and showed that cell death increased from 1 to 24 h. This study also looked at decreasing cell death, which will be discussed in the next section. Lewis et al.\textsuperscript{256} did not find more cell death away from surface fissures than in the controls after impacts with a peak stress of 53 MPa and surmised that cell death might only occur where the articular cartilage surface is disrupted.

Jeffrey et al.\textsuperscript{157,158} used a drop tower to impact chondral and osteochondral explants. In the first study, chondrocyte death increased linearly with increasing impact energy; energy was calculated from $E = mgh$, where $E$ is the energy, $m$ is the mass, $g$ is gravitational acceleration, and $h$ is the height.\textsuperscript{157} Estimation of the energy using this equation ignores losses due to rebound and any dampening. The fissures tended to be at 45° angles to the surface, as they were in the study by Silyn-Roberts et al.\textsuperscript{223} The water content increased for three days and remained at the high level, likely due to matrix disruption. The second study used pre-radiolabeled leucine to assay for protein
production and pre-radiolabeled sulfate to assay for GAG metabolism. The protein and GAG were pre-labeled, so only recently created protein and GAG was being followed. Both the control and the impacted specimens lost the pre-labeling quickly, although the impacted specimens may have lost more.

These experiments describe how impact and other compressive loads cause chondrocyte necrosis and apoptosis, matrix degradation, and other effects. One detail of explant studies to be noted is whether the explants are only articular cartilage or osteochondral. For impact studies, taking notice of whether the impact occurred on a whole joint, an osteochondral plug, or a cartilage explant is important. Likely these three possibilities offer respectively less measure of protection for the chondrocytes and their matrix. An osteochondral plug is unconfined compression, while a whole joint is closer to confined compression. Overall, the results of these experiments are in fair agreement as to the patterns of cell death that occur with fast and slow loading rates. In addition, these studies agree that impact causes an increased loss of GAG in the media, or an increase in GAG production that ends up in the media.

**DISCUSSION**

Impact of articular cartilage has been shown to result in a large array of acute and chronic degenerative effects. These effects can be ruinous to a patient who has suffered a car crash, sports injury, or other mishap, eventually leading to the development of post-traumatic arthritis. The cost in dollars is in the billions, and the cost in quality of life is enormous. Being able to prevent OA in even a small fraction of cases would result in great improvement in hundreds of thousands of lives, especially if the treatment were
simple and cost-effective. Studying impact is difficult for many reasons, not the least of which is the complexity of the actual mechanical event, which can be characterized by an energy level, a peak load, a time to peak load, duration of impact, and other metrics. This is further exacerbated by the fact that these mechanical metrics are known to vary 5 to 10% even in tightly controlled situations. This complexity makes comparisons problematic if two different studies do not use the same metrics to describe an impact. However, even with the difficulty in characterization and different models of impact damage to articular cartilage, much information is now known about the biomechanical, biochemical, and genetic sequelae.

*Effects of Impact*

Mathematical models of impact are usually computational because of the complexity of impact. The analytical models of articular cartilage contact mechanics are helpful in understanding the behavior of cartilage, which has a complex architecture with solid and fluid components. The computational models consist primarily of finite element models. Such models have shown that shear stresses, followed by tensile stresses of the solid matrix are likely the best predictors of articular cartilage fissuring. They have also established a baseline for the least complex mechanical model of articular cartilage that will provide agreement with experimental data.

Acute effects of impact involve matrix damage and histological changes. Studies of load attenuation have found articular cartilage has an effect, but that the knee meniscus attenuates force better. Also, these investigations showed cartilage to be stiffer with high loading rates. The viability of cells after impact shows a consistent pattern from study to
study, with diffuse chondrocyte death at low loading rates and more focal chondrocyte
death around fissures and in the superficial and middle zone for high loading rates.
Flexion angles and impact orientation were shown to have large effects on the damage
caused by impact. The load at which bone fractures or articular cartilage disrupts seems
to be different for different animal models, although the rabbit patello-femoral joint
shows bone and articular cartilage damage around 25-30 MPa. The acute effects also
included chondrocyte death in response to subfracture loads that did not damage the
surface of articular cartilage.

The in vivo models of impact produced much information about the natural
history of the resulting damage to the matrix and the chondrocytes. The articular
cartilage was fissured, fibrillated, and in some cases, crushed. The response of the
cartilage varied from getting thinner and softer to attempting repair. The subchondral
bone thickened in some models, but not in others. The question of whether or not
subchondral bone thickens before articular cartilage degeneration is still not fully
answered, but evidence exists on both sides of the question. These models showed pro-
inflammatory cytokines, soft tissue swelling on MRI, increased GAG release into media,
increased cAMP levels in the subchondral bone, and increased sulphate uptake (GAG
production). These all help draw the picture of what occurs post-impact. Most
importantly, these models showed how a single or repetitive impact leads to articular
cartilage degeneration and signs of OA.

Explant studies to date have mostly dealt with viability and matrix degradation.
Many experiments do not use impact, but rather lower rates of loading and hold the load
at a certain level. Static loading is likely used, at least in some cases, because it produced more cell death and these larger effects are easier to study. High rates of loading, which are more akin to impact, caused more matrix damage, while the low rates of loading produced diffuse cell death. Water content increased with impact and proteoglycan synthesis decreased with load in some studies.

Investigation of Treatments for the Prevention of Post-Traumatic Osteoarthritis

Several recent studies have searched for ways to alter the negative effects of impact loads on articular cartilage. D’Lima et al.\textsuperscript{165} explored several agents to prevent apoptosis in a statically loaded set of explants. They used human and bovine caspase inhibitor z-Vad.fmK, human and bovine IGF-1, and dexamethasone to attempt to decrease the apoptosis in the explants. Caspases are aspartate-specific cysteine proteases that mediate apoptosis. All of these treatments decreased the amount of apoptosis that occurred after loading. GAG is usually released into the media after loading, and the caspase inhibitor treatment also decreased the amount released, which could be because of less disruption or less production. The authors suggest that dexamethasone may decrease apoptosis by decreasing Fas mRNA. Another study using z-Vad.fmK found a 50% reduction in apoptosis following loading.\textsuperscript{159} Using this caspase inhibitor in human articular cartilage resulted in a decrease in apoptosis from 34% to 21% with a concomitant decrease in the release of GAG into the media.\textsuperscript{257} Apoptosis in this study was found to correspond with the amount of load, with higher loads showing more apoptosis in the superficial and middle zones, as seen with impact at a high loading rate.
These loads were not impact, but the method of reducing the amount of apoptosis would likely also work for impact and possibly slow or prevent articular cartilage degeneration seen in impact studies.

Other types of treatments may also work to ameliorate the detrimental effects of impact. Ewers and Haut\textsuperscript{258} used polysulphated GAGs (psGAGs) to attempt to alleviate the loss of mechanical properties of impacted rabbit knees. Using psGAGs prevented the decrease in biomechanical properties due to impact load. The treatment was intramuscular injections every four days for six weeks, which may not be amenable to human treatment. However, this treatment was based on a treatment of equine articular damage and appears quite successful at mitigating some of the impact damage\textsuperscript{259,260}. Phillips and Haut\textsuperscript{255} treated post-impact articular cartilage with a non-ionic surfactant called P188, which is a block polymer. They discovered that use of this surfactant caused less cell death in the superficial zone at 1 hr and less cell death in all zones at 24 hr. Thus, cell membrane rupture may be one mechanism by which impact causes chondrocyte death.

The experiments described in this review illustrate and detail the damage to articular cartilage that impact loads can cause. The different types of cartilage injuries, which include no surface disruption, surface disruption, or articular cartilage and bone disruption, will have different natural histories. Therefore, to prevent the degeneration of articular cartilage, different injuries must be studied. Several experiments, particularly with explants, have shown that impacts that do not fracture bone or disrupt the surface of cartilage can cause substantial cell death. Such cell death may lead to OA, and this
mechanism may explain the etiology of a proportion of primary OA. Also, this type of injury is likely the easiest to treat non-invasively by limiting cell death. To further understand this type of impact damage, more studies of the post-impact genetic sequelae would elucidate the biochemical process of biomechanical breakdown. Fehrenbacher et al.\textsuperscript{26} looked at the genetics of articular cartilage explants after a non-impact load and found that collagen I and II both decreased in response to load. While articular cartilage is not known to produce substantial amounts of type I collagen, more of the genetic effects in response to mechanical loading, impact in particular, will advance the knowledge of how to limit the negative effects. Intact articular cartilage is more resistant to impact loads than explants. Therefore, explant studies of the effects of impact on cartilage should be done by applying an impact before removing the cartilage as an explant. This method would allow highly controlled and repeatable impacts, more direct comparison to the \textit{in vivo} contact stress levels, and more correlation with mechanical impact response of the joint. The short-term response of the tissue tends to dominate the picture because once the tissue begins to break down no current treatment alters the rate of degeneration. Once the genetic, biochemical, and biomechanical progress of articular injuries are understood at baseline for a given explant model of articular cartilage impact injury, different treatments can be explored before moving into small and large animal models.

From Dr. Hunter's 1746 treatise on the diseases of articular cartilage to Dr. Magnuson's 1926 description of joint debridement and the contemporary studies of impact, research and medicine have attempted to forge solutions to the problem of articular cartilage degeneration and OA. Prevention of post-traumatic OA promises to
keep patients from years of pain and expense. If a trauma patient coming into an emergency department can be given an injection into the joint capsule or an oral pharmaceutical and decrease the chances of developing OA by a small percentage, thousands would benefit. If this treatment was extended to high-risk populations such as jack-hammer operators and college and professional athletes and worked, the benefits would increase exponentially.

Overall, these studies provide crucial information to understand the effects of impact and hopefully impede the damage impact can trigger. Future studies should include the recorded energy of the impact, as well as peak load, time to peak load, and, if possible, the contact stresses. Designing and testing treatments for prevention of post-traumatic arthritis should be a major goal of bioengineering. Better understanding of how growth factors interplay in impacted articular cartilage is also needed, both physiologically and in vitro. Treatment strategies would also benefit from additional information on the genetic regulation and influences in impacted cartilage.
ACKNOWLEDGMENTS

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Table 3. Summary of studies examining acute changes in cartilage after impact.

<table>
<thead>
<tr>
<th>Primary Author</th>
<th>Year</th>
<th>Load Application</th>
<th>Type of Loading*</th>
<th>Impact Characteristics Reported†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radin</td>
<td>1970</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PKL – 85 kg, 115 kg, 160 kg t-PKL – 1.5 ms, 1.5 ms, 1.6 ms</td>
</tr>
<tr>
<td>Radin</td>
<td>1970</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>N/A</td>
</tr>
<tr>
<td>Radin</td>
<td>1971</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PKL – 169, 191, 209, 275, 320, and 350N</td>
</tr>
<tr>
<td>Repp</td>
<td>1977</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PKS – 10 to 52 MPa SR – 500 to 1000/s E – 0.1 to 8.2 mJ/mm²</td>
</tr>
<tr>
<td>Finlay</td>
<td>1977</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PKS – 3 to 55 MPa E – 0 to 12 mJ/mm²</td>
</tr>
<tr>
<td>Finlay</td>
<td>1978</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PKS – 15 to 65 MPa E – 1.28 to 16.3 mJ/mm²</td>
</tr>
<tr>
<td>Finlay</td>
<td>1978</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PKS – 10 to 65 MPa</td>
</tr>
<tr>
<td>Finlay</td>
<td>1979</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PKS – 15 to 65 MPa SR – 500 to 1000/s E – 0 to 12 mJ/mm²</td>
</tr>
<tr>
<td>Chrisman</td>
<td>1981</td>
<td>Pendulum</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>Haut</td>
<td>1989</td>
<td>Free Flight Mass</td>
<td>Impact</td>
<td>PKL – 3.6 to 14.4 kN Dur – 8 to 23.5 ms</td>
</tr>
<tr>
<td>Silyns-Roberts</td>
<td>1990</td>
<td>Pendulum</td>
<td>Impact</td>
<td>E‘ – 3, 6, 9 J</td>
</tr>
<tr>
<td>Vener</td>
<td>1992</td>
<td>Materials Testing Machine</td>
<td>Impact</td>
<td>PKL – 0.5 to 2.0 kN PKS – 20 to 70 MPa Dur – 20 to 30 ms</td>
</tr>
<tr>
<td>Atkinson</td>
<td>1995</td>
<td>Free Flight Mass</td>
<td>Impact</td>
<td>PKL – 3.2 to 9.4 kN t-PKL – 5.3 = 3.1 ms E – 10 to 90 J</td>
</tr>
<tr>
<td>Fukuda</td>
<td>2000</td>
<td>Drop Tower</td>
<td>Unknown</td>
<td>PKS – 1 to 7 MPa</td>
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<tr>
<td>Duda</td>
<td>2001</td>
<td>Drop Tower</td>
<td>IC</td>
<td>E – 0.06 to 0.2 J Dur – 200 ms</td>
</tr>
<tr>
<td>Atkinson</td>
<td>2001</td>
<td>Free Flight Mass</td>
<td>Impact</td>
<td>PKL – 3.7 to 8.3 kN t-PKL – 3.4 to 7.8 ms E – 27.3 to 78.8 J Dur – 7.4 to 24.1 ms</td>
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<tr>
<td>Atkinson</td>
<td>2001</td>
<td>Free Flight Mass</td>
<td>Impact</td>
<td>PKL – 2.4 to 6.4 kN t-PKL – 3.9 to 9.5 ms E – 16.5 to 74.8 J Dur – 12.1 to 18.8 ms</td>
</tr>
<tr>
<td>Atkinson</td>
<td>2001</td>
<td>Free Flight Mass</td>
<td>Impact</td>
<td>PKL – 2.1 to 3.8 kN t-PKL – 2.6 to 5.4 ms E – 2, 11, 22 J</td>
</tr>
</tbody>
</table>

* Type of loading refers to impact versus injurious compression (IC), where the definition of impact was taken as time to peak load less than 30 milliseconds.
† Impact characteristics: PKS – peak stress (or PKL – peak load, where reported), t-PKS – time to peak stress (or t-PKL – time to peak load), SR – strain rates, E – Energy (E’ – calculated energy, not measured), Dur – duration of impact. The units were converted, where possible, to allow easy comparison. N/A – characteristics were not reported.
Table 4. Summary of in vivo models of cartilage impact.

<table>
<thead>
<tr>
<th>Primary Author</th>
<th>Year</th>
<th>Load Application Method</th>
<th>Type of Loading*</th>
<th>Impact Characteristics Reported†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simon⁴²</td>
<td>1972</td>
<td>Impact Table</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>Radin¹³</td>
<td>1973</td>
<td>Impulse Table</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>Dekel²⁰</td>
<td>1978</td>
<td>Impulse Table</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>Radin¹¹²</td>
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<td>Impulse Table</td>
<td>Unknown</td>
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</tr>
<tr>
<td>Donohue¹⁹</td>
<td>1983</td>
<td>Drop Tower</td>
<td>Impact</td>
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</tr>
<tr>
<td>Mazieres¹¹³</td>
<td>1987</td>
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<td>Impact</td>
<td>N/A</td>
</tr>
<tr>
<td>Thompson¹⁰⁸</td>
<td>1991</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PkL – 2.1 to 2.4 kN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t-PkL – 2 ms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dur – 4 ms</td>
</tr>
<tr>
<td>Newberry¹¹⁶</td>
<td>1997</td>
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<td>Impact</td>
<td>PkL – 0.56 kN</td>
</tr>
<tr>
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<td></td>
<td>t-PkL – 4.5 ms</td>
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<td></td>
<td></td>
<td></td>
<td>Dur – 10.9 ms</td>
</tr>
<tr>
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<td>Impact</td>
<td>PkL – 0.57 kN</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>t-PkL – 4.6 ms</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Dur – 11.2 ms</td>
</tr>
<tr>
<td>Newberry¹¹⁸</td>
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<td>Drop Tower</td>
<td>Impact</td>
<td>PkL – 0.15, 0.55 kN</td>
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<td>t-PkL – 1.9, 3.5 ms</td>
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<td></td>
<td></td>
<td>Dur – 8.5, 10.4 ms</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>E – 0.9, 6.3 J</td>
</tr>
<tr>
<td>Ewers¹¹⁹</td>
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<td>Drop Tower</td>
<td>Impact</td>
<td>PkL – 0.63 kN</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>t-PkL – 5.4 ms</td>
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<td></td>
<td>Dur – 13.4 ms</td>
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<td></td>
<td></td>
<td>E – 6.6 J</td>
</tr>
<tr>
<td>Ewers⁶⁷</td>
<td>2002</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PkL – 0.6 to 0.7 kN</td>
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<td>t-PkL – 3.7 to 4.5 ms</td>
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<td></td>
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<td>Dur – 10.5 to 11.6 ms</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>E – 6 J</td>
</tr>
<tr>
<td>Ewers²⁵</td>
<td>2002</td>
<td>Drop Tower</td>
<td>Impact and IC</td>
<td>PkL – 0.59 to 0.63 kN</td>
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<td></td>
<td>t-PkL – 4.2 to 60 ms</td>
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<td>Dur – 10.5 to 257 ms</td>
</tr>
<tr>
<td>Ewers²⁶</td>
<td>2002</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>PkL – 0.63 kN</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t-PkL – 4.5 ms</td>
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</tbody>
</table>

* Type of loading refers to impact versus injurious compression (IC), where the definition of impact was taken as time to peak load less than 30 milliseconds.
† Impact characteristics: PkS – peak stress (or PkL – peak load, where reported), t-PkS – time to peak stress (or t-PkL – time to peak load), E – Energy (E' – calculated energy, not measured), Dur – duration of impact. The units were converted, where possible, to allow easy comparison. N/A – impact characteristics were not reported.
**Table 5. Summary explanl models of cartilage impact.**

<table>
<thead>
<tr>
<th>Primary Author</th>
<th>Year</th>
<th>Load Application Method*</th>
<th>Type of Loading*</th>
<th>Impact Characteristics Reported†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeffery28</td>
<td>1995</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>Dur - 0.6 ms, E' - 0.05 to 1.96 J</td>
</tr>
<tr>
<td>Torzilli121</td>
<td>1996</td>
<td>MTM</td>
<td>Cyclic IC</td>
<td>PkS - 0.5 to 24 MPa</td>
</tr>
<tr>
<td>Farquhar27</td>
<td>1996</td>
<td>MTM</td>
<td>Cyclic IC</td>
<td>PkS - 0.5 to 50 MPa, Dur - 500 ms</td>
</tr>
<tr>
<td>Jeffrey29</td>
<td>1997</td>
<td>Drop Tower</td>
<td>Impact</td>
<td>E' - 0.05 to 1.96 J</td>
</tr>
<tr>
<td>Torzilli32</td>
<td>1999</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 0.5 to 65 MPa, Dur - 14 to 1857 ms</td>
</tr>
<tr>
<td>D’lima128</td>
<td>2001</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 7, 14, 23 MPa, Dur - 500 ms</td>
</tr>
<tr>
<td>Ewers130</td>
<td>2001</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 40 MPa, t-PkS - 45, 1000 ms</td>
</tr>
<tr>
<td>Quinn126</td>
<td>2001</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 3.5, 7, 14 MPa</td>
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<tr>
<td>Levin125</td>
<td>2001</td>
<td>MTM</td>
<td>Cyclic IC</td>
<td>PkS - 5MPa</td>
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<tr>
<td>Chen123</td>
<td>2001</td>
<td>MTM</td>
<td>Cyclic IC</td>
<td>PkS - 5MPa</td>
</tr>
<tr>
<td>Lewis132</td>
<td>2003</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 53 MPa, t-PkS - 250 ms</td>
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<td>Morel122</td>
<td>2003</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 14 MPa</td>
</tr>
<tr>
<td>Krueger129</td>
<td>2003</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 0.81 to 0.86 kN, t-PkS - 50, 1000 ms</td>
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<tr>
<td>Clements18</td>
<td>2004</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 5 MPa</td>
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<tr>
<td>Patwari124</td>
<td>2004</td>
<td>MTM</td>
<td>IC</td>
<td>PkS - 20 MPa</td>
</tr>
</tbody>
</table>

*MTM – Materials testing machine.

* Type of loading refers to impact versus injurious compression (IC), where the definition of impact was taken as time to peak load less than 30 milliseconds.

†Impact characteristics: PkS – peak stress (or PkL – peak load, where reported), t-PkS – time to peak stress (or t-PkL – time to peak load), E – Energy (E' - calculated energy, not measured), Dur – duration of impact. The units were converted, where possible, to allow easy comparison. N/A – characteristics were not reported.
Figure 17. Experimental instruments for applying mechanical impacts with short durations and high strain rates.

a) Free flight mass, b) drop tower, and c) pendulum.
Figure 18. Example of damage to the surface of articular cartilage caused by impact.

a) Healthy cartilage, b) low energy impact with a surface fissure and surface damage, and c) high-energy impact with fibrillation and a deep fissure that penetrates the cartilage half-way through the thickness.
CHAPTER 5: ARTICULAR CHONDROCYTE ISOLATION METHODS ALTER GENE EXPRESSION*

ABSTRACT

Tissue engineering of articular cartilage usually requires the isolation and culture of chondrocytes. Previous studies have suggested that enzymatic isolation may alter the metabolic activity and growth rate of chondrocytes. This study examined the effects of four common isolation protocols on chondrocyte gene expression, morphology, and total cell yield immediately following the digest (t = 0) and after two culture periods (24 h and 1 wk). Cartilage explants were digested using one of four protocols: a) 6 h collagenase digest, b) 22 h collagenase digest, c) 45 min trypsin digest followed by a 3 h collagenase digest, or d) 1.5 h pronase digest followed by a 3 h collagenase digest. Gene expression levels for GAPDH, collagen I, collagen II, aggrecan, superficial zone protein, matrix metalloproteinase-1, and tissue inhibitor of metalloproteinase-1 were measured at t = 0 h, 24 h, and 1 wk using quantitative reverse transcriptase-polymerase chain reaction. Cell yield was highest for the 22 h collagenase and pronase-collagenase digests. However, cells isolated in the 6 h collagenase digest had the fewest gene expression changes compared to cells in undigested tissue. For tissue engineering purposes, if the quantity of

chondrocytes desired is more important than deviation from native phenotype, then the 22 h collagenase digest will be most beneficial. Otherwise, the 6 h collagenase digest is preferable.
INTRODUCTION

Tissue engineering offers a promising solution for patients with damaged articular cartilage. As a tissue that has limited intrinsic ability to heal following injury, articular cartilage defects are often treated symptomatically. Older patients suffering from articular cartilage injury often undergo total joint replacement, thus incurring the risks associated with surgery, given their age and the chance of post-operative infection. Prospects in tissue engineering have improved vastly over the last several years with the development of various biodegradable scaffold designs that are seeded with chondrocytes and then either injected or implanted into the joint to restore function.

Articular cartilage consists of an extensive extracellular matrix (ECM) that contains primarily type II collagen and large aggregating proteoglycans. The ECM is produced and maintained by chondrocytes, and the tissue is relatively acellular. This paucity of cells (1-5% by volume) coupled with the tissue’s avascular nature, severely hinders the ability of articular cartilage to regenerate and maintain its unique function following injury. Integrins, specialized surface protein receptors on the chondrocyte, anchor the cell to the extracellular matrix. Studies have demonstrated that chondrocytes in situ must maintain normal attachment to the ECM through integrins in order to remain viable, maintain cell morphology, and promote homeostasis. Additionally, integrin binding stimulates intracellular signaling which has been demonstrated to affect gene expression and cell function. Articular chondrocytes have been found to rapidly dedifferentiate and lose their phenotype when cultured in monolayer. These cells produce proteins normally seen in fibroblasts or
prechondrocytes,\textsuperscript{279} presenting many problems for researchers and tissue engineers alike. Chondrocytes have also been observed to change from their characteristic round shape to a flattened, fibroblast-like shape following as few as four passages.\textsuperscript{282} Little is known about the mechanism of these changes during culture, although one prevailing theory is that the enzymatic digestion used to isolate and then passage these cells may contribute to altering the structure and function of chondrocytes.\textsuperscript{270,281,283}

Different protocols for the digestion of ECM in articular cartilage are used in the scientific community. The variability of digestion methods raise concern due to the varying specificities and efficiencies of the enzymes used to isolate chondrocytes.\textsuperscript{284} Few studies have been performed regarding the effects that enzymatic digestion procedures have on chondrocytes isolated from tissue. Consequently, little is known about the effect of these enzymes on cell viability and metabolism, cell surface proteins, and perhaps most importantly, the effect on cell phenotype and gene expression. Isolated chondrocytes are required in the seeding of tissue engineered constructs\textsuperscript{262,265,268} as well as for studies involving the physiology and biomechanics of single chondrocytes.\textsuperscript{59,285} Successful tissue engineered constructs require viable chondrocytes that can attach to a scaffold and develop a dense ECM. Damage to integrins on the cell membrane following enzymatic digestion could affect cell adhesion and alter the gene expression required for ECM protein synthesis.\textsuperscript{276} Furthermore, enzymatic digestion may introduce cell selection, as those cells which are both viable and adherent following isolation may in fact have proteinase-resistant or other properties causing the preferential survival and attachment.\textsuperscript{284}
Nearly all of the protocols developed for the enzymatic digestion of articular cartilage utilize collagenase\textsuperscript{277,281,284,286-288} to cleave native helical collagen fibrils to liberate cells from connective tissue.\textsuperscript{289} The type 2 collagenase used in the current study is derived from the bacteria \textit{Clostridium histolyticum} and, according to the manufacturer, contains high levels of the protease clostripain. Some isolation methods also utilize other enzymes such as pronase\textsuperscript{281,290-293} and trypsin\textsuperscript{265,284,289} to partially degrade proteoglycans within the matrix prior to collagenase digestion. This procedure is done to minimize exposure time to collagenase as well as decrease overall digestion time.

The goal of this study was to investigate the hypothesis that enzymatic digestion of articular cartilage would alter the gene expression profile of chondrocytes when compared to undigested tissue. In addition, this study sought to examine the effects of four common enzymatic digestion methods on chondrocyte morphology and investigate whether longer digest periods would increase cell yield. The current study was performed using serum-free media to minimize interference with the enzymatic digestion procedures and to prevent changes in gene expression due to growth factors contained within the serum.

\textbf{MATERIALS AND METHODS}

\textit{Articular Cartilage Harvest and Digestion}

This study examined the effects of the following enzymatic digestion protocols: a) 6 h collagenase digest, b) 22 h collagenase digest, c) 45 min trypsin digest followed by a 3 h collagenase digest, and d) 1.5 h pronase digest followed by a 3 h collagenase digest.
Table 6 lists the enzyme concentrations and duration of each digest. The values for enzyme concentrations and durations for all digests were based on published values. Collagenase type 2 (Worthington, Lakewood, NJ), crystallized trypsin (Sigma, St. Louis, MO), and type XIV bacteria-derived pronase (Sigma, St. Louis, MO) were dissolved in a culture medium composed of Dulbecco’s modified Eagle medium (Biowhittaker, Walkersville, MD) supplemented with 100 units/mL Penicillin (Biowhittaker), 100 μg/ml Streptomycin (Biowhittaker), 2.5 μg/mL Fungizone (Biowhittaker), 2 mM L-Glutamine (Biowhittaker), 0.1 mM non-essential amino acids (Invitrogen, Grand Island, NY), and 50 mg/L ascorbic acid. All digest media were filtered with a 0.2 μm filter (Steriflip, Billerica, MA) after the addition of enzymes. The first digest medium for each group was prepared prior to tissue harvest and incubated at 37° C, 10% CO₂.

Articular cartilage explants were harvested using sterile technique from the distal humerus of adult bulls within 24 h of slaughter. Samples were taken from a total of three animals. Full-thickness cartilage explants were removed from the joint in 6 mm discs using a dermatological biopsy punch and a no. 10 scalpel blade. Two undigested explants from each animal were frozen in RNAlater®-ICE (Ambion, Austin, TX) and used as the study control. The explants were each placed into one well of a 6-well plate containing 9 mL of digest solution, minced, and incubated for the specified time. The resulting cell suspension was centrifuged at 1800 rpm for 5 min at 25° C. After the cell suspension was centrifuged the cell pellets were placed in culture.
The trypsin-collagenase protocol and the pronase-collagenase protocol both involved a second digestion. The second enzymatic solution, containing collagenase, was prepared prior to the completion of the first digestion step and stored in an incubator. After completion of the first digestion, the cell suspension was centrifuged at 1800 rpm for 5 min at 25° C. The cell pellet and remaining tissue were then placed in one well of a 6-well plate with the second digest solution and incubated as before. Following the second digestion the cell suspension was again centrifuged, and the resulting cell pellet from the two-step digestions were saved for culture. Two explants from each animal were used for each digest protocol and the resulting cell suspensions were combined before culture.

**Cell Culture, Yield, and Viability**

The final cell pellet from each digest group was resuspended in 2.5 mL of culture medium without digestive enzymes, and the cell suspension was then divided into three groups. The first group of cells was immediately frozen in RNAlater®-ICE (t = 0) for gene expression testing. The second and third groups were cultured in 12-well tissue-culture-treated plates with 1 ml of media for 24 h and 1 wk, respectively. The approximate seeding concentrations (cells/well) were 76000, 420000, 280000, and 420000 for the 6 h collagenase, 22 h collagenase, trypsin-collagenase, and pronase-collagenase digests, respectively. Half of the media was changed every 48 h after the initial 24 h seeding time. At 24 h and 1 wk, pictures were taken of each well at 100X magnification.
The percentage of viable cells in each well was determined at 24 h and 1 wk using the trypan blue exclusion method. Cells were detached using a cell scraper and a 10 min treatment with 0.05% trypsin-EDTA (Invitrogen, Burlington, Ontario), centrifuged, and resuspended. The total cell number was determined using a hemocytometer. For quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), the cell suspension was again centrifuged, and the cell pellet was then frozen in RNAlater®-ICE. Statistical software (JMP IN 5.1, SAS Institute, Cary, NC) was used to perform a multifactor ANOVA with repeated measures on the cell yield results. If significance ($p < 0.05$) was found, a Tukey HSD post-hoc test was performed.

**RNA Isolation and Gene Expression**

RNA was isolated from undigested tissue samples and from each digest group at $t = 0$, 24 h, and 1 wk. The frozen cell pellets were homogenized, and the RNA was isolated using TriZol (Invitrogen, Carlsbad, CA). Total RNA isolated for each sample was measured using a spectrophotometer (Nanodrop, Wilmington, DE). The amount of RNA was normalized to 830 ng in the reverse transcriptase (RT) reaction. During the RT reaction, RNA was incubated with 1 mM dNTPs, 1 mM random hexamers, and Stratascript™ RT enzyme (Stratagene, La Jolla, CA) at 42° C for 60 min, followed by another incubation at 90° C for 5 min. qRT-PCR was performed on the cDNA from each sample for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen I (Col1), collagen II (Col2), aggrecan (AGC), superficial zone protein (SZP), matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1). The sequences for the primers and probes of all of the target genes are listed in Table 7. The
Multiplex Master Mix kit (Qiagen, Valencia, CA) and corresponding probes and primers were used for the qRT-PCR reaction, which was performed using a Rotor-Gene (Corbett Research, Sydney, Australia). The reaction consisted of a 15 min denaturing step followed by 45 temperature cycles. Each temperature cycle consisted of 15 s at 90° C followed by 30 s at 60° C. The fluorescence was measured at the end of each 30 s cycle.

Abundance values quantify the expression level of the gene of interest. Since a standard amount of RNA was used for each RT reaction, it was possible to compare gene expression quantitatively. Abundance levels for the gene of interest ($A_{GOI}$) were calculated from the take-off cycles of the gene of interest ($C_i$) using the following equation:

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

where $E_{GOI}$ is the efficiency of the gene of interest for the corresponding qRT-PCR reaction. Statistical significance for gene expression was determined using the procedure previously described for cell yield.

RESULTS

Morphology

Chondrocytes from all digest groups retained their characteristic rounded shape throughout the culture period and exhibited no gross physical differences between digest groups at $t = 24$ h (Fig 19. However, after 1 week in culture the chondrocytes digested in
trypsin-collagenase formed small clusters of cells (Fig 19). The cell clusters remained intact after detachment, but the individual chondrocytes still retained their rounded shape. No other digest groups exhibited any morphological differences between 24 h and 1 wk in culture. None of the groups appeared to produce ECM during the culture period.

**Cell Yield**

The 22 h collagenase and pronase-collagenase digest groups yielded the highest total cell number, while the 6 h collagenase digest yielded the lowest total number of cells (Fig 20. Intact tissue remained in the well after three digest protocols: 6 h collagenase, trypsin-collagenase, and pronase-collagenase. Collagenase digestion following both the trypsin and pronase pretreatments eliminated the remaining tissue. No second digestion followed the 6 h collagenase digestion, so the remaining tissue was discarded. Chondrocytes attached to the culture surface were counted at 24 h and 1 wk. The number of attached cells from the 22 h collagenase group significantly decreased between 24 h and 1 wk. All other digest groups exhibited a slight increase in the number of cells attached between 24 h and 1 wk, but the changes were not significant. Trypan blue exclusion determined that all cells remained alive throughout the culture period. The values for cell yield (mean x 10^3 ± S.E.) were: 253 ± 35 for 6 h collagenase, 1408 ± 38 for 22 h collagenase, 922 ± 48 for trypsin-collagenase, and 1383 ± 182 for pronase-collagenase.
Gene Expression

Differences in the gene expression profiles indicated that the 22 h collagenase, trypsin-collagenase, and pronase-collagenase digest protocols all caused significant changes in gene expression of isolated chondrocytes. Figure 21 shows that at \( t = 0 \) only the pronase-collagenase group exhibited GAPDH levels that were significantly different from all other groups. At both \( t = 24 \) h and 1 wk the pronase-collagenase digest group was no longer significantly different than the other digest groups. Since GAPDH abundance was not constant across all digest groups, gene expression data are reported as abundance normalized to the average abundance of undigested tissue, except in the case of MMP-1. The MMP-1 abundance was normalized to the average abundance of the 6 h collagenase at \( t = 0 \) because MMP-1 was not expressed in undigested tissue.

No significant changes were observed in the AGC abundance levels until 1 wk (Fig. 21b). Between 24 h and 1 wk the AGC abundance of the 22 hr collagenase group increased 3.5-fold. At 1 wk AGC expression was significantly higher than undigested tissue and the other digest groups. No significant changes were observed in the SZP abundance levels between any digest groups or time points.

All digests, except the pronase-collagenase digest, significantly decreased Col2 abundance at \( t = 0 \) (Fig. 21c). At 24 h, Col2 abundance of the pronase-collagenase group significantly decreased from its \( t = 0 \) value. At both \( t = 24 \) h and 1 wk, all digest groups exhibited Col2 abundance levels that were significantly lower than the undigested tissue. No Coll expression was observed for undigested tissue or any of the digest groups at the analyzed time points.
The ratio of MMP-1 abundance to TIMP-1 abundance is shown in Figure 22a. MMP-1 was not expressed in undigested tissue, making the ratio for the undigested control equal zero. The MMP-1/TIMP-1 ratio of all digest groups is close to zero at both \( t = 0 \) and 24 h. However, at 1 wk the MMP-1/TIMP-1 ratios of the trypsin-collagenase and pronase-collagenase groups increased significantly. TIMP-1 abundance for digest groups was not significantly different from that of undigested tissue at any time point. However, the MMP-1 abundance of the 22 h collagenase, trypsin-collagenase, and pronase-collagenase groups exhibited changes as shown in Figure 22b. The 22 h collagenase digest increased MMP-1 abundance by 6.5-fold between \( t = 0 \) and \( t = 24 \) h, and the trypsin-collagenase digest increased MMP-1 abundance 120-fold between \( t = 24 \) h and \( t = 1 \) wk, although neither of these changes were significant. In contrast, between \( t = 24 \) h and \( t = 1 \) wk, the pronase-collagenase digestion significantly increased MMP-1 abundance compared to the 6 h collagenase digestion.

**DISCUSSION**

The development of tissue engineered articular cartilage is still a work in progress. While newer methods of tissue engineering are being developed, they have one common requirement: viable cells that can produce extracellular matrix. We hypothesized that isolation methods used for chondrocytes would alter their gene expression, which was supported by our results. Additionally, the data suggest that different isolation methods produce varying cell yields and can alter cell morphology. These changes underscore the inherent differences among the proteolytic enzymes used in the digestion of articular cartilage. The present study was designed to utilize four
common enzymatic digestion procedures from literature. This was done to highlight changes in the gene expression profile of chondrocytes that underwent common digestion procedures. Cell yield was still accounted for in order to highlight the varying strengths of the digestions and compare our cell yield to results found in other studies. In subsequent studies, the amount of time and the concentration of enzymes used in the digestion procedure need to be optimized.

Both the 22 h collagenase and the pronase-collagenase digestion yielded the greatest number of cells. While the 22 h collagenase digest yielded nearly five times as many cells as the 6 h collagenase digest, it was done at one-third of the concentration (0.0015 g/mL for 22 h vs. 0.0045 g/mL for 6 h). This indicates that exposure time to the enzyme may have more impact on cell yield than concentration of the enzyme used in the digestion. In a similar study, Jakob et al.\textsuperscript{284} found comparable cell yield results. Morphology results indicated that, after 1 wk in culture, trypsin-collagenase digested cells aggregated and formed clumps. This result is interesting because clumps did not appear until 1 wk post-digestion, and the cell aggregation only occurred in the trypsin-collagenase group, perhaps suggesting that the adhesive properties of these cells promoted aggregation. If clumps of cells are desired for a specific tissue engineering application, this digestion procedure appears to provide such aggregation.

We found significantly different cell yields between the two collagenase digestions, and, with the exception of AGC abundance, no gene expression differences were seen between these two groups. After 1 wk in culture, the chondrocytes isolated using the 22 h collagenase digestion expressed a significantly greater amount of
aggrecan, a large proteoglycan and key component of the ECM. The expression of AGC at \( t = 24 \) h for cells isolated using the 22 h collagenase treatment trended upward relative to chondrocytes in the undigested control; however, this difference was not significant until \( t = 1 \) wk. The 13.5-fold increase of AGC expression at 1 wk for the 22 h collagenase digest may indicate an increased ECM production. However, a similar spike in AGC production was found in another study by Darling and Athanasiou following the first passage.\(^{280}\) AGC expression then fell off after successive passages, possibly indicating that a spike in AGC is an early feature of dedifferentiation.\(^{280}\) It is also noteworthy that no other isolated chondrocytes significantly decreased their aggrecan expression relative to the undigested control, suggesting that AGC expression was not affected negatively by enzymatic digestion at the analyzed time points. These data are in contrast to Col2 expression patterns, which are often used to measure the maintenance of the chondrocytic phenotype. Each of the enzymatic digestion procedures, except the pronase-collagenase method at \( t = 0 \), expressed significantly less Col2 compared to undigested control. Type II collagen accounts for over 90% of total collagen in the matrix\(^{129}\) and is an essential component in the development of articular cartilage. Decreased Col2 expression in isolated chondrocytes is a significant problem for those who work towards cartilage regeneration. While it is known that a decrease in Col2 accompanies dedifferentiation of chondrocytes,\(^{278,280,282,295}\) little is known about what may act as an impetus to this change. Monolayer cultures of chondrocytes rarely survive past a few passages before shifting their collagen synthesis to predominantly type I collagen, a characteristic of fibroblasts, fibrochondrocytes, and prechondrocytic cells.\(^{279,282}\) For three of the four enzymatic digestion methods, our data suggest that the significant decrease in Col2 expression at \( t =\)
0 could be the first step in a cascade of events that causes a change in chondrocyte phenotype when cultured in monolayer. While we never observed any Col1 expression in enzymatically isolated chondrocytes, our time points for qRT-PCR analysis were relatively early for Col1 expression.\textsuperscript{280} Additionally, since the cells were cultured in serum-free media, gene expression of Col1 was likely not promoted.

The two-step digestions represent an appealing alternative due to their decrease in procedure time and relatively high cell yields. However, the two-step protocols both produced the highest levels of MMP-1 abundance, and their MMP-1/TIMP-1 ratio was significantly higher than undigested tissue. A study by Mitrovic et al.\textsuperscript{283} found that pre-treatment with various proteolytic enzymes, including trypsin and pronase, modified the metabolic pattern of chondrocytes, even with relatively short periods of exposure of cells to the enzyme. In the same study, it was found that chondrocytes given a pronase pre-treatment demonstrated greater nucleic acid synthesis. The gene expression results show that pronase pre-treatment appears to have a stimulatory effect on GAPDH and Col2 expression at $t = 0$. It is interesting to note that studies have examined the effect of brief periods of enzymatic digestion on articular cartilage lesions to induce repair.\textsuperscript{273} Our data indicate that this treatment may have deleterious effects due to decreased Col2 expression. While increased MMP-1 expression does occur, indicative of matrix catabolism, the levels are typically accompanied by an increase in TIMP-1, its inhibitory analog. It is not until $t = 1 \text{ wk}$ that the ratio of MMP-1 to TIMP-1 changes significantly, and it is only for the two digestions that were pretreated with either trypsin or pronase. From the decrease in Col2 expression, coupled with the spike in AGC expression, it appears that an effort to stimulate matrix production by enzymatic digestion may cause
cells to alter their gene expression and develop ECM that is not similar to native tissue. Furthermore, the enzymatic digestions hydrolyze many of the surface proteins of chondrocytes, including integrins, ECM receptors, and other surface markers found on chondrocytes. These changes to the chondrocytes may also contribute to the observed dedifferentiation and the changes in gene expression.

As the baby-boomer generation ages there is a greater likelihood of patients developing osteoarthritis, thus increasing the need for replacement tissue. This study suggests that changes to the gene expression of isolated chondrocytes may affect the cell’s ability to produce ECM, maintain phenotype, and proliferate. A balance between cell yield and changes in gene expression exists and depends on length of digestion and specificity of the enzyme. For purposes of tissue engineering, higher cell yields were obtained using either a 22 h collagenase or pronase-collagenase digest, both of which altered gene expression the most. The 6 h collagenase digest resulted in chondrocytes that had a gene expression profile similar to cells in native tissue, but had a significantly lower cell yield. Ideally, a digest with high cell yield could be used in combination with an intervention that would promote maintenance of the native gene expression.
ACKNOWLEDGEMENTS

This study was supported, in part, by the U.S. Department of Transportation, National Highway Traffic Safety Administration Grant No. DTNH22-01-H-07551 and/or the Federal Highway Administration Grant No. FHWA ICRC(1) to the University of Alabama at Birmingham, Injury Control Research Center’s Southern Consortium for Injury Biomechanics. The authors also thank Gwen Hoben, Chris Revell, Roman Natoli, and Kate Nanney for editorial assistance.
Table 6. Concentration of each enzyme and the duration of each step

The concentration of each enzyme and the duration of each step in the digest are shown. The second digest was applied to the same tissue and cell sample immediately after the end of the first digest.

<table>
<thead>
<tr>
<th>First Digest</th>
<th>Concentration (g/mL)</th>
<th>Time (h)</th>
<th>Second Digest</th>
<th>Concentration (g/mL)</th>
<th>Time (h)</th>
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<tr>
<td>Collagenase</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagenase</td>
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<tr>
<td>Pronase</td>
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<td>Collagenase</td>
<td>0.0040</td>
<td>3</td>
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</table>

*0.0625 g Trypsin and 0.0073 g EDTA
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<tr>
<th>Gene (accession number, product size)</th>
<th>Forward Primer (5' – 3')</th>
<th>Reverse Primer (5' – 3')</th>
<th>Probe (5' – 3')</th>
<th>Dye Quencher</th>
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</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (U85042, 86bp)</td>
<td>ACCCTCAAGATTTGTCAGCAA</td>
<td>ACGATGCCAAGTGGTCA</td>
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<td>FAM</td>
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<tr>
<td>Collagen type II (NM_174520, 69bp)</td>
<td>CCTCCTGCACCACAACCTTGGTT</td>
<td>AACGGTGCTTTCCACCTTC</td>
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<td>ROX</td>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (U85042, 86bp)</td>
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<td>ATGACAACCTGGCTCCCACACC</td>
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<tr>
<td>Collagen type I (X02420, 97bp)</td>
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<td>TGGAGTTCCATTTTCACCAG</td>
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<td>Quasar 670</td>
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<td>Aggrecon (U76615, 76bp)</td>
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<td>GCTACCTGACCCCTCAC</td>
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<tr>
<td>Superficial Zone Protein (AF056218, 77bp)</td>
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<tr>
<td>Matrix Metalloproteinase I (X74326, 82b)</td>
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Table 7. qRT-PCR target gene sequences, dyes, and quenchers.
Figure 19. Chondrocyte morphology.

(a) Chondrocytes isolated by a pronase-collagenase digest after 1 wk in culture. All chondrocytes exhibit the same rounded shape. (b) Chondrocytes isolated using the trypsin-collagenase digest after 1 wk in culture. Cells formed small clusters.
Figure 20. Total cell yield for each digest protocol.

Total cell yield (mean ± S.E.) for each digest protocol. Groups not connected by the same letter are significantly different from one another ($p < 0.05$). Total cell yield was highest for the 22 h collagenase and pronase-collagenase digests. (C6 = 6 h collagenase digest, C22 = 22 h collagenase digest, TC = trypsin/collagenase digest, and PC = pronase/collagenase digest).
Figure 21. Normalized abundances (mean ± S.E.) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aggrecan, and collagen II.

(a) Normalized abundances (mean ± S.E.) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The pronase-collagenase digest at t = 0 exhibited a significantly higher level of GAPDH expression. (b) Normalized abundances (mean ± S.E.) of aggrecan (AGC). The 22 h collagenase digest at t = 1 wk exhibited a significantly higher level of AGC expression than any of the other samples. (c) Normalized abundances (mean ± S.E.) of collagen II (Col2). The Col2 levels of the undigested tissue were significantly higher than the Col2 levels expressed by each of the digest groups, except the pronase-collagenase digest at t = 0. Abundances for a particular gene and digest were normalized to the average abundance for that gene in undigested tissue. Groups not connected by the same letter are significantly different from one another (p < 0.05). (C6 = 6 h collagenase digest, C22 = 22 h collagenase digest, TC = trypsin/collagenase digest, and PC = pronase/collagenease digest).

Normalized GAPDH Abundance

![Normalized GAPDH Abundance Graph]

(a)
Normalized AGC Abundance

Normalized Col2 Abundance
Figure 22. MMP-1/TIMP-1 ratio expressed as abundance (mean ± S.E.).

(a) MMP-1/TIMP-1 ratio expressed as abundance (mean ± S.E.). The trypsin-collagenase and pronase-collagenase digest groups have a significantly higher MMP-1 to TIMP-1 ratio at t = 1 wk. (b) MMP-1 abundance (mean ± S.E.) normalized to the 6 h collagenase digest at t = 0. Groups not connected by the same letter are significantly different from one another (p < 0.05). (C6 = 6 h collagenase digest, C22 = 22 h collagenase digest, TC = trypsin/collagenase digest, and PC = pronase/collagenase digest).
CHAPTER 6: GENE EXPRESSION, BIOCHEMICAL, AND BIOMECHANICAL CHANGES IN ARTICULAR CARTILAGE EXPLANT CULTURE

ABSTRACT

Explant culture has many advantages for studying the effects of different interventions on articular cartilage, as well as studying its physiology. The amount of control over the environment that cartilage explant culture provides is beneficial for comparing conditions, such as different mechanical histories and environments or different bioactive molecules. In this study, the biochemistry, gene expression, and biomechanics of articular cartilage explants in culture are investigated. Explants were cultured for 0, 1, and 4 weeks and tested using biochemical assays for DNA, hydroxyproline, and glycosaminoglycans, real-time reverse-transcriptase polymerase-chain-reaction, and creep indentation. Creep indentation testing allowed acquisition of the aggregate modulus, the permeability, and Poisson’s ratio. The glycosaminoglycan content, relative expression of superficial zone protein, and aggregate modulus all decreased significantly from week 0 to week 4 ($p < 0.05$), while the relative expression of

type I collagen increased significantly. These results parallel disuse atrophy studies and indicate that articular cartilage explants under tissue culture conditions exhibit slow changes in extracellular matrix and mechanical integrity, which become significant by 4 weeks. These changes are consistent with an alteration in phenotype from hyaline articular cartilage to fibrocartilage.
INTRODUCTION

Native physiological processes to repair articular cartilage breakdown are usually inadequate to result in a long-term successful healing, unless the extracellular matrix restoration processes outperform the breakdown processes. The native mechanisms to repair articular cartilage usually result in tissue that is more fibrous and less durable. Many processes of mechanical, inflammatory, infectious, and autoimmune injury, as well as other pathologies have the potential to cause cartilage failure. One method of studying the progression of cartilage breakdown with a high degree of environmental control, while still addressing numerous areas of inquiry is by using cartilage explant culture.

Explant culture has been used to study cartilage breakdown under many conditions, including biochemical and biomechanical factors. For example, fibronectin fragments in explant culture were shown to cause extracellular matrix components to be released into the media. In another study, the inflammatory cytokine combination of tumor necrosis factor-α with oncostatin M was examined on cartilage explants, showing increased collagen release from the extracellular matrix. The effects of interleukin-1 and other drugs on healthy and diseased articular cartilage were also studied, finding that the enzymatic activity of cartilage was decreased by anti-inflammatory drugs and that interleukin-1 caused a greater increase in enzyme activity in diseased cartilage.

Several investigators have researched the effects of cyclic compressive loading on articular cartilage using explant culture. These studies found cyclic
loading could have advantageous or detrimental effects on articular cartilage, depending on the loading conditions. High-frequency (0.01 – 1 Hz) cyclic compression was found to stimulate collagen and glycosaminoglycan synthesis, while low frequencies had a negative effect on synthesis when applied at 25% compression. A similar study found synthesis was spatially dependent on the frequency of compression. At 0.01 Hz, the periphery of the articular cartilage explants had increased synthesis compared to the center. Static compression was shown to decrease proteoglycan synthesis, while dynamic compression increased proteoglycan synthesis. Dynamic shear loading of articular cartilage explants was also shown to increase proteoglycan and protein synthesis.

Other studies found higher levels of compression loading to be detrimental. Cyclic loading from 0.5 to 50 MPa every 5 s for 30 min on explants that were cultured for up to 10 d resulted in increased water and fibronectin content, similar to changes seen with osteoarthritis. Stresses of 0.5 to 24 MPa at 1 Hz for 2 – 24 h on articular cartilage explants for 24 h. resulted in decreased proteoglycan synthesis. Cyclically compressed cartilage explants were cultured for up to 21 d with and without a ring of attached, surrounding cartilage that was not cyclically compressed. The study found that cell death spread from the loaded to the unloaded cartilage. However, if the ring of unloaded cartilage was cut away immediately after compression, cell death only occurred in the loaded portion. A similar experiment exploring the role of nitric oxide and caspases in cell death found that these two compounds were not the cause.

A different set of explant studies explored how a single impact or compression load affects articular cartilage. One study applied a single 250 ms
duration compression of 53 MPa and cultured explants for 18 h and 4 d.\textsuperscript{256} This investigation illustrated cell death around fissures on the articular cartilage surface. A similar study compressed bovine articular cartilage explants with a fast (900 MPa/s) or a slow rate (40 MPa/s) of loading and cultured them for 4 d.\textsuperscript{254} This study also found concentrated cell death around surface fissures of the high-loading-rate explants. Low-loading-rate explants had an increased overall cell death that was diffusely distributed throughout the tissue. Cartilage explants that were loaded to 7, 14, and 23 MPa before culturing for 2 d resulted in a dose-response increase in GAG release into the media and apoptosis.\textsuperscript{252} Another investigation found an impact stress threshold of 15-20 MPa, above which the articular cartilage matrix was ruptured and cell death occurred.\textsuperscript{161} Other studies found decreased cell synthesis, matrix loss, and cell death due to a single impact or compressive load using articular cartilage explants.\textsuperscript{157,158,250}

In addition to studying cartilage degradation, explant culture has been used to evaluate ways the degenerative processes can be reversed or ameliorated. The caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp (zVAD.fmk) was used to prevent up to 50% of apoptosis in articular cartilage that had been compressively loaded.\textsuperscript{159,257} Dexamethasone and IGF-I were also discovered to decrease the rate of apoptosis, but to a lesser degree.\textsuperscript{165} A non-ionic surfactant known as P188 was utilized to prevent cell death in articular cartilage explants following impact.\textsuperscript{255} This surfactant is thought to repair the cell membrane by replacing damaged components.

Many investigations using explant culture have been performed to study a wide variety of degeneration or disease prevention modalities. These studies have often
concentrated on a particular aspect of explant culture, such as biochemistry of the extracellular matrix or biomechanical properties of the explant or gene expression changes. Few studies have looked at a broad range of measurements, including changes at the tissue, cellular, and gene expression levels. To more fully characterize explant culture, this study quantifies changes in gene expression, biochemistry, and biomechanics of articular cartilage explants. The goal of this study is to understand the comprehensive changes that articular cartilage undergoes when taken out of its environment and without any mechanical stimulation. This study may also serve as a baseline for examination of a myriad of interventions to alter tissue pathophysiology.

EXPERIMENTAL PROCEDURES

Cartilage Harvest

Proximal ulnas from bulls were acquired within 24 hrs of slaughter. The surfaces of the proximal ulnas were then harvested using sterile technique. This joint surface is relatively flat, as that of the human tibial plateau. Explants of 6 mm were taken using a dermatological punch without any bone from the center of the medial portion of the joint surface. These explants were then washed with PBS and placed in medium, which contained Dulbecco’s Modified Eagle Medium (DMEM) with Glutamax™ (Biowhittaker, Maryland) supplemented with 100 units/ml Penicillin (Biowhittaker), 100 mcg/ml Streptomycin (Biowhittaker), 10% Fetal Bovine Serum (FBS) (Biowhittaker), 2.5 mcg/ml Fungizone (Biowhittaker), 0.1 mM non-essential amino acids (NEAA) (Invitrogen, Grand Island, NY), and ascorbic acid (50 mg/L).
The explants were cultured separately in 6-well plates at 37°C in a 10% CO₂ incubator for 1 and 4 weeks. Half of the medium was changed every other day. When harvested, the medium was carefully removed from the well and the samples were weighed before being processed.

**RNA Isolation and Real Time Reverse Transcriptase-Polymerase Chain Reaction**

The explants were placed in -80°C RNAlater ICE (Ambion, Austin, TX) and frozen at -20°C for no longer than three weeks. RNA was then isolated using TriZol Reagent (Invitrogen, Grand Island, NY). Briefly, the explants were snap-frozen in liquid nitrogen and ground with a mortar and pestle that had been pre-chilled with liquid nitrogen. The ground explant was then placed into 3 ml of TriZol Reagent and homogenized using a Polytron PT1200 homogenizer with a 12mm Polytron aggregate (Kinematica, Switzerland) at 25,000 rpm. Chloroform in the amount of 0.6 ml was added to the sample and was then centrifuged at 12,000g for 15 min, and the top, aqueous layer was removed. The RNA was precipitated with 1.5 ml isopropanol, centrifuged, washed with 2 ml of 75% ethanol, and then the RNA pellet was dissolved in RNase free water.

The RNA was reverse transcribed to DNA using Stratascript™ First Strand Synthesis System (Stratagene, La Jolla, CA). Briefly, random primers were added to the RNA at 65°C for 5 min and cooled to room temperature before buffer, RNase block, dNTPs, and enzyme were added. The mixture was then incubated at 42°C for 1 hr, quenched at 90°C for 5 min, and frozen at -20°C until real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) was performed.
qrtRT-PCR was performed for collagen I (Col1), collagen II (Col2), superficial zone protein (SZP), aggrecan (AGC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), matrix metalloproteinase I (MMP-I), and tissue inhibitor of MMP-I (TIMP-I). The rRT-PCR was run for three genes at a time in the groups of a) Col1, Col2, GAPDH, b) SZP, AGC, GAPDH, and c) MMP-I, TIMP-I, GAPDH. For each gene a forward primer and reverse primer, and a gene-specific probe were used. The primers and probes are listed in Table 8, and the primers were designed from bovine and human mRNA sequences from The National Center for Biotechnology Information (NCBI). After a 15 min activation step at 95°C, rRT-PCR was performed using HotStarTaq Master Mix Kit (Qiagen, Valencia, California) with a 15 sec denaturing step and a 30 sec annealing/extension step at 60°C after which the fluorescence reading was taken. The concentration of the MgCl was 3.5 mM for all reactions.

Relative Expression

Gene expression was normalized by using the take-off cycle number (Ct) for each gene and comparing it to the housekeeping gene, GAPDH. Although the expression of GAPDH was not compared directly in this study, our laboratory has demonstrated that the expression level of GAPDH in middle/deep chondrocytes is constant in articular chondrocytes with and without growth factors.300

The efficiencies and take off cycle for each gene were normalized to the efficiency and take off cycle of the housekeeping gene GAPDH. The efficiencies were found by running standard curves for the rRT-PCR tests. The relative expression of each gene of interest (R_{GOI}) was found using the equation301:
\[ R_{GOI} = \frac{(1 + E_{\text{GAPDH}})^{C_{i,\text{GAPDH}}}}{(1 + E_{\text{GOI}})^{C_{i,\text{GOI}}}} \] (4)

Where \( E_{\text{GAPDH}} \) and \( E_{\text{GOI}} \) are the efficiencies of the housekeeping gene (GAPDH) and the gene of interest, and \( C_{i,\text{GAPDH}} \) and \( C_{i,\text{GOI}} \) are the take off cycle numbers of the housekeeping gene (GAPDH) and the gene of interest, respectively. Any number higher than one illustrates that the mRNA of the gene of interest is created in greater amount than that of the housekeeping gene. GAPDH is assumed to be constant from cell to cell, so the relative expression is quantified by normalizing to GAPDH. The relative abundance of gene expression was found for AGC, SZP, type II collagen, type I collagen, MMP-1, and TIMP-1.

**GAG, Hydroxyproline, and DNA Assay**

Once explants were harvested, they were weighed before and after lyophilization for 2 d, allowing the dry and wet weights to be acquired. To each sample, 1.5 ml of a papain solution was added that contained 125 \( \mu \text{g/ml} \) of papain (Sigma, St. Louis, Missouri) in 50 mM phosphate-buffered solution with a pH of 6.5 with 2mM of N-acetyl cysteine (Sigma, St. Louis, Missouri) and EDTA (Sigma, St. Louis, Missouri). The explants were digested at 60 °C for 12 hrs. If residual tissue remained, the explants were left for another 2 hrs.

GAG content was tested using a 1,9-dimethyl methylene blue (DMMB) colorimetric assay. The GAG concentration was determined using standards and
dimethylene blue from a Blyscan kit (Accurate Chemical and Scientific Corp., Westbury, NY).

The amount of collagen present in the explants was found by using a modified chloramine T and dimethylaminobenzaldehyde assay.\textsuperscript{302} The sample from the papain digest was hydrolyzed in 4 N NaOH at 121 °C for 1 hr before being neutralized and put in a buffer with 50g citric acid, 120g sodium acetate trihydrate, 34g sodium hydroxide, and 34 ml of glacial acetic acid. Briefly, the samples and standards were reacted with chloramine T (1.14g Chloramine T in 20.7 ml of water, 26 ml of isopropanol, and 53.3 ml of the buffer) for 20 min at room temperature. Then, 1ml of a dimethylaminobenzaldehyde mixture (15 g of dimethylaminobenzaldehyde dissolved in isopropanol after adding 26 ml of 60% perchloric acid) was added. Afterward, the samples and standards were read at 550 nm absorbance. Type II collagen standards were used instead of hydroxyproline, which requires a multiplier that ranges from 8-10.

The number of cells in the explants was acquired by measuring the amount of DNA and normalizing using wet weight. The DNA measurement was performed using a reaction between PicoGreen and DNA from a kit (Molecular Probes, Eugene, Oregon). Briefly, 50 \( \mu \)m of sample was taken and added to 100 \( \mu \)m of tris buffer, to which 150 \( \mu \)m of PicoGreen solution was added.

\textit{Creep Indentation Biomechanical Testing}

After harvest or removal from culture, mechanical testing samples were wrapped in gauze, soaked in phosphate-buffered saline with protease inhibitors (10 mM N-
ethylmaleimide, 5 mM benzamidine, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and frozen at -20 °C until testing. To thaw out the samples, they were placed in phosphate-buffered saline with protease inhibitors, as mentioned above, for at least 1 hr at room temperature to equilibrate. An automated creep indentation apparatus was used to determine the compressive creep and recovery behavior of the cartilage explants. 303,304

Each sample was attached to a metal surface with a thin layer of cyanoacrylate glue and immediately submerged in phosphate-buffered saline with protease inhibitors. After being attached to the base plate and submerged, the samples were allowed to equilibrate for at least 20 min. The sample was placed under the loading shaft such that the surface was perpendicular to the shaft using a spherical joint. The automated creep indentation apparatus automatically loaded and unloaded the specimen to obtain the creep and recovery behavior. A tare load of approximately 0.005 N (0.5 g) followed by a test load of 0.01 N (2 g) were applied to the sample with a 0.8 mm diameter, flat-ended, rigid, porous tip while the creep behavior was recorded. The porosity of the tip was 50% with approximately 50 μm pore diameter. Tissue strain remained below 10% for these tests. The automated creep indentation apparatus yielded a curve of the creep and recovery behavior. Specimen thickness was measured using a needle probe, a force transducer and a linear variable differential transformer (LVDT). The needle was moved downward while the force was recorded. When the needle contacted the tissue, a change in the force was noted, along with the needle’s vertical position. When the needle contacted the stainless steel, the force increased to a greater extent and the vertical position was again noted. The difference between these two positions provided the thickness of each sample.
To calculate the material properties from the creep curve, a finite element, non-linear optimization model was utilized. This method allowed the aggregate modulus, Poisson’s ratio, and permeability to be determined using the linear biphasic theory. To use this method, an initial estimate of the material properties was obtained using a semi-analytical, semi-numeric biphasic model.

**Statistical Analysis**

A sample size of n = 6 was used for the biomechanics, n = 5 for biochemistry, and n = 3 for gene expression. Each sample was from a different animal. A multifactor ANOVA with repeated measures was performed on the mechanical properties, the biochemistry, and the rtRT-PCR results using statistics software (JMP IN 5.1, SAS Institute Inc, Cary, NC). If significance existed a Tukey HSD post-hoc was performed with p < 0.05 being considered significant.

**RESULTS**

**Gene Expression Changes**

There were small, but statistically significant temporal changes in gene expression of the explants. Figure 23 demonstrates the relative abundance for type I and type II collagen. Type I collagen significantly increased in week 4, when compared with weeks 0 and 1 (p < 0.05). While the relative abundance at week 4 was 0.019 ± 0.010, the expression at weeks 0 and 1 was not detectable. Collagen type II showed a non-significant downward trend in the mean from week 0 to week 4. The values of the
relative abundance of the gene expression of type II collagen were 115 ± 101 at week 0, 45 ± 29 at week 1, and 11 ± 6 at week 4. The decrease in the mean was an order of magnitude, but the standard deviations were the same order of magnitude as the mean. Type II collagen had the largest relative abundance of gene expression of the six genes assayed.

The relative abundance of aggrecan increased at week 1 and then decreased below the initial level (Fig. 24a). The values for the relative abundance of aggrecan were 0.047 ± 0.047 at week 0, 0.164 ± 0.100 at week 1, and 0.014 ± 0.016 at week 4. The initial upward trend at week 1 in gene expression was accompanied by a downward trend in GAG content, as demonstrated later. The gene expression of SZP significantly decreased from week 0 to week 4 (Fig. 24b). While the values of the relative abundance were not large, the mean decreased one order of magnitude at week 1 and at week 4. The values for the relative abundance of SZP were 0.431 ± 0.301 at week 0, 0.049 ± 0.021 at week 1, and 0.004 ± 0.003 at week 4. The relative abundance at week 4 was barely within detectable limits.

The gene expression of MMP-1 and TIMP-1 did not show any significant changes, though MMP-1 expression did not show up until week 4 (Fig. 25a and 25b). The relative abundance for MMP-1 was undetectable at weeks 0 and 1, and 0.080 ± 0.130 at week 4. TIMP-1 did not show any discernable pattern over the course of the study. The relative abundance values for TIMP-1 were 0.54 ± 0.45 at week 0, 0.82 ± 0.76 at week 1, and 0.75 ± 0.54 at week 4.
Cellularity, GAG, and Collagen Content

The cellularity of the cartilage explants measured using the PicoGreen assay did not significantly change either as a function of culture time or among animals throughout the study. The number of cells per mg was relatively constant, although the standard deviation for this measurement was large. The number of cells per mg was $23,200 \pm 14,700$ at week 0; $22,200 \pm 10,800$ at week 1; and $22,400 \pm 11,000$ at week 4.

The wet weight percentage of the GAG content from the explants, obtained using the DMMB assay, was significantly different between week 0 and week 4, and week 1 and 4. Figure 26 illustrates the mean and standard deviation of the GAG content. The GAG content of the explants had already trended down by 0.5% at week 1, but was not significant at that point. From week 0 to week 4 the GAG content dropped significantly, losing 38% of the initial amount. No statistically significant differences among animals occurred.

The loss of GAG was accompanied by a downward trend in the amount of collagen, which was measured using the hydroxyproline assay. Figure 26b shows the wet weight percentage of the collagen content for weeks 0, 1, and 4. The collagen content decreased 6% after 1 week, and 22% after 4 weeks, although neither drop was statistically significant. The collagen content of one of the animals was statistically different from two of the other animals, which might explain why the standard deviations, ranging from 18% to 35% of the mean, were high. It should be noted that the significant loss of GAG and the downward trend in collagen were not manifested in any gross morphological changes in the explants.
**Biomechanical Properties**

The biomechanical properties, as quantified with creep indentation testing methodology, exhibited significant differences. Figure 27 shows the aggregate modulus and the Poisson’s ratio values for weeks 0, 1, and 4. Figure 27a illustrates the significant decrease in the aggregate modulus from week 0 to week 4. The aggregate moduli were $622 \text{ kPa} \pm 169 \text{ kPa}$ for week 0, $517 \text{ kPa} \pm 100 \text{ kPa}$ for week 1, and $460 \text{ kPa} \pm 173 \text{ kPa}$ for week 4. The aggregate modulus dropped 16%, 11%, and 26% from weeks 0 to 1, weeks 1 to 4, and weeks 0 to 4, respectively. The inter-animal variability was significant. Two animals were significantly different from one of the animals. The inter-animal variability may also partially account for the large standard deviations seen in the permeability and Poisson’s ratio values.

The Poisson’s ratio and the permeability were not significantly different between weeks 0, 1, or 4. The Poisson’s ratio is shown in Figure 27b, which illustrates the large standard deviation at each time point. The values of the mean and standard deviation for the Poisson’s ratio were $0.13 \pm 0.10$ for week 0, $0.11 \pm 0.08$ for week 1, and $0.15 \pm 0.07$ for week 4. Permeability was not significantly different between the time points, either. While the standard deviations were large, the mean of the permeability trended down from week 0 to 1 and week 1 to 4. The values for the permeability were $9.30 \pm 6.28 \left(10^{-14} \text{ m}^4/\text{N s}\right)$ for week 0, $7.75 \pm 5.90 \left(10^{-14} \text{ m}^4/\text{N s}\right)$ for week 1, and $7.24 \pm 5.62 \left(10^{-14} \text{ m}^4/\text{N s}\right)$ for week 4.
DISCUSSION

Explant culture is a valuable tool for studying different aspects of articular cartilage and chondrocytes temporally under controlled culture conditions. As illustrated in this study, quantitative gene expression, biochemical characteristics, and biomechanical properties of articular cartilage change in significant ways as a function of time in culture. The gene expression changes demonstrate evidence of phenotypic change, and the biochemical and biomechanical properties decrease in tandem. Together, the results parallel previous studies on cartilage disuse, but changes in the explants occur slowly enough to explore interventions that prevent baseline degradation, or explore interventions that will allow further understanding of the pathophysiology of articular cartilage injury and its amelioration.

The gene expression changes of type I and type II collagen may indicate phenotypic changes, also reflected in changes in collagen content. Furthermore, these changes are accompanied by a decrease in the aggregate modulus of the extracellular matrix. Specifically, the reduction of collagen type II expression parallels the decrease in total collagen content. The level of expression of type I collagen significantly increased, but was several orders of magnitude lower than that of type II collagen. Combined with the fact that articular cartilage does not initially contain type I collagen, type I collagen expression is not likely to substantially contribute to the measured collagen content and its decrease. Type I collagen expression was significant at week 4, which is a sign of phenotypic change or de-differentiation, as type I collagen is found in wound responses of cartilage, as well as in chondrocytes that have been passaged in cell culture.²,³⁰⁶ These
changes in gene expression may be one of the responses by articular cartilage explants in reaction to insufficient mechanical stimulation for a period of time.

The decrease in SZP gene expression, in conjunction with the decrease in type II collagen expression and increase in type I collagen expression, is another sign of phenotypic change. SZP was initially found in the superficial zone of articular cartilage and thought to signify differentiation from fetal to adult cartilage, as well as to function in prevention of cell adhesion to the articular surface.\textsuperscript{307,308} In this study, SZP decreased quickly at week 1, and was almost undetectable at week 4. Thus, the biochemical and biomechanical changes the explants undergo might be more sensitively detected or predicted by a drop in SZP than in the other genes tested. A reduction in SZP expression was seen in the previously mentioned passage study, disappearing by the third passage.\textsuperscript{306} The disappearance of SZP expression in this study indicates that the chondrocytes, even in their native matrix, start to show phenotypic change, although at a slower rate than in monolayer culture.

Unlike SZP expression, the upward trend in aggregcan from week 0 to week 1 illustrates that aggregcan expression is not a sensitive marker for phenotypic change. This is accentuated also by the fact that as aggregcan expression increased, GAG content decreased. This change has also been seen in studies on chondrocytes in monolayer culture, where the middle/deep zone of cartilage showed a spike in aggregcan expression after the first passage that dropped off in successive passages.\textsuperscript{306} While several explanations may be employed as the cause for this spike in gene expression, the end
results indicate that aggrecan expression does not work well as a marker for chondrocytic phenotype or de-differentiation.

The expression levels of MMP-1 and TIMP-1 are indicative of articular cartilage breakdown. More specifically, an increase in MMP-1, a collagenase, without a relative increase in TIMP-1, a collagenase inhibitor, likely signals the beginning of cartilage degeneration. The relative abundance of TIMP-1 was constant throughout the study, but, like type I collagen, MMP-1 expression was undetectable until week 4. The MMP-1/TIMP-1 ratio is 0 for weeks 0 and 1, which suggests that MMP-1 is minimally contributing to the matrix breakdown. At week 4, the ratio increases to 10%, indicating that the expression of MMP-1 is starting to increase with respect to TIMP-1 expression and might speed collagen degradation. The increasing trend of the ratio of MMP-1/TIMP-1 and the expression of MMP-1 will likely be hastened by a variety of injury modalities, such as a mechanical impact or an aggravating bioactive agent, such as interleukin-1β.

The significant decrease in the GAG content is consistent with previous studies that measured GAG synthesis or loss into the media. However, the loss of collagen content, which was temporally constant in this study, cannot be readily compared to other studies, as tissue collagen content is usually not reported. GAG has been investigated in other cartilage explant studies that vary from 4 hrs to 15 d. Most of these experiments measure GAG that has been released into the media or use radiolabelled GAG precursors to measure synthesis. These studies found that GAG release into the media decreased up to 4-5 d with the largest amount released in the first day. The
present study illustrates longer-term effects on the GAG and collagen content of explants, showing a larger per week drop in wet weight percentage of GAG in the first week, which agrees with the aforementioned results. The current results illustrate that GAG release continues to 4 weeks, but more slowly during the latter three weeks. In contrast to the rate of GAG loss, the decrease in collagen content is consistent, resulting in a 0.8% wet weight loss the first week and an average of 0.7% wet weight loss each of the following weeks. Other studies have looked at water content as an indicator of collagen damage, \(^1\) loss of radiolabel as a measure of protein loss, \(^2\) or hydroxyproline released in the media, \(^3\) but do not usually report collagen loss of the tissue content. Our results indicate that both GAG and collagen contents of the explants decrease with time.

The biomechanical properties of the articular cartilage explants are temporally consistent with the biochemical and gene expression changes. The current results correspond well to the established direct correlation between GAG content and compressive stiffness of articular cartilage. \(^4\) The drop in the aggregate modulus mirrors the decrease in the collagen and GAG content, as well as the decrease in the gene expression of type II collagen. The significant decrease in GAG content and downward trend in collagen content probably coincided with some structural changes in the matrix. The decrease in the aggregate modulus was likely due to both a decrease in the wet weight percentage of the matrix constituents, as well as changes in how these constituents were organized. The permeability and Poisson's ratio were likely less affected by the decrease in GAG and collagen content.
The results of this investigation mirror those found in articular cartilage disuse studies. For example, in a comparison of animal models of osteoarthritis and disuse atrophy, similarities between disuse and osteoarthritis were identified. Furthermore, the ratio of proteoglycans to collagen was decreased at week 4 in both groups. The data of the present study correspond well with these results, where GAG content decreased at a faster rate than the collagen content. Other studies of articular cartilage disuse in vivo have also found a decrease in the proteoglycan content or an increase of GAG release into synovial fluid, both of which agree well with the current explant results. In another study of disuse atrophy, an increase in matrix metalloproteinases was seen, while a decrease in TIMP levels was found. The current investigation found no change in the level of TIMP-1, but did show an increase in MMP-1 expression.

Interventions that have been used in the past on articular cartilage have been either mechanical or chemical and have had positive or negative effects. Biochemical agents that have been used previously have been shown to cause matrix components to be released into the media, including fibronectin fragments and tumor necrosis factor-α. Anti-inflammatory molecules have been found to decrease enzyme activity. Degenerative mechanical interventions include impact and injurious compression, while dynamic compression and dynamic shear have been shown to be beneficial. Results from the current study found explant culture alone caused several degenerative results that might be accelerated by the aforementioned injurious phenomena or decelerated using ameliorative methods, such as growth factors or cyclic mechanical stimulation. Articular cartilage explant culture, as described in this study, could be utilized to explore these injury and treatment modalities.
This study is the first to comprehensively characterize long-term articular cartilage explant culture at multiple levels of investigation, from transcription to translation to function. Using real time RT-PCR, protein measurements, and creep indentation, it is shown that under conditions of free-swelling in culture, articular cartilage explants undergo significant changes reminiscent of early degeneration. The study results offer further insight into the interrelationship between gene expression, biochemical properties of the matrix, and matrix integrity. These baseline data allow the examination of different intervention modalities on articular cartilage using explant culture. Using these data, hypotheses can be better formulated as to what might happen given a particular mechanical or biochemical intervention, which may be considered beneficial, detrimental, or both.
ACKNOWLEDGMENTS

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**Table 8.** Target gene sequences, dyes, and quenchers.

<table>
<thead>
<tr>
<th>Gene (accession number, product size)</th>
<th>Forward Primer (5' – 3')</th>
<th>Reverse Primer (5' – 3')</th>
<th>Probe (5' – 3')</th>
<th>Dye Quencher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (U85042, 86bp)</td>
<td>ACCCTCAAGATTGTCAGCAA</td>
<td>ACGATGCCAAAGTGGTCA</td>
<td>CCTCCTGCACCCAACAATGCTT</td>
<td>FAM</td>
</tr>
<tr>
<td>Collagen type II (NM_174520, 69bp)</td>
<td>GCAGGAAGGTCATCTGGA</td>
<td>ATGACAACCTGGCTCCCAACACC</td>
<td>ACGGTTGGCCTCCACTTC</td>
<td>BHQ-2</td>
</tr>
<tr>
<td>Collagen type I (X02420, 97bp)</td>
<td>CATTAGGGGTCAACAATGGTC</td>
<td>TGGAGTTCCATTTTCACCAG</td>
<td>ATGGATTGAAGGACACGCTTGGT</td>
<td>Quasar 670</td>
</tr>
<tr>
<td>Aggrecan (U76615, 76bp)</td>
<td>GCTACCCCTGAACCTTCATC</td>
<td>AAGCTTCTGGGATGTCCAC</td>
<td>TGACGCGATCTCTACACAGGTA</td>
<td>BHQ-2</td>
</tr>
<tr>
<td>Superficial Zone Protein (AF056218, 77bp)</td>
<td>CACCCTCAGGTACACTACACA</td>
<td>TCACCTTAAACTTCATATTGAGGA</td>
<td>CCCGTACAGTCCCTTATCAAGACA</td>
<td>ROX</td>
</tr>
<tr>
<td>Matrix Metalloproteinase I (X74326, 82b)</td>
<td>AAAATGCTGGAGGTATGATGA</td>
<td>AATTCCCGGAAGTCTTCTG</td>
<td>TCCATGGATGCAGGTATCCACAA</td>
<td>BHQ-2</td>
</tr>
<tr>
<td>Tissue Inhibitor of Matrix Metalloproteinase I (NM174471, unknown)</td>
<td>GAGATCAAGATGACTAAGATGTTCA</td>
<td>GGTGATAGATGAACCGGATG</td>
<td>AGGTTCCAGTGCTTGAGGGATG</td>
<td>ROX</td>
</tr>
</tbody>
</table>

*All sequences are 5'-3'*
Figure 23. Relative gene expression (mean and standard deviation) of: a.) collagen type I and b.) collagen type II.

The symbol * denotes week 4 is significantly different from weeks 0 and 1 (p < 0.05).
**Figure 24.** Relative gene expression (mean and standard deviation) of: a.) aggrecan and b.) superficial zone protein.

The symbol * denotes week 4 is significantly different from week 0 (p < 0.05).
Figure 25. Relative gene expression (mean and standard deviation) of: a.) matrix metalloproteinase 1 and b.) tissue inhibitor of matrix metalloproteinase 1.
Figure 26. Mean and standard deviation of the wet weight percentage of the GAG and collagen content of the explants at 0, 1, and 4 weeks.

a.) The GAG content of the explants significantly decreased from week 0 to week 4 and week 1 to week 4 (p < 0.05). b.) The collagen content trended down from 0 to 4 weeks, but was not significant. Symbols * and † denote statistically significance (p < 0.05).
Figure 27. Biomechanical properties (mean and standard deviation) of the explants at 0, 1, and 4 weeks.

a.) The aggregate modulus of the explants at 0, 1, and 4 weeks. The symbol * denotes statistical significance between week 0 and week 4 (p < 0.05). b.) Poisson’s ratio of the explants at 0, 1, and 4 weeks.
CHAPTER 7: DESIGN, VALIDATION, AND UTILIZATION OF AN ARTICULAR CARTILAGE IMPACT INSTRUMENT*

ABSTRACT

Background. This paper describes the development and use of an instrument to mechanically impact articular cartilage and record the event using a piezoelectric accelerometer, as well as post-impact characterization of the tissue.

Method of Approach. Following design and fabrication of the instrument and data acquisition system, viscoelastic rubber was impacted for the purpose of validation. Then the instrument was used to impact bovine articular cartilage. Two levels of impact (Low: 6 cm drop height, 18.4 N tup; High: 10 cm drop height, 27.8 N tup) were chosen such that the former did not show gross damage upon inspection, while the latter showed substantial gross damage. Peak stress, time to peak stress, and impact duration were taken from data recorded by the instrument.

Results. Three cartilage biomechanical properties (aggregate modulus, Poisson’s ratio, and permeability) were acquired by creep indentation, and tissue morphology rated on a standardized scale was also determined. When subjected to the highest level of

impact, corresponding to 2.78 J, articular cartilage showed statistically significant (p < 0.05) differences in all three impact metrics and morphology. This high level of impact also resulted in a 37% decrease in the tissue’s aggregate modulus. Lower drop heights resulted in more consistent impact curves, demonstrated less standard deviation, and did not change the tissue’s biomechanical properties.

Conclusions. These results show that the device can apply repeatable, statistically different impact levels on both viscoelastic rubber and articular cartilage. With the instrument and techniques described in this study, articular cartilage can be subjected to specific levels of impact in order to study injury biomechanics of the tissue at various levels of mechanical damage.
INTRODUCTION

Articular cartilage is sensitive to mechanical loading. While a certain amount of loading is necessary for maintenance of the tissue's matrix, too much loading can cause injury.\textsuperscript{2} Instances of discrete impact loading, such as sports injuries, car crashes, and falls involve high strain rates that can damage articular cartilage, leading to post-traumatic osteoarthritis (OA), the main cause of secondary OA.\textsuperscript{177,179,181,184,203} While gross tissue damage may be apparent after the application of a massive force, smaller insults incurred over the course of a lifespan may cause subtler changes in articular cartilage, eventually leading to clinically significant damage. Further understanding of the link between impact loading and subsequent development of post-traumatic OA will permit more focused investigations of the degenerative processes, thus leading to the development of clinical interventions, either ameliorative or preventative.

Characterizing a mechanical impact is nontrivial and requires several metrics for full description. Common measurements include peak stress or load, time to peak stress, and duration.\textsuperscript{188} Characterization efforts are further complicated by changes in the energy absorption rate of the surface being impacted, which may alter the shape of the impact force-time curve. Impact mass and either drop height or velocity are also often described, as well as contact area, which is inherent in any measurement of stress. Aspden \textit{et al.}\textsuperscript{169} define impact loads as those which are not physiological. Physiological loads incurred during routine activity\textsuperscript{190} and aerobic dance\textsuperscript{192} have been measured to be up to three times body weight. Impact loads, however, have times to peak load of less than 30 msec, and stress rates of greater than 1000 MPa/s. Loads characterized by
metrics outside these parameters are classified as either injurious compression or physiological. Due to the differences in the thickness and biomechanical properties of articular cartilage among animals, species, and joints, a strict upper limit for peak stress is likely dependent on the situation as well as the strain-rate involved.

Several studies, which cover a range of research areas, have explored injurious compression and impact loading using different methods to apply the load. Measurements can be taken by applying impact loads directly to the articular cartilage surface or indirectly by striking an exterior tissue such as the anterior patella and using pressure sensitive film to characterize the peak stresses. Methods of applying a direct impact include a free flying mass, a pendulum, and a drop tower. While hydraulic materials testing machines are usually used to apply injurious compression, they are not able to apply a high enough rate of stress to deliver an impact load. A study by Ewers et al. used a drop tower with a force transducer and a linear voltage differential transformer (LVDT) to apply and measure an impact load to the patello-femoral joints of rabbits. They found that a single impact load caused cartilage softening without subchondral bone thickening. Radin et al. also performed a study using a drop tower to investigate the force-attenuating effects of synovial fluid, cartilage, and bone. They found that cancellous bone and articular cartilage have similar force-attenuation characteristics. Using a pendulum impactor and an in vivo rabbit model, Borrelli et al. illustrated that cartilage can withstand a single impact load that has a peak load up to 45% of the subchondral bone fracture load without showing structural or matrix damage after 12 months. Silyn-Roberts and Broom used a pendulum impactor to apply repeated impacts to the articular surface of a bovine femur to investigate the fracture behavior.
These studies illustrate different methods for applying impact, as well as the diversity of experiments that can be performed to explore the effects of mechanical impact.

The present study describes the design of an adaptable impact instrument and tissue preparation method to apply consistent, repeatable impacts to the surface of articular cartilage, in this case proximal bovine ulnas. All impact events were recorded using an accelerometer allowing the necessary measurements to be acquired. The instrument was validated using well-characterized, standard rubber at five impact levels. Then, two levels of mechanical impact were performed on articular cartilage, the effects of which were characterized using creep indentation testing and a morphological grading scale.

**MATERIALS AND METHODS**

*Impact Instrument Design and Components*

A drop tower impact instrument was designed and constructed from 304 stainless steel and off-the-shelf parts and electronics. The impact instrument, named the Chondropaktor, has several components (Fig. 28), including a linear bearing with a slide plate (Thompson industries, Port Washington, New York) a specimen clamp (Fig. 29), an impact tip and tip interface (Fig. 30), a base, and an air table (Kinetic Industries, Cambridge, Massachusetts). The base consists of two 0.15 cm (3/8") thick stainless steel plates bolted together with one of the plates bent at a right angle. The linear bearing consists of two cylindrical guides with frictionless bearings attached to a slide plate that
holds the impact mass. Different amounts of mass can be attached to the slide plate, which acts as the tup, or impacting mass.

The specimen clamp, shown in Figure 29, was constructed of 304-stainless steel plate, bolts, wing nuts, washers, and adjustable leveling feet. The base measures 20.3 cm² and was constructed to be able to hold two specimens at a time. The large bolts in the middle of the plate allow the rectangular strip of stainless steel plate (equipped with two non-threaded holes) to slide onto them. The rectangular strip of stainless steel has three set screws in the center to allow for the curvature of a specimen. The sample was placed on the base of the specimen clamp and firmly secured by alternately tightening the wing nuts on the large bolts and set screws. All components of the specimen clamp were designed to be autoclaved.

The impact tip and tip interface, shown in Figure 30, are custom-machined out of polished 304 stainless steel rod and plate. Different sets of impact tips and interfaces were machined to allow for an adjustable contact area. The impact tips fit snugly into a stainless steel pipe which is threaded on the outside and screws into tapped, partial-thickness holes in the plate. The tips measure 5 mm and 3 mm in diameter at their ends, which are rounded with a 0.1 mm radius to prevent damage caused by sharp edges. This study only utilizes the 5 mm diameter tips, which have a contact area of 19.63 mm². The impact tips and tip interfaces were designed to be compatible with an autoclave.
Data Acquisition and Interface

To collect data, the instrument uses a data acquisition card (National Instruments, Austin, Texas), a personal computer (Dell Computer Corporation, Austin, Texas), and an accelerometer with power supply (Endevco, San Juan Capistrano, California). The piezoelectric accelerometer is rigidly affixed to the impact tip interface. The weight of all of the components and the diameter of the impact tip are input for each impact level. The acquisition software (LabView custom VI program) calculates the mass, force, and stress based upon these input variables. Impact events are captured at 10 kHz, and all of the data are displayed graphically, along with the 30 msec interval that contains the impact. The peak acceleration and peak stress are also shown. After the impact, a MATLAB (Mathworks, Natick, MA) script is run on each impact to record the peak stress, time to peak stress, and duration. Each curve is visualized to ensure that only the impact event is included in the analysis.

Validation

To validate the instrument, viscoelastic rubber was mechanically impacted at five impact levels, three of which used a low tup weight of 18.4 N and two that used a high tup weight of 27.8 N. Ten impacts at each level were performed and the peak stress, time to peak stress, and duration of each were recorded. The two high tup weight impacts were chosen to be close to the same energy level (calculated using $E = mgh$, where $E$ is the energy, $m$ is the mass, $g$ is gravity, and $h$ is the drop height) as two of the low tup weight impacts. These validation impact levels were chosen to be near the energy and drop heights of the articular cartilage impacts, as well as demonstrate the ability to apply
a range of impact levels. The low tup weight impact energies were 0.55 J, 1.10 J, and 2.02 J, while the high tup weight impact energies were 1.03 J and 2.06 J.

**Tissue Harvest**

Bovine proximal ulnas, which have a relatively flat articular surface (Fig. 31) similar in shape to the human tibial plateau, were harvested from the elbow joint of mature heifers. The specimens were acquired within 4 h of slaughter and harvested within 24 h. After removing the surrounding tissue, the proximal ulna was cut parallel to the articular surface while the distal humerus was still attached, functioning to protect the articular surface and keep it hydrated. Next, the distal humerus was removed and the olecranon of the ulna was cut off, which resulted in a relatively flat specimen. The articular surface was covered with sterile gauze. Finally, the test sample was placed into the autoclaved specimen clamp, covered with sterile towels, and taken to the impact instrument. The specimen was kept hydrated at all times with phosphate-buffered saline (PBS) until the explant was removed.

**Articular Cartilage Impact**

The clamp with a processed specimen in place (Fig 29) was put in the impact instrument. After the site for impact was selected a small autoclaved aluminum cylinder, was used to orient the site perpendicular to the impact tip. The impact tip was then placed on the selected impact location. The impact tip interface, with attached accelerometer, was then placed on the top of the tip. The tup was raised to the specified height (6 cm for the 18.4 N tup weight and 10 cm for the 27.8 N tup weight) and
subsequently dropped onto the impact interface. For this study the former impact level will be referred to as Low and the latter as High. These levels were obtained by impacting cartilage at several different heights and tup weights to find a low impact level that did not visibly damage the cartilage and high impact level that caused grossly identifiable damage.

After impact, articular cartilage explants of 5 mm were removed steriley from the medial surface of the joint using a dermatological punch and scalpel. These explants were then washed with PBS and processed for use in either histology or biomechanical testing.

*Morphology and Histology*

Morphology was performed by grossly inspecting the surface of the cartilage after impact and after application of India ink. The India ink was diluted 1:3 and 2 ml was applied to the articular surface while PBS-soaked gauze was lightly brushed across the surface. A picture of the impact site was taken under uniform lighting conditions and a morphological scale was used to rate six specimens at each impact level (Table 9). The tissue morphology scale was a measure of the amount of damage to the cartilage with 0 being healthy and 3 being completely destroyed. The India ink staining was rated from 0 to 3 for much heterogeneity existed and how intensely the stain varied from surrounding tissue. The surface regularity scale was from 0 (smooth) to 3 (most of the area of impact being irregular or uneven). The surface indentation was scored immediately after impact, while tissue damage, surface morphology, and India ink staining heterogeneity was
scored by two independent, blinded observers. These morphology scores from the two observers were averaged before comparison of control, Low and High impact levels.

Histology was performed on impacted and control samples. The specimens were fixed in 10% phosphate-buffered formalin and immediately frozen in cryoembedding medium. The frozen specimens were then sectioned at 14 μm, and were fixed again with 10% phosphate-buffered formalin. They were stained with safranin O and fast green to observe glycosaminoglycan distribution.\textsuperscript{316}

\textit{Creep Indentation Biomechanical Testing}

After harvest, explants to be used for biomechanical testing were wrapped in gauze, soaked in phosphate-buffered saline with protease inhibitors (10 mM N-ethylmaleimide, 5 mM benzamidine, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and frozen at -20 °C until testing. They were thawed for at least 1 hr at room temperature in the same phosphate-buffered saline with protease inhibitor solution mentioned above to equilibrate. An automated creep indentation apparatus was used to determine the compressive creep and recovery behavior of the cartilage explants, including the aggregate modulus, permeability, and Poisson’s ratio.\textsuperscript{303,304}

Each sample was attached to a metal surface with a thin layer of cyanoacrylate glue and immediately submerged in phosphate-buffered saline with protease inhibitors. After being attached to the base plate and submerged, the samples were allowed to equilibrate for at least 20 min. The sample was placed under the loading shaft such that the surface was oriented perpendicular to the shaft using a small steel cylinder. The
automated creep indentation apparatus automatically loaded and unloaded the specimen to obtain the creep and recovery behavior. A tare load of approximately 0.005 N (0.5 g) followed by a test load of 0.01 N (2 g) was applied to the sample with a 0.8 mm diameter, flat-ended, rigid, porous tip while the creep behavior was recorded. The porosity of the tip was 50% with approximately 50 μm pore diameter. Tissue strain remained below 10% for these tests. The automated creep indentation apparatus yielded a curve of the creep and recovery behavior. Specimen thickness was measured using a needle probe, a force transducer and an LVDT. The needle moved downward using an adjustable electric motor while the force was recorded. When the needle contacted the tissue, a change in the force was noted, along with the needle’s vertical position. When the needle contacted the stainless steel, the slope of the force increased to a greater extent and the vertical position was again noted. The difference between these two positions provided the thickness of each sample.

To calculate the material properties from the creep curve, a finite element, non-linear optimization model was utilized.\textsuperscript{304} This method allowed the aggregate modulus, Poisson’s ratio, and permeability to be determined using the linear biphasic theory. An initial estimate of the material properties was obtained using a semi-analytical, semi-numeric biphasic model before using the finite element method.\textsuperscript{305}

\textit{Statistical Analysis}

A sample size of \( n = 10 \) was used for the impact measurements on both the viscoelastic rubber and the articular cartilage and \( n = 6 \) for biomechanics and tissue morphology. A multifactor ANOVA with repeated measures was performed on the
impact measurements, mechanical properties, and the morphology score using statistics software (JMP IN 5.1, SAS Institute Inc, Cary, NC). If significance existed ($p < 0.05$) a Tukey HSD post-hoc was performed.

RESULTS

*Impact Measurements - Viscoelastic Rubber*

The impact instrument recorded five different levels of impact on viscoelastic rubber, and the results are shown in Table 10. In each column, the measurements not connected by the same superscripted letter are statistically significant ($p < 0.05$). Two different tup weights were used to impact the viscoelastic rubber, 18.4 N and 27.8 N. The three levels of impact for the 18.4 N tup weight (drop heights of 3 cm, 6 cm, and 11 cm) were all found to have statistically significant differences in the peak stress. The standard deviations were low for these impacts, ranging from 2 to 3% of the mean value. The relationship between drop height and peak stress was linear for the low tup weight impact levels, had a slope of 0.389, and an $R^2$ value of 1.00. The high tup weight impacts (drop heights of 4 cm and 8 cm), had slightly higher standard deviations, but were still both under 7% of the mean. Nonlinear relationships were observed between peak stress and both the energy and the drop height.

The time to peak stress was significantly different between the 3 cm impact level and all other impact levels, including the high tup weight impact levels. The times to peak stress of the 4 cm and 8 cm high tup weight impact levels and the 11 cm low-tup
weight impact level were not significantly different from each other. The durations of impact of the high tup weight impacts were both statistically significant from all of the other impact levels. The low tup weight impact durations, while showing some significant differences among the levels, were all around 5 msec, while the high tup weight impacts had shorter durations.

Figure 32 illustrates sample impact traces on the viscoelastic rubber for the 3 cm and 11 cm low tup weight impacts (Fig 32a and 32b) and both of the high tup weight impacts (Fig 32c and 32d). From these traces, the general forms of the impacts can be seen. The low tup weight impacts in Figures 32a and 32b both show the same basic curve, but the 11 cm impact level tended to have a small peak superimposed on the impact curve near the beginning. The high tup weight impacts seen in Figures 32c and 32d also show the same general shape, although the 4 cm impact has a sharper peak. The time to peak stress for both of these impacts is similar.

**Impact Measurements - Articular Cartilage**

Table 11 lists the peak stress, time to peak stress, and total duration of impact for the two impact levels on articular cartilage, which are all significantly different. When compared to the ranges of viscoelastic rubber impact levels, the times to peak stress and durations are brief. In particular, the duration of the articular cartilage impact levels are shorter than the duration of the briefest impact level on viscoelastic rubber. The peak stress of the High articular cartilage impact level has a notably larger standard deviation than the impact levels on viscoelastic rubber and the Low impact level on articular cartilage.
Figures 32e and 32f illustrate sample impacts for both the Low and High level of impact on articular cartilage. The Low impact level had more variable shapes and was more likely to have multiple peaks, although the largest peak was easily apparent. The High impact level generally had only a single peak, had less variability, and was shorter in duration.

**Morphology and Histology**

Figure 33 shows samples of the morphology and histology of the mechanically impacted articular cartilage. Histology staining with safranin-O (saf-O)/fast-green stain is on the left and India ink staining of the cartilage surface is shown on the right with a white circle denoting the area of impact. The histological appearance of control cartilage (Fig. 33a) is similar to that of Low impact level cartilage (Fig. 33b) with a few notable differences. The superficial layer is less homogeneous in the Low impact sample and retains more of the fast-green counter stain than unimpacted control. The surface is also less regular and slightly flattened, although this is not apparent from the India ink gross morphology. The High impact level on articular cartilage, seen in Figure 33c, shows an area of tissue destruction, which is representative of the dark areas in the gross morphology picture next to it. While this histology section is more severely damaged than most, it does illustrate different modes of damage, including fissures, fibrillation, and gross tissue destruction.

The gross morphology score for the two different impact levels and control specimens are shown in Figure 34. The morphological scaling was from Table 9, and scores had a possible range from 0 to 12. The High impact level was significantly
different from both the Low impact level and control, and the mean score of the High impact level was about 4 times the mean score of the control. The Low impact level and control were not significantly different. The scores were $2.1 \pm 1.7$ for the control, $1.6 \pm 1.0$ for the Low impact level, and $8.5 \pm 1.0$ for the High impact level. The scores given ranged from 0 to 10, with the lowest score a high impact level specimen received being 8.

**Biomechanical Properties**

Significant differences were noted between the aggregate modulus of the High impact cartilage, and the control and Low impact tissues (Fig 35). The values of the aggregate modulus were $812 \text{kPa} \pm 153 \text{kPa}$ for the control, $820 \text{kPa} \pm 205 \text{kPa}$ for the Low impact level, and $509 \text{kPa} \pm 115 \text{kPa}$ for the High impact level. The aggregate modulus of the High impact level was 37% less than the mean of the control. The low impact level and control had similar aggregate moduli.

The Poisson’s ratio and permeability of articular cartilage were not statistically different, although the Poisson’s ratio trended higher with higher impact level. The values for the Poisson’s ratio were $0.164 \pm 0.008$ for the control, $0.171 \pm 0.012$ for Low level impact, and $0.177 \pm 0.014$ for the High impact level. The permeability was $3.50 \pm 1.77 \text{(x10}^{-14} \text{ m}^4/\text{N s})$ for the control, $6.10 \pm 2.88 \text{(x10}^{-14} \text{ m}^4/\text{N s})$ for the Low impact level, and $3.26 \pm 1.46 \text{(x10}^{-14} \text{ m}^4/\text{N s})$ for the High impact level.

**DISCUSSION**

The Chondropaktor instrument described here enabled the application of discrete levels of repeatable and quantifiable mechanical impacts to the surface of articular
cartilage to examine corresponding biomechanical and morphological alterations. While two studies have found a softening of the compressive modulus after 4.5 months using an in vivo rabbit model,\textsuperscript{155,243} we believe that this study is the first to show that a High impact level on articular cartilage immediately decreases the aggregate modulus, as measured by creep indentation, by 37\% compared to the control sample. Although the tissue did not show any difference from control after application of Low level impact, the morphological scale of the High impact increased almost 400\%. The Low level impact did not show any difference from the control when comparing the morphological scale or the biomechanical properties. The impact levels in the current study meet the definition set forth by Aspden et al\textsuperscript{169} in that they are well under the time to peak stress and are above the rate of stress application specified for impact as opposed to injurious or physiologic compression.

The impact instrument design allowed for the careful characterization of the effects of impact and of the impact event, which is shown here to depend upon not only the drop height and weight, but also on the consistency and material properties of the impact surface. Mechanical impact of viscoelastic rubber illustrated that impacts on a material with consistent properties are more repeatable than impacts on a soft tissue with inter-animal variability. The stress-time curves and associated metrics allow for characterization of different impact levels on a given material. In general, the low drop height and low energy impacts produced by the device tend to generate more consistent forces as characterized by the peak stress and shape of the force-time curve. The lower standard deviations of the peak stresses for the low tup weight viscoelastic impacts illustrate the relative uniformity of the low tup weight impact levels. The High impact
level of articular cartilage showed the largest standard deviation for peak stresses as compared to the Low level and all impact levels of viscoelastic rubber. These data illustrate that higher impact levels are more complex and variable events, and that variation in the material properties of articular cartilage leads to larger variation in peak stress. The time to peak stress decreased significantly for articular cartilage between the Low and High level and was decreasing with increased tup weight for the viscoelastic impacts. This change demonstrates that stiffer materials (articular cartilage stiffness is greater than viscoelastic rubber) and higher energy impacts decrease the time to peak stress and, consequently, increase the rate of stress application.

High level impact on articular cartilage demonstrates distinct, repeatable levels of tissue damage, as evaluated by the biomechanical properties, morphological scale, and histology. The morphology score significantly increased. The histology of the High impact level illustrated substantial damage over at least a third of the area. The damage included fissures, fibrillation, and, in some cases, displacement of portions of tissue from the matrix. The fissures and fibrillation were likely the reason that the aggregate modulus decreased 37% from control. Based on the significance of these changes, the High level of impact damage would very likely lead to post-traumatic OA.

The Low impact level on articular cartilage, however, yields results similar to the control, in terms of biomechanical properties, morphology, and histology. The morphology score for the Low cartilage impact level and control cartilage were similar, while the High level morphology score was significantly higher. The only appreciable difference between the Low level and control was the superficial zone, which was less
uniform and slightly compressed in the Low level impact. The biomechanical properties were also similar, particularly the aggregate modulus. However, the tissue may still have been damaged in other ways that would only show up acutely with gene expression or temporally with biochemical and biomechanical testing.

The Chondropaktor instrument is a valuable tool for applying impacts that cause damage similar to articular cartilage injuries that can lead to post-traumatic OA. The design provides a flexible platform to obtain further data in this area, and is easily adaptable to other experimental techniques. For example, the impact mass interface could be extended further than its present 15 cm distance from the linear bearing to allow more freedom of specimen placement. Also, perpendicular orientation of impact can be ensured despite variable geometry of either the cartilage surface or the clamping area by adjusting the height and pitch of the specimen clamp.

It is envisioned that this impact instrument will be useful in delivering impacts that replicate articular cartilage injuries that can lead to post-traumatic degenerative changes. Using the method set forth here, the sequelae of these impact events can be characterized in terms of gross morphology and biomechanical behavior of the tissue, matrix constituents and organization, dynamic homeostasis of pro- and anti-inflammatory cytokine production and responses to them, and reactive chondrocyte gene expression. The impact levels on articular cartilage used are examples of an injury level known to lead to OA (High) and an injury level thought to cause damage that is undetectable at the time, but may cause long-term cartilage degeneration (Low). The tissue response to the Low impact level was not detectable through gross morphology or biomechanical testing,
which are the only currently available methods of clinical OA assessment other than nonspecific symptoms of pain and decreased range of motion. Thus the Low level could be studied to further our understanding of the long-term consequences of impact from gene expression to tissue-level behavior. Low level impact could also be used to investigate ways to ameliorate any degenerative changes thereby preventing development of post-traumatic OA. Such investigations would lead to more sophisticated clinical assessment and treatment of the painful and ubiquitous sequelae of articular cartilage damage.
ACKNOWLEDGEMENTS

This study was supported, in part, by the U.S. Department of Transportation, National Highway Traffic Safety Administration Grant No. DTNH22-01-H-07551 and/or the Federal Highway Administration Grant No. FHWA ICRC(1) to the University of Alabama at Birmingham, Injury Control Research Center's Southern Consortium for Injury Biomechanics. The authors would like to thank Angela Lea Scott and Christopher Jordan Kicklighter for editorial expertise, Roman Natoli and Gwen Hoben for assistance with the morphology scaling, and Maurice Kasper and James Holub of Kasper's Processing Plant for assistance with tissue procurement.
Table 9. Morphological grading scale.

<table>
<thead>
<tr>
<th>A. Tissue morphology</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy, no damage</td>
<td>0</td>
</tr>
<tr>
<td>Some tissue damage</td>
<td>1</td>
</tr>
<tr>
<td>Extensive damage</td>
<td>2</td>
</tr>
<tr>
<td>Complete tissue destruction</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. India ink staining</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous</td>
<td>0</td>
</tr>
<tr>
<td>Some staining heterogeneity</td>
<td>1</td>
</tr>
<tr>
<td>Moderate increased staining</td>
<td>2</td>
</tr>
<tr>
<td>Markedly increased and irregular</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Surface regularity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth</td>
<td>0</td>
</tr>
<tr>
<td>Small area of irregular surface</td>
<td>1</td>
</tr>
<tr>
<td>Moderate area of irregular surface</td>
<td>2</td>
</tr>
<tr>
<td>Most of surface irregular</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D. Surface indentation*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No indentation</td>
<td>0</td>
</tr>
<tr>
<td>Indentation lasts longer than 30 s</td>
<td>1</td>
</tr>
<tr>
<td>Indentation lasts longer than 1 min</td>
<td>2</td>
</tr>
<tr>
<td>Indentation lasts longer than 2 min</td>
<td>3</td>
</tr>
</tbody>
</table>

| Total                     | 12 (Max) |

* - This scale was assessed at the time of impact only. The other scales were performed by two different, blinded researchers.
Table 10. Impact measurements for each of six levels of impact on viscoelastic rubber.

In each column, every value that is not connected by the same superscripted letter is significantly different (p < 0.05).

<table>
<thead>
<tr>
<th>Tup Weight N</th>
<th>Drop height cm</th>
<th>Energy J</th>
<th>Peak stress (Mean ± S.D.) MPa</th>
<th>Time to peak stress (Mean ± S.D.) msec</th>
<th>Duration (Mean ± S.D.) msec</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.4</td>
<td>3</td>
<td>0.55</td>
<td>1.46 ± 0.03&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.53 ± 0.35&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.11 ± 0.28&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.10</td>
<td>1.67 ± 0.04&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.83 ± 0.13&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.99 ± 0.17&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>2.02</td>
<td>2.03 ± 0.06&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.65 ± 0.07&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>5.41 ± 0.12&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>27.8</td>
<td>4</td>
<td>1.03</td>
<td>4.28 ± 0.28&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.60 ± 0.08&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>3.10 ± 0.40&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.06</td>
<td>5.56 ± 0.19&lt;sup&gt;E&lt;/sup&gt;</td>
<td>0.50 ± 0.07&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.00 ± 0.20&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 11. Impact measurements for each of two levels of impact on articular cartilage.

In each column, every value that is not connected by the same superscripted letter is significantly different (p < 0.05).

<table>
<thead>
<tr>
<th>Tup Weight</th>
<th>Drop height</th>
<th>Energy</th>
<th>Peak stress (Mean ± S.D.) MPa</th>
<th>Time to peak stress (Mean ± S.D.) msec</th>
<th>Duration (Mean ± S.D.) msec</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.4</td>
<td>3</td>
<td>0.55</td>
<td>1.46 ± 0.03&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.53 ± 0.35&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.11 ± 0.28&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.10</td>
<td>1.67 ± 0.04&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.83 ± 0.13&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.99 ± 0.17&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>2.02</td>
<td>2.03 ± 0.06&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.65 ± 0.07&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>5.41 ± 0.12&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>27.8</td>
<td>4</td>
<td>1.03</td>
<td>4.28 ± 0.28&lt;sup&gt;D&lt;/sup&gt;</td>
<td>0.60 ± 0.08&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>3.10 ± 0.40&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.06</td>
<td>5.56 ± 0.19&lt;sup&gt;E&lt;/sup&gt;</td>
<td>0.50 ± 0.07&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.00 ± 0.20&lt;sup&gt;D&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Figure 28. The impact instrument with an adjustable specimen clamp and a proximal bovine ulna.

The linear bearing (A) has an attached sliding plate (B) that can have variable amounts of mass attached. The base (E) consists of two pieces of plate 304 stainless steel. The specimen clamp (D) holds a bovine ulna, while the impact tip and tip interface (C) are perpendicular to the articular surface.
Figure 29. The specimen clamp with a harvested, prepared bovine ulna.

The specimen clamp has four adjustable leveling feet (A), one at each corner. A strip of stainless steel (B) slides onto two large bolts, one at each end. Three set screws (C) in the middle of the strip of stainless steel allow for variable geometry.
Figure 30. One of the impact tips a) assembled and b) disassembled to be sterilized.

The impact tip (C) is stainless steel rod that has been lathed to 5 mm (right) or 3 mm (left) in diameter. The impact interface consists of a small plate (A) that has a partial thickness, tapped-hole that a piece of threaded stainless steel pipe (B) screws into. The impact tip fits snugly into pipe.
**Figure 31.** Two views of a proximal bovine ulnar articular surface.

a) superior aspect, and b) anterior aspect. The articular cartilage surface is flat, similar to that of the proximal human tibia.
Figure 32. Sample of the force-time curve for a mechanical impact on viscoelastic cartilage and b) the articulating surface of the proximal bovine ulna.
Figure 33. Histology and morphology of articular cartilage explants
a) control, b) low impact level, and c) high impact level. The morphology pictures were
taken after application with a pipette of 2 ml of India ink that was diluted 3:1 in PBS.
The white circle indicates the 5 mm diameter area of impact.
Figure 34. Morphological rating of the articular cartilage using the scale in Table 1.

The rating was performed by two blinded researchers. * - denotes statistical significance ($p < 0.05$).
**Figure 35.** Aggregate modulus of the mechanically impacted articular cartilage.

The aggregate modulus of the mechanically impacted articular cartilage at high (27.8 N tup, 8 cm height, 2.06 J) and low (27.8 N tup, 4 cm height, 1.03 J) levels. * - denotes statistical significance (p < 0.05).
CHAPTER 8: ACUTE EFFECTS OF MECHANICAL IMPACT ON ARTICULAR CARTILAGE*

ABSTRACT

The subclinical damage caused to articular cartilage by acute impact loading can manifest years later. While a motor vehicle accident or sports injury may not lead to immediate, gross tissue damage, patients who experience this type of impact load may still develop osteoarthritis, a major degenerative condition that current clinical tools can do little to predict or prevent. By exploring the acute effects of impact on articular cartilage, this investigation aims to provide a broader picture of cartilage response, thus leading to a better understanding of pathological impact loading. Articular cartilage was subjected to two levels of impact (High, 2.8 J, 6.7 MPa, and Low, 1.1 J, 3.4 MPa), and the acute sequelae, including changes in gene expression profile, glycosaminoglycan release, creep indentation biomechanical properties, and viability were recorded at 24 hours. It was noteworthy that upon mechanical insult, the Low impact group exhibited no morphological differences with explant controls, unlike the High impact group, which was significantly four-fold larger. In contrast, the cell death for the High (18.8% ± 3.7%) and Low (9.9% ± 1.0%) impact level explants and the explant controls (1.8% ± 0.8%) were all statistically different. The High impact level decreased superficial zone protein

gene expression, and increased aggregan and matrix metalloproteinase-1 expression, while the Low impact level increased the expression of collagen I. Also, the High impact level aggregate modulus decreased significantly by 40%, while the Low impact group's modulus was unchanged. These findings support the hypothesis that high impact loads to articular cartilage result in immediate, tissue-level change, while low impact loads do not result in gross change, but rather in more subtle alterations that may mark the beginning of a degenerative cascade.
INTRODUCTION

Osteoarthritis (OA), the second most common cause of disability in the United States, affects over 6% of the U.S. population and 50% of the people over age 65. Clinically, OA is classified as either primary (idiopathic) or secondary. Most cases of secondary OA are due to trauma or injury, but a proportion of primary OA may be due to a past injury, when one considers that OA can take many years to develop. Much of the trauma that leads to OA probably occurs in workplace or automobile accidents, which number in the millions annually.

The process by which post-traumatic OA results from injuries to articular joints is not fully understood. Because these incidents occur discretely, the acute changes to cartilage tissue are thought to be significant in understanding the pathology that may eventually develop. Studies into the short-term effects of impact or compression help guide the search for therapies that may prevent a degenerative cascade that will lead to OA and disability.

Early studies of the acute effects of impact found that cartilage is much stiffer under higher strain rates, such as those seen in impact. Other studies have characterized the acute effects of different impact levels, including matrix disruption, surface fissuring and subchondral bone fracture, chondrocyte death, and decreased proteoglycan synthesis. Repo and Finley used autoradiography to show that chondrocyte death did not occur until near fracture levels of the subchondral bone, around 25 MPa. Duda et al. found that articular chondrocyte...
death occurred at lower stresses, 3 MPa and lower, and that increasing energy of impact correlated to increasing cell death, especially in the superficial zone. The finding that chondrocytes died at low impact levels despite no visible damage underscores the fact that current clinical and arthroscopic methods are not always able to assess early degenerative processes. Another study found sub-impact loading of bovine articular cartilage at 3.5 and 14 MPa caused cell death in the superficial zone. This study also found that glycosaminoglycan (GAG) release from the matrix increased with increasing strain rates. DiMicco et al. looked at GAG release in the 72 h following impact and found the largest amount occurred over the first 24 h. D'Lima et al. used static compression of 14 MPa for 500 ms on human articular cartilage explants and found increased GAG release as compared to control. They also found evidence of apoptosis in one-third of the articular chondrocytes. One study that examined the gene expression of MIG-6, a transduction factor, after cyclical injurious compression, found that this factor increases four-fold after impact.

More information on the response of the biomechanical integrity of the articular cartilage matrix and gene expression would be useful to understand and combat the acute negative consequences of impact loading. Profiles of multiple genes after mechanical impact would also increase the knowledge of acute effects. By exploring the acute effects of impact on a number of tissue properties, including gene expression, GAG release, biomechanics, and histology and viability, this investigation hopes to provide a broader picture of acute mechanical impact, leading to an increased capability to predict and prevent the long term effects of pathological impact loading. In this study, articular cartilage was subjected to two levels of impact (Low, 1.1 J, and High, 2.8 J) in order to
identify and characterize acute changes to the tissue; “acute” here is defined as 24 h. Following gross morphology examination, the release of GAGs into the media was measured over 24 h. The creep indentation biomechanical properties and gene expression of the baseline, explant control, Low, and High impact samples were also assayed. Further, gene expression abundance was measured for collagen I (Col1), collagen II (Col2), aggrecan (AGC), superficial zone protein (SZP), matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1). Based on our pilot studies, we hypothesized that High impact would result in immediate, tissue-level change, while Low impact would not result in gross change, but rather lead to more subtle alterations perhaps marking the beginning of degenerative osteoarthritis.

**MATERIALS AND METHODS**

*Tissue Harvest*

Following sterile procedures, proximal bovine ulnas were harvested from the elbow joint of mature steers. Elbows were obtained from a local abattoir within 4 h of slaughter and harvested within 18 h. The specimen was cut parallel to the articular surface (leaving 1 cm of bone) using a reciprocating saw (Ryobi, Hiroshima, Japan) and a sterile saw blade. The exposed articular surface was covered with sterile gauze and kept hydrated at all times with phosphate-buffered saline (PBS). Finally, the test sample was placed into an autoclaved specimen clamp, covered with sterile towels, and taken to the impact instrument. Aseptic procedures were followed throughout the experiment.
Articular Cartilage Impact

The specimen clamp with the test sample in place was positioned in the impact instrument (Fig. 36). The impact instrument, named the Chondropaktor, has several components, including a linear bearing with a slide plate (Thompson Industries, Port Washington, NY), a stainless steel custom-machined specimen clamp, an impact tip and tip interface, a base, and a vibration-damping air table (Kinetic Industries, Cambridge, MA). After the site was selected for impact, a small cylinder was used to orient the surface perpendicular to the impact tip. An impact tip, 5 mm in diameter, was then placed on the selected location. The impact tip interface, with attached accelerometer (Endevco, San Juan Capistrano, CA), was then placed on the top of the tip. The impacting mass (tup) was raised to the specified height (6 cm for the 18.4 N tup weight and 10 cm for the 27.8 N tup weight) and subsequently dropped onto the impact interface. For this study the former impact level will be referred to as Low and the latter as High. These impact levels were selected by impacting cartilage at several different heights and tup weights to find a low impact level that did not visibly damage the cartilage and a high impact level that caused grossly identifiable damage.

Explant Processing

After impact, articular cartilage explants of 8 mm diameter, with the impact area in the center, were removed from the medial surface of the joint using a dermatological punch and scalpel. They were then washed twice with PBS, and placed into 12-well plates for 24 h. The baseline explants were processed for use in either histology or biomechanical testing. Explants were cultured for 24 h in 3 ml of media that contained
Dulbecco's Modified Eagle Medium (DMEM) with Glutamax™ (Invitrogen, NY) supplemented with 100 units/ml Penicillin (Biowhittaker, MD), 100 µg/ml Streptomycin (Biowhittaker), 50 µg/ml Gentamycin (Invitrogen), 50 µg/ml Kanamycin (Sigma, MO), 10% Fetal Bovine Serum (FBS) (Gemini Bioproducts, CA), 2.5 mcg/ml Fungizone (Biowhittaker), 0.1 mM non-essential amino acids (Invitrogen), and 50 mg/L ascorbic acid. The explants remained in culture for 24 h and were then processed, as follows: For histology and morphology, the entire explant was utilized. For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and creep indentation, the impacted area of the explant (5mm diameter) was removed, leaving a 5 mm explant and an outer ring. Then, a 3 mm diameter specimen was taken from the 5 mm explant, leaving a 3 mm diameter explant plus an inner and outer ring. The inner ring, which contained the impact zone, and the outer ring, containing tissue adjacent to the impact zone, were both used for qRT-PCR. The 3mm explant for biomechanical testing was frozen in normal saline with protease inhibitors (10 mM N-ethylmaleimide, 5 mM benzamidine, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (Sigma)) at -20 °C until testing. The two rings for qRT-PCR were stored at -20° C in RNALater (Ambion, TX) until the RNA was reverse-transcribed to DNA. Baseline samples were not cultured or mechanically impacted.

*Morphology and Histology*

Morphology was performed on the surface of India ink stained articular cartilage using a standardized scale for explant control, Low, and High impact levels. The scale was the sum of four metrics that were scored from 0 to 3, including surface indentation,
tissue damage, surface morphology, and India ink staining heterogeneity. The higher the score, the more damage the cartilage had grossly sustained. Safranin-O, fast-green staining was performed on 12 μm thick cryosections of the explants, while a Live/Dead® viability stain (Molecular Probes, Eugene, OR), was performed on 0.5mm slices of explants taken directly out of culture. The viability stain was performed on explants in their wells by cutting them into 0.5 mm sections with a scalpel and adding 0.5 μl Calcein-AM and 2 μl Ethidium homodimer-1 per ml of complete media. The samples were then incubated at 37°C for 20 min. Viability pictures were taken using an epi-fluorescence microscope (Zeiss, NY).

**RNA Isolation and qRT-PCR**

RNA isolation was performed for the baseline and explant control samples simultaneously. RNA was isolated for the following six conditions: baseline and explant control, Low impact within the impact zone (Low-in), Low impact adjacent to the impact zone (Low-out), High impact within the impact zone (High-in) and high impact adjacent the impact zone (High-out). Chondrocytes were homogenized in TriZol (Invitrogen) using a Polytron homogenizer (Kinematica, Switzerland). The procedure employed for RNA isolation was from the manufacturer. Total RNA isolated for each sample was measured using a spectrophotometer (Nanodrop, DE). RNA sample sizes were normalized by using 500 ng of RNA in the reverse transcriptase (RT) reaction, and all samples were reverse-transcribed to DNA at the same time. The RT reaction consisted of 1mM dNTPs, 1mM random hexamers, RNAsen Block and StrataScript RT enzyme (Stratagene, Inc., CA) incubated at 42°C for 60 min, followed by incubation at 90°C for 5
min. qRT-PCR was performed on the cDNA from each sample for Col1, Col2, AGC, SZP, MMP-1, and TIMP-1. Sequences for the primers and probes of Col1, Col2, AGC, and SZP were developed by our lab previously.\textsuperscript{320} Sequences for the primers and probes of TIMP-1 and MMP-1 are listed in Table 12. Multiplex Master Mix (Qiagen, CA) and corresponding probes and primers were used for the qRT-PCR reaction. qRT-PCR was performed using a Rotor-gene (Corbett Research, Australia) and consisted of a 15-min denaturing step followed by 45 temperature cycles. Further, each temperature cycle consisted of 15 s at 90°C followed by 30 s at 60°C. Fluorescence was measured at the end of each 30 s cycle.

The amount of RNA used for each RT reaction was equal, thus making it possible to compare gene expression quantitatively using abundance. Abundance levels of the gene of interest ($A_{GOI}$) were calculated from the take-off cycles for the gene of interest ($C_i$) using the following equation:

$$A_{GOI} = \frac{1}{(1 + E_{GOI})^{C_i}} \quad (2)$$

where $E_{GOI}$ is the efficiency of the gene of interest calculated from the standard curve of each qRT-PCR reaction. Abundance values of the gene of interest quantify the expression level.

\textit{GAG Release}

Following 24 h of incubation, the media were removed from all wells and stored separately at –20°C. After thawing the media in a water bath each sample was mixed
thoroughly before removing 25 μl for testing. GAG content was tested using a 1,9-dimethyl methylene blue (DMMB) colorimetric assay (Blyscan kit, Accurate Chemical and Scientific Corp., NY).

Creep Indentation Biomechanical Testing

Before each creep indentation test, an explant was thawed for 1 h at room temperature in the normal saline with protease inhibitor solution. An automated creep indentation apparatus was used to determine the compressive creep and recovery behavior of the cartilage explants, including the aggregate modulus, permeability, and Poisson’s ratio.\(^{303,304}\)

An explant was attached to a flat stainless steel surface using a thin layer of cyanoacrylate glue and immediately submerged in normal saline with protease inhibitors. Samples were allowed to equilibrate for 20 min in the solution. Using a small steel cylinder, the explant was positioned under the loading shaft such that the surface was oriented perpendicular to the shaft. A tare load of approximately 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, porous-tipped indenter. The porosity of the tip was 50% with an approximately 50 μm pore diameter. The creep indentation apparatus automatically loaded and unloaded the specimen and recorded the creep and recovery behavior. The creep and recovery curves were stored for later analysis. Final tissue strain was under 10% for these tests.
Specimen thickness was measured using a needle probe technique, as reported previously.\textsuperscript{321} To calculate the material properties from the creep curve, a semi-analytical, semi-numeric biphasic model was used.\textsuperscript{305} This methodology allows the aggregate modulus, Poisson’s ratio, and permeability to be determined using the linear biphasic theory.

**Statistical Analysis**

The impact measurement levels, Low and High, had a sample size of $n = 8$ each and were compared with a Student’s t-test. A sample size of $n = 5$ was used for viability data via the Live/Dead® Stain. For gene expression, GAG release, and creep indentation biomechanics, a sample size of $n = 5$ was used based on a power analysis of previous work ($\alpha = 0.05$ and $\beta = 0.2$). Samples were taken from five different animals. Statistical software (StatView, Abaqus Concepts, CA) was used to perform a multifactor ANOVA with repeated measures on the gene expression results. If significance (measured at a level of $p < 0.05$) was found, a Student-Newman-Keuls post-hoc test was performed.

**RESULTS**

**Impact Measurements**

The impact instrument recorded two different levels of impact. The measurements describing the Low and High impact levels are listed in Table 13. In each column, the measurements not connected by the same superscripted letter are statistically
significant. The three impact measurements, peak stress, time to peak stress, and duration all showed statistically significant differences. The peak stress of the High impact level had a higher standard deviation (2.1 MPa) than the Low impact level standard deviation (0.8 MPa).

*Morphology, Histology, and Viability*

Figure 37 shows histology, as well as viability staining for the explant control, Low, and High impact samples. The histology pictures (Fig. 37a) show that the explant control looked healthy, while the High impact sample had noticeable fissures and fibrillation. Other high impact samples had surface rupture that penetrated into the middle/deep zone of the cartilage. The histology of the Low impact samples did not show any obvious differences from the explant control, except a slight thinning or irregularity of the superficial zone visible only with histology. It should be noted that gross morphology scores of the explant control and Low impact level were not statistically significant and were 2.1 ± 1.7, and 1.6 ± 1.0, respectively. The High level impact was statistically different from both the explant control and the Low impact level with a score of 8.5 ± 1.0. For the viability staining (Fig. 37b), green indicates live cells and red indicates dead cells. The red fluorescent dye (Ethidium homodimer-1) was bound to the DNA of cells without an intact membrane, while the green fluorescent dye (Calcein-AM) was activated in the cytoplasm of live cells. Explant control sample viability staining illustrated very little cell death. The Low impact viability sample demonstrated cell death mostly concentrated in the superficial layer with fewer dead cells in the middle/deep layer. The high impact sample had obvious gross damage, as well as
much more cell death clustered in the upper third of the explant. Cell death was also apparent in the deeper parts of the explant. The percentage of dead cells in the explants for the different conditions is statistically significant (Fig. 38). The High impact level had 1.9 times the amount of cell death as the Low and about one order of magnitude more than the explant control, while the Low impact level contained 5.5 times more cell death than the explant control.

**Gene Expression**

Gene expression results are shown in Figures 39, 40, and 41. Figure 39 illustrates the gene expression for SZP and AGC. High impact level had a significantly higher abundance of SZP outside of the impact zone than inside. Low impact level showed the same trend, though not significantly. A general trend of increasing AGC abundance can be seen in Figure 39b from explant control to Low to High, though it was not significant. AGC abundance of the High impact level outside the impact zone was significantly increased compared to the baseline and explant control, as well as the Low impact level inside the impact zone.

Collagen II abundance was decreased for all conditions at 24 h compared to baseline (Fig. 40a). However, collagen I expression was significantly higher for the Low impact conditions, both inside and outside the impact zone (Fig. 40b). For collagen I expression, the High impact outside condition was greater than the High impact inside condition by an order of magnitude, but both conditions are much less than for Low impact. Significantly, the Low impact conditions are two orders of magnitude greater than the explant control, and one order of magnitude greater than the baseline.
The MMP-1 abundance is shown in Figure 41. Inside the High impact zone gene expression was significantly higher than all other conditions except outside the High impact zone and inside the Low impact zone. Inside the high impact zone MMP-1 expression is more than six times greater than the explant control, while the baseline did not show any MMP-1 expression. The baseline and explant control both have the smallest mean abundance for MMP-1 expression, less than the Low impact both inside and outside of the impact zone. No significant differences were seen among TIMP-1 gene expression abundance.

**GAG Release**

GAG release into the media for the Low and High impact level was statistically significant from the explant control though not significantly different from each other (Fig. 42). Standard deviation of the explant control sample was smaller than either the High or the Low condition. High and Low impact levels of GAG released into the media were 1.9 and 2.1 times the mean value of the explant control, respectively.

**Biomechanics**

Figure 43 shows the aggregate moduli of the baseline, and explant control, Low, and High impact levels. The aggregate modulus for the High impact level is statistically different from all other conditions and is about 60% of the value of baseline or explant control. The values for the aggregate modulus are 701 kPa ± 98 kPa for the baseline, 654 kPa ± 128 kPa for the explant control, 560 kPa ± 156 kPa for the Low impact level, and 402 kPa ± 32 kPa for the High impact level. The Poisson’s ratio values were similar
among conditions. They were $0.165 \pm 0.010$ for the baseline, $0.163 \pm 0.011$ for the explant control, $0.167 \pm 0.014$ for Low level impact, and $0.163 \pm 0.005$ for the High impact level. The permeability of the High impact was more than twice the permeability of the explant control, and the Low impact permeability was 1.9 times the explant control, though neither significant. The permeability was $3.16 \pm 2.46 \times 10^{-14}$ m$^4$/N s for the baseline, $2.40 \pm 0.86 \times 10^{-14}$ m$^4$/N s for the explant control, $4.57 \pm 2.76 \times 10^{-14}$ m$^4$/N s) for the Low impact level, and $5.03 \pm 2.32 \times 10^{-14}$ m$^4$/N s for the High impact level.

**DISCUSSION**

This study characterized the acute effects of two different levels of impact on cartilage at 24 h for the first time using a broad analysis of gene expression, histology and viability, biochemistry, and creep indentation biomechanics. The hypothesis was that high impact would immediately demonstrate gross morphological change, while low impact would not show immediate change, but would show evidence indicative of degradation. As expected, the High level of impact resulted in acute gross damage of articular cartilage, both in terms of fissures and surface disruption, as well as biomechanical integrity, which decreased by 40%. In contrast, Low level impact cartilage was indistinguishable from the explant control in terms of gross morphology, though histology showed slight variation in the superficial zone. Additionally, Low impact had no effect on mechanical properties. Yet, while morphologically and biomechanically equivalent, the Low impact tissue, the baseline, and the explant control showed several notable differences. For instance, viability staining of Low impact cartilage demonstrated a five-fold increase in cell death over explant control. High
impact demonstrated a two-fold increase in cell death over the Low impact tissue. GAG release for the Low impact group was more than double that of the explant control. Additionally, Low impact gene expression showed upregulation of collagen type I compared to explant control, a finding which signifies phenotypic change. The gene expression profile of High impact cartilage was indicative of the initiation of ECM breakdown with MMP-1 increasing. While the Low impact level did not show gross damage or biomechanical differences, the GAG release, viability staining, and gene expression profile all imply that a degenerative process is beginning to take hold.

The High impact gene expression profile correlates to visual evidence of cartilage damage, while the Low impact profile showed changes in absence of gross change. Tissue impacted at Low levels reacted by upregulating a dedifferentiation marker, collagen type I, while High impact showed markers of immediate ECM breakdown. The inside and outside Low impact collagen type I abundance were orders of magnitude greater than explant control. This may signify the beginning of an early attempt to heal with more fibrous tissue, one hallmark of an ultimately unsuccessful articular cartilage healing response. The High impact zone gene expression includes significantly increased levels of MMP-1 compared to explant control, indicating that ECM breakdown enzymes are upregulated quickly. Additionally, the MMP-1 expression of the area adjacent to the High impact zone is increased five-fold over the explant control. SZP, a protein thought to be involved in reducing friction, was decreased in the High impact zone, and this decrease has also been shown to be a sign of early phenotypic change. While not significant, this same trend with SZP was also seen in the Low impact. AGC abundance showed an increasing trend from explant control to Low to High, and the
outside zone of the High impact was significantly increased. AGC has been shown to increase initially in the process of phenotypic change, indicating that High impact levels affect adjacent tissue.

High and Low impact both released more than twice as much GAG than the explant control. Jeffrey et al.\textsuperscript{158} found that radiolabelled GAG precursors were lost in greater amounts with greater impact levels, but this was over a two week period. Torzilli et al.\textsuperscript{161} mechanically impacted bovine cartilage from 0.5 MPa to 65 MPa and found that proteoglycan synthesis decreased after 24 h. The current study found increased GAG release that was not correlated with impact level. Also, the increased aggregcan expression found in this study may or may not translate into increased GAG because it may take longer than 24 h to become apparent at the protein level. DiMicco et al.\textsuperscript{317} tested for GAG release after injurious compression of immature bovine cartilage and found that GAG release is highest in the first 24 h, but falls off to control levels afterward. This study mirrors that finding with impact as the injury rather than compression.

Biomechanical properties of the High impact samples were 60\% of the baseline and explant control, while Low impact and explant control conditions were not significantly different. Either the Low impact samples had not sustained enough damage or the degeneration cascade was not fast enough to be apparent in the aggregate modulus in the first 24 h. Thus, biomechanical testing of articular cartilage was unable to differentiate explant control cartilage from the Low impact acutely.
Histological evaluation illustrated no differences between Low impact and explant control, while the viability stain illustrates significantly increased cell death in the Low impact. High impact is shown to contain gross damage and substantially more cell death than the Low impact condition. Repo and Finley\textsuperscript{144} determined that increasing impact of human articular cartilage resulted in increasing cell death using tritiated proline. Lewis et al.\textsuperscript{256} used a viability stain to show that mechanically impacted articular chondrocyte cell death occurs around matrix disruptions, as well as preferentially in the superficial zone. The results of the current study reflect both of these findings. The High impact viability stain shows the cell death clustered both around matrix disruptions and in the superficial zone. The Low impact showed cell death to be almost solely in the superficial zone, and showed five times more cell death.

The changes to tissue after a high level impact are readily apparent. However, with identical morphology and biomechanics, tissue impacted at low levels appears equivalent from a clinical examination standpoint to explant control samples. Neither a physical exam nor arthroscopy would detect the changes that occur acutely during such injuries. Yet the gene expression profile, viability staining, and GAG release of the Low impact data suggest the beginning of a degenerative cascade. Taken to longer time points, tissue subjected to low impact would likely start to lose biomechanical integrity and show some histological alteration. In addition, cell death may increase and the gene expression profile may shift more toward ECM degeneration. High impact will likely fare even worse, thus, in the long term we can see a role for both Low and High impact injuries in the pathological process of OA, though post-traumatic OA prevention is initially likely to be more fruitful investigating Low impact subclinical injuries.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Gene* (accession number, product size)</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Dye</th>
<th>Quencher</th>
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<tr>
<td>Matrix</td>
<td>CAATGCTGGAGGTATGATGA</td>
<td></td>
<td></td>
<td>Quasar 670</td>
</tr>
<tr>
<td>Metalloproteinase I (X74326, 82b)</td>
<td>AATCCGGGAAGTCTTCTG</td>
<td></td>
<td></td>
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<tr>
<td>Tissue Inhibitor of Matrix</td>
<td>TCCATGGATGCAGGTTATCCAAA</td>
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<td>Metalloproteinase I (NM174471, unknown)</td>
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<td></td>
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<td></td>
<td>AGGGTTCAGTGCTTGAGGGATG</td>
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</tbody>
</table>

*All sequences are 5’-3’*
Table 13. Impact measurements for the Low and High levels.

<table>
<thead>
<tr>
<th>Impact Level</th>
<th>Tup Weight</th>
<th>Drop height</th>
<th>Energy</th>
<th>Peak stress (Mean ± S.D.)</th>
<th>Time to peak stress (Mean ± S.D.)</th>
<th>Duration (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>18.4</td>
<td>6</td>
<td>1.1</td>
<td>3.4± 0.8^A</td>
<td>0.7± 0.3^A</td>
<td>1.7± 0.5^A</td>
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<tr>
<td>High</td>
<td>27.8</td>
<td>10</td>
<td>2.78</td>
<td>6.7± 2.1^B</td>
<td>0.4± 0.1^B</td>
<td>0.9± 0.3^B</td>
</tr>
</tbody>
</table>

For each column, the numbers with different superscripted letters, 'A' and 'B', are statistically significant (p < 0.05).
**Figure 36.** The impact instrument.

The impact instrument is shown consisting of a linear bearing, impact weight (tup), specimen clamp, impact tip, and impact tip interface. The instrument records peak stress, time to peak stress, and duration of impact.
**Figure 37.** Histology and viability of mechanically impacted articular cartilage.

a) histology and b) viability staining are shown for the explant control (left), low impact (center), and high impact (right) conditions. All images were taken at 100X.
Figure 38. Percent of live cells for each condition.

The percent of live cells for each condition show that the low and high impact are significantly different from each other and the explant control ($p < 0.05$, n=5). Significance on the graphs is denoted by the letters (A - C) above each bar; the values are significantly different from one another when the letters above the condition are different.
Figure 39. Gene expression is illustrated for a) SZP and b) AGC, expressed as mean abundance and standard deviation.

Significance on both graphs is denoted by the letters (A and B) above each bar; the values are significantly different from one another when the letters above the condition are different. (Baseline cartilage has not been cultured or mechanically impacted; Low, in – Low impact, inside impact zone; Low, out – Low impact, outside impact zone; High, in – High impact, inside impact zone; High, out – High impact, outside impact zone).

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**SZP Abundance**

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**AGC Abundance**
**Figure 40.** Gene expression is illustrated for a) collagen type I and b) Collagen type II, expressed as mean abundance and standard deviation.

Significance on both graphs is denoted by the letters (A - C) above each bar; the values are significantly different from one another when the letters above the condition are different.
Figure 41. Gene expression is illustrated for a) MMP-1 expressed as mean abundance and standard deviation.

Significance on both graphs is denoted by the letters (A and B) above each bar; the values are significantly different from one another when the letters above the condition are different.
Figure 42. GAG release for the Low and High impact conditions.

The GAG release for the Low and High impact conditions are both statistically (p < 0.05) increased compared to the explant control condition. The values are statistically different from one another when the letters above the condition are different.

24 hr Media GAG Release

<table>
<thead>
<tr>
<th>Condition</th>
<th>GAG (ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Explant Control</td>
<td>66±17A</td>
</tr>
<tr>
<td>Low</td>
<td>140±53B</td>
</tr>
<tr>
<td>High</td>
<td>126±40B</td>
</tr>
</tbody>
</table>
Figure 43. The biomechanical properties of the baseline, explant control, Low, and High impact conditions.
CHAPTER 9: TEMPORAL EFFECTS OF MECHANICAL IMPACT ON ARTICULAR CARTILAGE*

ABSTRACT

Pathologic impact loading of articular cartilage can lead to post-traumatic osteoarthritis years after an initial injury even if the immediate damage is not grossly apparent. The goal of this study is to comprehensively characterize the temporal effects of two different levels of impact loading on articular cartilage (Low, 1.1 J at 3.7 MPa and High, 2.8 J at 6.5 MPa) in order to better understand how clinically silent impact injuries can lead to degeneration of the tissue. After mechanical impact, bovine articular cartilage explants were cultured for 1 or 4 weeks. Cell viability, morphology, gene expression profiles, extracellular matrix biochemistry, and creep indentation biomechanical properties were assayed. Morphology scores after impact found no difference between Low and baseline, but were significantly increased for High impact. High and Low impacted tissues had significantly more GAG released to the media during week 1 compared to later weeks and incurred more cell death than culture controls at weeks 1 and 4. The wet weight percent GAG of the High (3.5 ± 1.6%) and Low (3.9 ± 1.1%)

impact areas was significantly less than baseline (5.5 ± 0.6%) cartilage at week 4. Additionally, the impacted areas demonstrated suppressed aggrecan expression at week 1. The aggregate modulus of the Low and High impact explants was significantly decreased compared to the culture control at both weeks 1 and 4. These results support the hypothesis that impact loads that do not cause gross damage still result in significant degeneration in articular cartilage. The degenerative cascade brought about by impact that does not result in clinically identifiable damage may help explain many cases of primary osteoarthritis. The results from this study may help identify strategies to decrease the prevalence of osteoarthritis.
INTRODUCTION

Osteoarthritis (OA) causes significant pain and suffering to individual patients, and the economic burden of this disease to society\textsuperscript{172} is great. It is estimated that one in six Americans have OA, making it one of the leading cause of disability in the United States.\textsuperscript{315} OA is clinically divided into two types: primary (cases with no known cause) and secondary. It is believed that a majority of secondary OA is attributable to traumatic joint injury, known as post-traumatic OA, that may have occurred years previously.\textsuperscript{164,322,323} Impact injuries, such as those that happen during motor vehicle collisions and falls have been implicated in the development of post-traumatic OA, though the precise pathophysiology is not fully understood.\textsuperscript{143,315}

Impact loading of articular cartilage leads to post-traumatic OA through its effects on the cells and extracellular (ECM) matrix of the tissue. In such cases, cartilage function is believed to deteriorate as a result of chondrocyte death,\textsuperscript{324} changes in the biochemical characteristics of the ECM,\textsuperscript{325} and overall weakening of the tissue’s biomechanical properties.\textsuperscript{243,326,327} Several studies have shown that the level of impact or compression correlates with increasing cell death and degradative changes in the ECM.\textsuperscript{157,162} Another study has shown that injurious compression correlates with detrimental changes in the biomechanical properties of cartilage.\textsuperscript{327} While these adverse changes have been noted, the majority of explant studies have looked at the acute effects of impact loading.
With regard to the temporal effects of impact loading, several explant studies have investigated the degree of cell death following impact. Lewis et al.\textsuperscript{256} impacted intact mature bovine patella at 53 MPa over 250 ms and cultured the specimens for 5 days. The study found increased cell death in areas adjacent to impact induced surface disruptions. In another study, Chen et al.\textsuperscript{247} cyclically loaded canine cartilage explants at 0.3 Hz to 5 MPa at a rate of 60 MPa/s. Explants were cultured out to 6 days and the study found apoptosis resulted in 68% cell death in one group. Using the same tissue source and cyclic loading protocol as Chen et al., Levin et al.\textsuperscript{249} cultured explants for 3 weeks to investigate whether cell signaling from inside the impact area affected adjacent tissue. They found that cell death not only increased with time in culture, but was highest inside the impact area and spread to adjacent tissue. These studies illustrate the damaging effects impact or compression can have on articular cartilage.

Studies investigating the biochemical content of the ECM have found that impact causes glycosaminoglycans (GAGs) to be released into the culture media and chondrocyte proteoglycan synthesis to decrease. A drop tower was used in one study to apply several levels of impact to bovine articular cartilage explants to assess matrix loss and synthesis.\textsuperscript{158} During two weeks of culture, results indicated that both collagen and GAG were lost to the culture media, and an initial decrease in ECM synthesis recovered by day 3. Quinn et al.\textsuperscript{250} looked at sub-impact loading of bovine osteochondral explants. They too found that GAG was released into the media during four days of culture and that proteoglycan synthesis decreased with increasing peak stress. DiMicco et al.\textsuperscript{317} subjected bovine cartilage explants to injurious compression (50% strain, 100%/s) and found that GAG release was highest during the first 4 hours post-injury. However,
between 24 and 72 hours the amount of GAG released became similar to control. In the same study, they found that a matrix metalloproteinase (MMP) inhibitor was effective in decreasing GAG release but only after the first 24 hours. The authors concluded that early GAG release was the result of mechanical damage and not the result of MMP enzymatic degradation.

Another approach used to investigate the temporal sequelae of impact loading is the use of in vivo models. Compared to explant studies, these investigations offer less environmental control, although the conditions more closely match native physiology. Thompson et al.\textsuperscript{232} used a drop tower to apply a 2170 N load over 2 ms to rigidly held canine patellofemoral joints and studied the effects using histology out to 24 weeks. At six months, the histological findings represented OA-like changes consisting of decreased GAG staining, new bone formation in the subchondral region, and surface fibrillations. In a 36 month study by Ewers et al.,\textsuperscript{155} a single impact was applied to the patellofemoral joint of rabbits. Histological analysis showed fissures in the impacted cartilage compared to contralateral controls as well as a time dependent thickening of subchondral bone. Using a transversely isotropic poroelastic model, the authors also found that the compressive modulus of the impacted tissue decreased and the permeability increased.

The above studies underscore the detrimental long-term effects that impact loading can produce. To further understand, and eventually prevent post-traumatic OA, better characterization of the biomechanical behavior of articular cartilage post-impact loading is needed. In particular, information in regard to impact loading regimes that do not cause gross identifiable damage would be helpful in understanding the possible
causes of 'idiopathic' or primary OA. To date, there is little work investigating gene expression following impact loads. The characterization of temporal gene expression profiles may aid in discovering clinical tools to prevent or manage post-traumatic OA. In this study we investigate the comprehensive changes that take place in articular cartilage at 1 and 4 weeks following impact loading at two levels. Our approach involves quantifying cell death, changes in gene expression, alterations in the biochemical composition of the ECM, and changes to the tissue’s biomechanical properties. We hypothesize that low levels of impact that do not result in immediate tissue changes will begin a degeneration cascade that will appear and worsen in culture. In contrast, we expect that high levels of impact will result in immediate changes that will persist and become exacerbated. Understanding the temporal effects of impact loading will ultimately help to elucidate the processes that occur in post-traumatic OA and point to future avenues of inquiry to reverse or prevent this disease.

MATERIALS AND METHODS

Tissue Harvest and Sample Preparation

Bovine heifer elbows were acquired from a local abattoir within 4 hours of slaughter and harvested within 18 hours. The harvested specimens consisted of the articular cartilage surface accompanied by approximately 1cm of underlying bone from the proximal ulna. Sterile procedures were used throughout tissue harvest and impact. The distal portion of the proximal ulna was cut from the specimen parallel to the articular surface using a reciprocating saw (Ryobi, Hiroshima, Japan) and a sterile blade. Sterile
gauze was then placed over the exposed cartilage surface and the olecranon process of the ulna was removed from the specimen with the reciprocating saw. The above process resulted in a relatively flat specimen that resembles a human tibial plateau. The specimens were washed with sterile PBS and then transferred onto a sterile specimen clamp (Fig. 44).

Articular Cartilage Impact

The specimen clamp was positioned in the impact instrument (Chondropaktor) as shown in figure 44. The Chondropaktor has several components: 1) a linear bearing with a slide plate (Thompson Industries, Port Washington, New York), 2) a 5 cm diameter impact tip and tip interface, and 3) a base which sits on an air table (Kinetic Industries, Cambridge, Massachusetts). Different levels of impact are achieved by varying the drop height and tup weight (impact weight). For this study two levels of impact were employed, which will 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 impact level did not visibly damage the cartilage whereas the High impact level caused grossly identifiable damage. To ensure a perpendicular impact, a small sterile cylinder was placed on the cartilage surface and the specimen clamp was adjusted to orient the chosen site perpendicular to the impact tip. The impact tip interface and attached accelerometer (Endevco, San Juan Capistrano, California) were placed on the tip before impact, and then each impact was performed and recorded.
**Explant Processing and Culture**

After impact, 8 mm explants were removed from the cartilage surface using a dermatological punch and a #10 scalpel blade. The punch was centered over the impact site, and the scalpel was used to separate the cartilage from the underlying bone. Baseline explants were taken from each animal and processed on the same day as harvest without any impact or time in culture. For each animal the control, Low impact, 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 Glutamax™ (Invitrogen, New York) supplemented with 100 units/ml Penicillin (Biowhittaker, Maryland), 100 µg/ml Streptomycin (Biowhittaker), 50 µg/ml Gentamycin (Invitrogen), 50 µg/ml Kanamycin (Sigma, Missouri), 10% Fetal Bovine Serum (FBS) (Gemini Bioproducts, California), 2.5 µg/ml Fungizone (Biowhittaker), 0.1 mM non-essential amino acids (Invitrogen), and 50 mg/L ascorbic acid. The explants were cultured for either 1 or 4 weeks with media changes every 2-3 days. The media collected during changes were stored by both week and animal at -20°C.

After either 1 or 4 weeks in culture, the explants were processed for viability staining, quantitative reverse transcription polymerase chain reaction (qRT-PCR), biochemistry, and biomechanics assays. A 5mm punch was taken from the center of the 8mm explant yielding an outer ring (outside of the impact area). The outer ring was cut in half, with half stored in RNALater® (Ambion, Austin, TX) at -20°C until RNA isolation began. The other half of the outer ring was weighed and lyophilized for at least 48 hours. From the center of the 5mm punch, a 3mm punch was taken, wrapped in gauze
soaked with normal saline with protease inhibitors (10 mM N-ethylmaleimide, 5 mM benzamidine, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), and stored at -20°C until creep indentation biomechanical testing. The remaining inner ring (inside the impact area) was cut in half and processed in the same manner as the outer ring.

**Viability Staining and Morphology**

Cell viability staining, using the Live/Dead® assay (Molecular Probes, Eugene, Oregon), was performed on 0.5mm thick sections of explants taken directly from culture. The 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°C. Viability pictures were taken using an epi-fluorescence microscope (Zeiss, New York), and images were analyzed with ImageJ (National Institutes of Health, Maryland). Morphological scores of these levels of impact were performed using a scale from 0 to 12, with 12 being the most damage. Ratings from 0 to 3 of surface indentation, tissue damage, surface morphology, and India ink staining heterogeneity were combined to obtain the final score.

**RNA Isolation and the qRT-PCR**

RNA isolation for the baseline, 1 week, and 4 week samples was performed simultaneously. RNA was isolated from 11 different groups: baseline, weeks 1 and 4 culture controls (CC), and weeks 1 and 4 Low impact inside the impact zone (LI), Low impact outside the impact zone (LO), High impact inside the impact zone (HI), and high impact outside the impact zone (HO). Cartilage tissue was homogenized in TriZol (Invitrogen, Carlsbad, CA) using a Polytron homogenizer (Kinematica, Switzerland) and
RNA was isolated using the manufacturer's protocol. When completed, the RNA concentration of each sample was determined using a spectrophotometer (Nanodrop, Wilmington, DE). RNA sample sizes were normalized to 300 ng of RNA for use in the reverse transcriptase (RT) reaction, and all 1 and 4 week samples were reverse-transcribed to DNA at the same time. The RT reaction consisted of 1mM dNTPs, 1mM random hexamers, RNAsa Block, and Stratascript RT enzyme (Stratagene, Inc. LaJolla, CA) incubated at 42°C for 60 min, followed by incubation at 90°C for 5 min.

qRT-PCR was performed on the cDNA derived from each sample for the following genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen I (Col1), collagen 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 for Col1, Col2, AGC, and SZP have been previously developed in our lab. The primer and probe sequences for MMP-1 and TIMP-1 are shown in Table 14, and all primers and probes were used with the Multiplex Master Mix kit (Qiagen, Valencia, CA). The qRT-PCR reaction was performed using a Rotor-Gene™ (Corbett Research, Sydney, AU) and consisted of a 15 min denaturing step followed by 45 temperature cycles. Each temperature cycle consisted of 15 s at 90°C followed by 30 s at 60°C. Probe fluorescence was measured at the end of each 30 s cycle.

Abundance values for the gene of interest quantify its expression level. The amount of RNA used for each RT reaction was equal, making it 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:

\[
A_{GOI} = \frac{1}{(1 + E_{GOI})^{C_i}}
\]

where \( E_{GOI} \) is the efficiency of the gene of interest.

**Biochemical Characterization**

Biochemical assays were performed for GAG, collagen, and total DNA content. For all assays, samples were digested in 2 ml papain solution\(^{220}\) for a minimum of 18 hours or until no visible tissue remained. GAG content was tested using a 1,9-dimethylmethylene blue colorimetric assay (Blyscan\(^{TM}\) Sulfated GAG Assay kit, Accurate Chemical and Scientific Corp., Westbury, NY). The GAG assay was performed on all digested tissue samples and on all collected media samples. Collagen content was measured using a modified chloramine T and dimethylaminobenzaldehyde colorimetric assay.\(^{328}\) SIRCOL Collagen Assay (Accurate Chemical, Westbury, NY) was used as the standard. Total DNA content of each sample was also assayed (Quant-iT\(^{TM}\) PicoGreen\(^{®}\) dsDNA Assay Kit, Molecular Probes, Eugene, Oregon). Cell number for a sample was calculated assuming 7.8 pg of DNA per chondrocyte and normalized per gram of tissue.

**Creep Indentation Biomechanical Properties**

Prior to mechanical testing, the 3 mm samples were thawed for 1 hour at room temperature in normal saline with protease inhibitors. An automated creep indentation
apparatus was used to determine the compressive creep and recovery behavior of the cartilage explants.\textsuperscript{303,304}

Each sample was attached to a flat stainless steel surface with a thin layer of cyanoacrylate glue and immediately submerged in normal saline with protease inhibitors, after which it equilibrated for 20 min. The sample was then placed under the loading shaft, and the cartilage surface was aligned perpendicular to the shaft using a small steel cylinder. The creep indentation apparatus 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, porous tip. The tip porosity was 50\%, with a pore diameter of 50 μm. Specimen thickness was measured using a needle probe, a force transducer, and a linear variable differential transformer.\textsuperscript{321} To calculate the specimens material properties, a semi-analytical, semi-numeric, linear biphasic model was used.\textsuperscript{305} This method allows the aggregate modulus, Poisson’s ratio, and permeability to be determined.

\textit{Statistical Analysis}

A sample size of $n = 5$ was used for viability data via the Live/Dead® Stain and for gene expression. For GAG, collagen, DNA, and creep indentation measurements, a sample size of $n = 6$ was used. Sample sizes for gene expression and biomechanics were based upon a power analysis of previous data with $\alpha = 0.05$ and $\beta = 0.2$. A multifactor ANOVA with repeated measures (StatView, Abaqus Concepts, Berkeley, CA) was
performed on all data. If significance ($p < 0.05$) was found, a Student-Newman-Keuls post-hoc test was performed.

**RESULTS**

*Impact Measurements*

The Chondropaktor was able to consistently reproduce two distinct levels of impact. Table 15 characterizes the Low and High impact levels in terms of the following metrics: peak stress, time to peak stress, and duration of impact. Each of these measurements is significantly different when comparing the Low and High levels. The High level of impact delivers more than 2.5 times the energy of the Low impact.

*Viability and Morphology*

Figure 45 shows 100X magnification pictures of viability-stained specimens. Dead cells fluoresce red and living cells fluoresce green. Cell death increased both with time in culture and level of impact. At baseline, almost all of the chondrocytes were alive; however, when comparing baseline and week 1 and 4 culture controls, (Figs. 45a, 45b and 45c, respectively) an increasing number of red cells appear, indicating that cell death occurred over time in culture. Cell death over time in culture controls was generally limited to the superficial zone, while in the Low (Figs. 45c and 45f) and High (Figs. 45d and 45g) impact groups cell death was more widespread. Additionally, impact caused increased cell death in the deeper zones of cartilage, which was most notable for the week 4 High level (Fig. 45g), and to some extent the week 4 Low level (Fig. 45f).
Also, there is a paucity of cells in the superficial zone for the High impact level, especially at week 4. Of note, the morphology scores for baseline and Low impact explants were not significantly different from each other (2.1 ± 1.7 and 1.6 ± 1.0, respectively), but were significantly less than the High impact (8.5 ± 1.0).

Figure 46 displays the percentage of dead cells for each group. Significantly increased cell death occurred in the explants with the High impact level and increased amount of time spent in culture. At week 1, the percentage of cell death in the High impact group was significantly greater than both culture control and Low impact groups. At week 4, both Low and High impact group cell death was significantly greater than the culture control and their respective week 1 values.

_qRT-PCR_

A one-way ANOVA test on GAPDH abundance showed no significant difference among the experimental groups in this study (p = 0.78). The baseline level of Col2 was significantly higher than all week 1 and 4 conditions. No other significance was found for Col2 expression.

For Col1, AGC, SZP, TIMP-1, and MMP-1/TIMP-1, abundances were normalized by dividing the abundance of a given gene for a given group by the average abundance of that gene at baseline. Baseline and week 1 culture control (CC), LI, and LO samples showed little Col1 expression, whereas the week 1 High impact, inside and outside groups, had over 800 times the expression level compared to baseline (Fig. 47a). By week 4, all conditions, except HI, expressed significantly more Col1 than their week 1
counterparts. Further, week 4 LO and HO groups were significantly greater than the week 4 CC. Figure 47b illustrates the normalized abundance for AGC expression. The week 1 CC, LO, and HO groups expressed significantly more AGC than all other groups.

Figure 48a shows the normalized abundance of SZP for baseline and the week 1 time point. At baseline there was significantly more SZP expression than for any of the week 1 groups. TIMP-1 expression was significantly increased in the week 1 CC and HO groups and elevated 8 fold (although not significantly) in the LO group (Fig. 48b) compared to baseline. TIMP-1 expression inside the impact area was not significantly different than baseline. The same trends for week 1 SZP and TIMP-1 were seen at week 4. Figure 49 shows the ratio of MMP-1 to TIMP-1 gene expression. The ratio was significantly greater for the week 1 HI group compared to all other groups; however, by week 4 the ratio had decreased back to the level of the culture controls.

**Biochemistry**

GAG released into the media was significantly greater for the High impact level compared to both the culture control and Low impact groups at week 1 (Fig. 50a). For the High impact group, it was 1.6 and 2.0 times larger than the Low and culture control groups, respectively. This difference was not observed during later weeks. Furthermore, figures 50b and 50c show that the majority of GAG release occurred during the first week for the Low and High impact levels. The High impact group released 2.5 times more GAG during the first week than for any subsequent week. In contrast, for the culture control explants, there was not a significant difference in GAG release during week 1 compared to later weeks. For weeks 1, 2, 3, and 4 the culture controls measured 425 ±
171, 216 ± 117, 292 ± 97, and 252 ± 107 μg GAG, respectively. Figure 51 shows the GAG content of the explants for the Low and High impact groups. At week 4, the inside and outside areas for both levels of impact were significantly lower than baseline. Also, for High and Low level GAG content, the impact zone was lower than the outside zone at both weeks 1 and 4, though not significantly.

Cell number, as measured by DNA content, inside the impact area (LI and HI) was significantly decreased compared to outside the impact area (LO and HO) and CC at week 1, but not at week 4 (Fig. 52). There was a decrease in the number of cells present as time in culture increased, evidenced by each group having a lower cell count at week 4 compared to week 1. This was significant for the CC, LO, and HO groups, but not for the LI and HI groups. At baseline there were 53.5 ± 10.2 million cells per gram of tissue. The cell numbers (x10⁶) per gram of tissue for week 1 were 62.2 ± 14.4 (CC), 38.4 ± 6.4 (LI), 59.7 ± 9.1 (LO), 37.6 ± 5.1 (HI), 59.0 ± 5.2 (HO). At the 4 week time point cell numbers (x10⁶) per gram of tissue were 45.4 ± 5.4 (CC), 38.7 ± 11.4 (LI), 44.1 ± 10.0 (LO), 31.9 ± 5.4 (HI), 42.7 ± 5.4 (HO). Collagen content, measured as wet weight percent, decreased at week 1 compared to baseline for all groups. The area inside the High impact had significantly more collagen at week 4 compared to week 1. At baseline the wet weight percent of collagen was 13.8 ± 1.5. At the 1 week time point the CC measured 12.3 ± 1.4 %, LI was 12.4 ± 3.5 %, LO was 11.6 ± 1.1 %, HI, 10.8 ± 1.2 %, and HO, 11.7 ± 1.4 %. For week 4, CC, LI, LO, HI, and HO were 14.0 ± 0.4 %, 14.0 ± 1.9 %, 14.4 ± 1.8 %, 14.0 ± 1.0 %, and 14.0 ± 1.8 %, respectively.
Biomechanics

In general, cartilage stiffness decreased over time. Within week 1, the aggregate modulus of the High impact group was significantly lower than the Low impact group. Further, at weeks 1 and 4, the Low and High impact groups’ aggregate modulus was significantly lower than culture control (CC). Figure 53a shows the aggregate modulus for the experimental conditions tested. The aggregate modulus at baseline measured 673 ± 93 kPa. At week 1 the aggregate modulus of the Low impact group (501 ± 190 kPa) was significantly lower than the CC group (729 ± 234 kPa), and the High impact modulus (266 ± 68 kPa) was significantly lower than the Low impact modulus. At week 1, Low was 69% of CC and High was 53% of Low. At week 4, the aggregate modulus of the Low group (183 ± 37 kPa) was significantly lower than the control (392 ± 83 kPa), measuring 47% of its value. The stiffness of the High impact group measured 149 ± 55 kPa; however, it is noteworthy that the significant difference between the Low and High impact groups seen at week 1 disappeared by week 4. The CC and Low impact aggregate moduli decreased significantly between weeks 1 and 4. The modulus of the High impact group did not change significantly between weeks 1 and 4, although it did trend downward.

Figure 53b displays the permeability of articular cartilage for each experimental group. The permeability of the Low impacted specimens at week 1 was significantly greater than all other groups by at least 2 fold. At baseline, the permeability was 2.32 ± 1.53 (x10^{-14} m^4/N s). At week 1 the permeability of the CC, Low impact, and High impact samples were 2.62 ± 1.63 (x10^{-14} m^4/N s), 5.61 ± 2.47 (x10^{-14} m^4/N s), and 2.23 ±
1.58 (x10^{-14} m^4/N s) respectively. At week 4 the permeability of the CC, Low impact, and High impact samples were 2.40 ± 1.18 (x10^{-14} m^4/N s), 1.15 ± 0.53 (x10^{-14} m^4/N s), and 1.12 ± 0.60 (x10^{-14} m^4/N s) respectively. Poisson’s ratios were 0.158 ± 0.010, 0.164 ± 0.009, 0.160 ± 0.011, 0.162 ± 0.010, 0.161 ± 0.011, 0.173 ± 0.013, 0.183 ± 0.013 for baseline, week 1 CC, Low, and High, and week 4 CC, Low, and High, respectively.

**DISCUSSION**

The results presented here describe the temporal effects of mechanical impact on articular cartilage. They also, for the first time, investigate gene expression profiles and creep indentation biomechanics, correlated with cell viability, GAG release, and biochemistry data. The hypothesis of this study was that the High impact level, which caused gross damage, would show changes at both week 1 and 4, but worsen over time. In addition, the Low impact level, though not causing gross damage, would begin a degeneration cascade that would become apparent over time in culture.

Our results support this hypothesis, with data from the various assays combining to demonstrate that High and Low impact injuries to articular cartilage both produce similar degenerative changes over a period of 4 weeks, even though Low impact produces no initial gross damage. Mechanical properties of the tissue, while different for High and Low impact 1 week post-impact, are equivalent at 4 weeks. At week 4, the Low and High impact explants were equivalent, measuring 27% and 22% of the baseline, respectively. This is a loss of three-quarters of the tissue’s original mechanical integrity. The GAG content of High and Low samples showed the same trend. At week 4, both inside and outside the impact zone, GAG was significantly decreased from baseline. This
was not the case at week 1. GAG release data also showed the Low impact samples to be similar to the High impact samples, evidenced by both having significantly more GAG released during the first week compared to the following 3 weeks. Further, at week 1, the measured DNA content showed that tissue areas inside the Low and High impacts had a significantly decreased number of cells from baseline and culture controls, as well as a higher percent of cell death. The area inside the impact zone for both Low and High levels are also similar in that AGC and TIMP-1 gene expression is suppressed compared to culture controls at week 1. Taken together, these results show that the Low impact explants have deteriorated substantially, nearly to the level of the High impact explants by week 4.

The impact levels in this study meet the definition set forth by Aspden et al.\textsuperscript{169} in that they occur in less than 30 ms and are greater than a stress rate of 1000 MPa/s. Other studies have used higher peak stresses, but they report the impact measurements in various ways. Repo and Finley\textsuperscript{144} found the peak stress threshold for gross damage of articular damage to be 25 MPa, while Clements et al.\textsuperscript{147} found cell death to begin at 6 MPa and gross damage to occur at 14 MPa. Haut\textsuperscript{146} found 25 MPa to cause subchondral bone fracture. Further, Duda et al.\textsuperscript{224} also found cell death at peak stresses as low as 3 MPa. Thus, while the peak stresses (3.7 MPa and 6.5 MPa) used in this study were not as high as some studies, the differences are likely due to the very high stress rates (at least 4600 MPa/s), as well as inherent differences between explant studies and animal models.

To the authors' knowledge this is the first study to look at gene expression profiles following articular cartilage impact. One study did note MIG-6, a signal
transduction factor, showed increased expression after cyclical compressive loading.\textsuperscript{319} In the present study, the MMP-1/TIMP-1 ratio was increased at week 1 inside the high impact zone. This suggests that a high level of mechanical insult can tilt the reparative-degradative balance toward degeneration, though, in general, impact seems to suppress gene expression within the impact zone. For both Low and High levels, TIMP-1 and AGC expression were decreased inside the impact area compared to outside. This may represent a decreased ability of the impact area to attempt repair, or the surrounding area is attempting to heal itself. Our finding that the impact area affected the surrounding tissue is consistent with a previous study.\textsuperscript{249} Additionally, increased AGC expression at week 1 may be represented by the increase in GAG released to the media during the first week. The decrease in AGC expression at week 4 also corresponds to decreased GAG release occurring in later weeks. The Coll increase at week 4 compared to week 1 and the SZP decrease from baseline at week 1 are signs of phenotypic change.\textsuperscript{306}

The majority of GAG released to the media occurred during the first week for both Low and High impact levels. DiMicco et al.\textsuperscript{317} found similar results, observing that GAG release after injurious compression was highest during the first 24 hours. While the present study looks at 1 and 4 weeks, our findings, combined with those of DiMicco et al.,\textsuperscript{317} suggest a therapeutic window for any intervention targeting GAG breakdown post-injury. Significantly decreased GAG content was observed when comparing week 4 Low and High impact levels to baseline. It should be noted that GAG content took 4 weeks to become significant, while GAG release into the media was significant at week 1. Thus, GAG release into culture media is more sensitive than measuring GAG content to detect ECM degradation. While the amount of GAG in the tissue is due to a balance of loss and
production, Quinn et al.\textsuperscript{250} found decreased synthesis in response to tissue insult. In the light of increased AGC expression, a plausible alternative is that increased MMP levels cause more collagen matrix breakdown, freeing GAG from the tissue. Collagen content of explants in the High impact, inside area group was significantly increased at week 4 compared to week 1. Given that collagen II expression decreased while collagen I increased, the increase in total collagen in the high impact area may represent an accumulation of collagen type I, suggesting fibrocartilage repair.\textsuperscript{2} Alternatively, this result could be due to the loss of other ECM constituents faster than collagen loss, which would increase the percent collagen in the explant.

The biomechanical properties of the explants are difficult to directly compare to previous studies due to differing measurement techniques; although, in general, the results are consistent with prior work. The aggregate modulus of the Low and High impact explants were decreased at weeks 1 and 4 significantly from culture controls and baseline. At week 1, the High impact aggregate modulus was significantly less than the Low impact, while the culture control and baseline aggregate modulus were equivalent. At week 4, the culture control was significantly less than the week 1 culture control and baseline, but was still 58\% of the baseline value. Notably, the Low and High impact at week 4 were approximately a quarter of the baseline value and not significantly different from each other. Ewers et al.\textsuperscript{155} applied a 625 N single impact peak load to rabbit patello-femoral joints and mechanically tested the rabbit cartilage out to 36 months. They found a 28\% and 35\% reduction in elastic moduli at 4.5 months from a poroelastic model and stress relaxation testing. This reduction persisted, but did not worsen, beyond this point. After a single injurious compression load on bovine cartilage, that ranged in
strain rate from 0.01 /s to 1/s and 12 MPa to 24 MPa respectively, Kurz et al.\textsuperscript{327} used confined compression testing to investigate the compressive stiffness 6 hours or 3 days later. They found that the compressive modulus decreased for the higher strain rates, but no significant differences existed between 6 hours and 3 days. While not directly comparable, the results of the current study generally agree with those of Ewers et al.\textsuperscript{155} in that the creep indentation aggregate modulus may have reached a lower limit by week 4. The sequential decrease of the Low impact aggregate modulus to the level of the High impact modulus between weeks 1 and 4 suggests that the degeneration cascade took longer to occur, but ultimately resulted in tissue that is mechanically equivalent to High impact and likely to fail.

Viability results showed that many cells died inside the High and Low impact area during the first week, and even more at week 4. The staining showed that much of the cell death occurred in the superficial zone upper portion of the explant. Chen et al.\textsuperscript{247} made similar findings after subjecting cartilage to cyclical compression. This study also agrees with Chen et al., in that more middle/deep cell death occurred in the impacted explants with increasing impact level. Torzilli et al.\textsuperscript{161} and Lewis et al.\textsuperscript{256} also found the superficial zone was more sensitive to loading. Interestingly, the superficial zone in the week 4 High impact viability stain showed few cells, living or dead, suggesting that the superficial layer of cartilage can no longer sustain viable cells. However, clusters of dead cells are seen in and near the superficial zone for both the Low and High impact week 4 viability staining. These clusters are starting to form in the week 1 and appear to progress to the middle/deep zone. The week 4 Low impact viability staining looks to be following the same course. At week 1, DNA content showed significantly decreased cell
numbers in the impact zone, even though the assay measured both live and dead cells. Thus, the High and Low impact areas had fewer cells, as well as a higher percentage of dead cells, both of which decrease the potential for repair. The Low impact percent cell death increased over three-fold from week 1 to week 4, while the High impact percent cell death increased less than two-fold. While viability of the Low impact was still significantly lower than High at week 4, it appears that the Low impact explants are close to the same level of degradation as the High impact explants. Low impact explants followed the same course of degeneration as those subjected to High impact, demonstrating a combination of increased cell death, loss of ECM, decreased biomechanical integrity, and similar histological cell death patterns.

From gene expression to GAG release and from ECM protein levels to biomechanical properties, the results of this study characterize the temporal effects of impact injury to articular cartilage and support our initial hypotheses. Though both Low impact and baseline tissue were morphologically equivalent initially, Low impact explants exhibited temporal behavior that was comparable to those of High impact. Overall, the High impact level caused initial tissue damage at week 1, which worsened by week 4. While at week 1 the Low impact tissue was not observed to be as damaged as High group, at week 4 the degenerative cascade of Low impact explants had reached that of the High impact explants in terms of biomechanics, GAG release, and gene expression profile. Further, Low impact samples displayed the same tableau of temporal cell death pattern and cell viability as the High. From the results presented in this investigation, it follows that interventions to slow or prevent the early changes associated with Low impact may be able to rescue the tissue from the ensuing degeneration cascade. These
interventions may translate into clinical tools to decrease the prevalence of post-traumatic osteoarthritis.
ACKNOWLEDGEMENTS

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Table 14. qRT-PCR target gene sequences, dyes, and quenchers for MMP-1 and TIMP-1.

<table>
<thead>
<tr>
<th>Gene* (accession number, product size)</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
<th>Dye</th>
<th>Quencher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix</td>
<td>CAAATGCTGGAGGTATGATGA</td>
<td></td>
<td></td>
<td>Quasar 670</td>
<td></td>
</tr>
<tr>
<td>Metalloproteinase I (X74326, 82b)</td>
<td>AATTCCGGGAAAGTCTTCTG</td>
<td></td>
<td></td>
<td>BHQ-2</td>
<td></td>
</tr>
<tr>
<td>Tissue Inhibitor of Matrix Metalloproteinase I (NM174471, unknown)</td>
<td>TCCATGGATGCAGGGTATCCCAA</td>
<td>GAGATCAAGATGACTAAGATGTTCAA</td>
<td>GGTGTAGATGAACCGGATG</td>
<td>ROX</td>
<td>BHQ-2</td>
</tr>
</tbody>
</table>

*All sequences are 5'-3'
Table 15. Impact measurements for the Low and High levels are listed in the table below.

<table>
<thead>
<tr>
<th>Impact Level</th>
<th>Tup weight</th>
<th>Drop height</th>
<th>Energy</th>
<th>Peak stress (Mean ± S.D.)</th>
<th>Time to peak stress (Mean ± S.D.)</th>
<th>Duration (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>18.4</td>
<td>6</td>
<td>1.1</td>
<td>3.7 ± 0.4(^A)</td>
<td>0.8 ± 0.2(^A)</td>
<td>1.7 ± 0.4(^A)</td>
</tr>
<tr>
<td>High</td>
<td>27.8</td>
<td>10</td>
<td>2.8</td>
<td>6.5 ± 1.6(^B)</td>
<td>0.6 ± 0.2(^B)</td>
<td>1.0 ± 0.2(^B)</td>
</tr>
</tbody>
</table>

For each column the numbers with different superscripted letters, ‘A’ and ‘B’, are statistically significant (p < 0.05).
Figure 44. The impact instrument.

A) linear bearings, B) impact weight (tup), C) impact interface with attached accelerometer, D) impact tip, E) specimen clamp with specimen held in place, and F) base sitting on an air table.
Figure 45. Viability staining for each experimental group.

a) Baseline, b) week 1 culture control, c) week 1 Low impacted specimen, d) week 1 High impacted specimen, e) week 4 culture control, f) week 4 Low impacted specimen, and g) week 4 High impacted specimen. Red indicates dead cells and green indicates living cells. Magnification is 100X for each image. Baseline explants were neither cultured nor impacted. Culture controls were not impacted.
Figure 46. Viability staining.

Results from the viability staining expressed as mean ± S.D. percent dead cells. Groups not connected by the same letter are significantly different from one another (p < 0.05).
Figure 47. Gene expression levels for a) collagen I (Col1) and b) aggrecan (AGC).

Values are normalized abundance expressed as mean ± S.D. Within a gene, groups connected by the same letter are not significantly different from each other. (CC = Culture control, LI = Low impact inside the impact zone, LO = Low impact outside the impact zone, HI = High impact inside the impact zone, HO = High impact outside the impact zone)

Col I Normalized Abundance

AGC Normalized Abundance
**Figure 48.** Gene expression levels for week 1 a) superficial zone protein (SZP) and b) tissue inhibitor of matrix metalloproteinase-1 (TIMP-1).

Values are normalized abundance expressed as mean ± S.D. Within a gene, groups connected by the same letter are not significantly different from each other.
**Figure 49.** The ratio of MMP-1 to TIMP-1 gene expression expressed as mean ± S.D.

Groups connected by the same letter are not significantly different from each other.

**MMP-1/TIMP-1 Normalized Abundance**
Figure 50. Data for GAG released into the media displayed as mean ± S.D.

a) Comparison of culture control, Low, and High impact levels at week 1, b) Comparison of GAG release per week for the Low impact level, c) Comparison of GAG release per week for the High impact level. Groups not connected by the same letter are significantly different from one another (p<0.05).
Figure 51. Wet weight % of GAG in the explant

a) Low and b) High impact groups. Numbers are mean ± S.D. For both levels, the week 4 inside and outside areas are significantly different from baseline.
Figure 52. DNA content in the explants.

The number of cells is in millions per gram of tissue. Groups not connected by the same letter are significantly different from one another. DNA content presented as mean ± S.D.
Figure 53. Comparison of mean ± S.D. of aggregate modulus and permeability.

a) aggregate modulus and b) permeability at week 1 and week 4 (CC = Culture control, L = Low impact level, H = High impact level). Groups connected by the same letter are not significantly different from each other (p < 0.05).
DISCUSSION

This thesis seeks to further understand articular cartilage through the completion of two major objectives. The first is a cellular approach, investigating the pathology and physiology of cartilage through the development, validation, and utilization of an imaging technique capable of characterizing the three-dimensional geometry of single chondrocytes, fibroblasts, and protein-coated surfaces. This methodology will enhance the ability of researchers in the field to understand and even modify the behavior of single chondrocytes, leading eventually to improved cell seeding and tissue engineering. The second objective is a tissue level approach, investigating the pathology and physiology of cartilage subjected to impact injuries, particularly those that lead to osteoarthritis. By characterizing the acute and temporal tissue response to two levels of impact loading, this work illuminates the processes by which a subclinical injury can lead to long term tissue degeneration, and further elucidates the way in which cartilage reacts to more acutely damaging impacts. Knowledge gained herein complements and enhances previous studies by characterizing a wide array of tissue responses, from gene expression to biomechanics to biochemistry, in order to gain a more comprehensive understanding of cartilage injury.

The first objective, the cellular approach, utilized vertical scanning interferometry (VSI) to image all salient features of the three-dimensional geometry of single cells and characterize bioactive surfaces (Chapters 2 and 3). VSI was able to acquire nanometer precision geometries of single chondrocytes and fibroblasts in a simple format, allowing fast computation of the cell’s volume, surface area, height, and diameter. In addition, the
three-dimensional ‘height map’ image of these cells was not constrained to any necessary symmetry, but was able to mathematically measure and visually represent any geometry. The advantages of VSI for single cell imaging include speed (2μm/s scan rate of depth), precision (2 nm resolution), and width of scan (the entire visual field of a microscope). This technology was taken a step further and used to image surfaces that were coated with extracellular matrix (ECM) proteins, and then to image fibroblasts seeded onto these surfaces. The cell processes (extensions) of the fibroblasts were imaged and measured as efficiently as the chondrocyte geometry.

Use of these measurements allowed comparison of different conditions under which the cells were placed, including seeding time and surface coating. Protein-coated surfaces could also be qualitatively and quantitatively differentiated, and the physiology of articular chondrocyte attachment and spreading was described. In addition, the technology allowed for the production of the first set of real-time pictures of a chondrocyte exposed to air as it dehydrated. Because of the level of detail acquired by VSI, variation in the bioactive surface characteristics could be seen, including the coating thickness. Notably, the qualitative differences between the proteins (aggrecan, decorin, and collagen II) and application techniques could be distinguished. Fibroblasts were seeded onto these surfaces in an attempt to chondroinduce them (tilt their phenotype toward chondrocytes). VSI was able to correlate protein-coated surface characteristics with the degree to which fibroblasts develop a rounded, chondrocytic morphology, indicative of dermal fibroblasts expressing chondrocytic markers.
The potential applications of VSI to further explore articular cartilage physiology and pathology are considerable. Geometries acquired by VSI of single cells could be directly entered into cytomechanical models for elucidation of biomechanical properties and stress fields. Cell attachment and spreading characteristics, as well as general geometric descriptions, could be studied in finer detail for an array of cell types, including those with highly complex geometries, such as neurons and glial cells. Data from cell attachment and spreading studies could help assess biocompatibility issues. Cell substrata could also be further explored with VSI to identify surfaces that induce chondrocytes to behave in a desired manner, such as form clusters or attach in a specific way to biomaterials. Advances in this technology, including an in situ VSI instrument, would allow three-dimensional “movies” of chondrocytes as they attach and spread on a substrate. Finally, VSI could be used to explore chondrocytes taken from mechanically damaged tissue, to further understand the effects of articular cartilage injuries such as impact.

The second objective of this thesis involved building a robust impact instrument and utilizing it to understand mechanical impact on articular cartilage with its accompanying acute and temporal sequelae. This tissue-level approach to cartilage pathology and physiology aimed to create a methodology for protecting diarthrodial joints from impact loads, translating clinically into the prevention of post-traumatic osteoarthritis. Understanding how articular cartilage responds to both enzymatic digestion (Chapter 5) and time in culture (Chapter 6) imparted a base of knowledge for this investigation. Building and validating the impact instrument allowed application and characterization of repeatable, consistent impact levels (Chapter 7). The design of
this instrument permitted two levels of impact to be defined and investigated: a) Low impact, which did not initially cause any initial visual damage compared to baseline articular cartilage and b) High impact, which caused immediate gross damage. The acute (Chapter 8) and temporal (Chapter 9) effects of pathologic mechanical impact were then comprehensively investigated.

Subjected to time in culture and enzymatic digestion, articular cartilage demonstrated several key changes. Explant culture was shown to cause small but significant changes, which made this model advantageous for studying impact. Further, articular cartilage explant culture was found to be similar to in vivo disuse studies in that, over time, biochemical and biomechanical changes occurred. The cultured tissue did not display drastic changes, but the biomechanical properties and glycosaminoglycan content significantly decreased over four weeks. At week 4, the tissue started to express a small amount of type I collagen and matrix metalloproteinase 1 (MMP-1), signs of phenotypic change and matrix breakdown, respectively. In contrast, chondrocyte gene expression demonstrated alterations immediately upon enzymatic digestion. For instance, a pronase-collagenase digestion caused an upregulation of glyceraldehyde-3-phosphate dehydrogenase, which was evident right away. The other three digestion methods all decreased type II collagen immediately, and the tissue demonstrated further change in monolayer culture. This portion of the investigation confirms that both explant culture and enzymatic digestion affect the physiology of articular cartilage. The results illustrate that although articular cartilage explants change in culture, the change is limited in scope, allowing for the addition of mechanical impact as a testing modality.
The pathological effects of mechanical impact on articular cartilage resulted in a cascade of degeneration, the pace of which was dependent upon impact level. Acutely, the gross morphology and biomechanical properties of Low level impact were identical to baseline articular cartilage. However, the High level impact immediately showed surface disruption, quantified by a morphology score that was four times higher than both baseline and Low impact tissue. High impact tissue also had a significantly lower aggregate modulus immediately and at 24 h. By week 1, the aggregate modulus of High impact samples had deteriorated to a third of the culture control samples and continued to decrease (though not significantly) thereafter. Thus, the High impact explants showed immediate signs of morphological, biochemical, biomechanical deterioration and genetic alteration, and then continued to worsen. The Low impact, while starting out clinically equivalent to baseline, followed a similar course of degeneration.

Immediately after injury and at 24 h, the Low impact explants were clinically identical to baseline. Yet even at these early time points, some subtle alterations were already present. The histology demonstrated slight variation in the superficial zone, the gene expression of type I collagen was significantly increased at 24 h, and glycosaminoglycan release was significantly increased over the explant control. While these differences were not extensive, they signaled the onset of a degenerative process. Over four weeks, the Low impact explants exhibited the same pathological changes as the High impact. At week 4, Low impact explants had an equivalent decrease in aggregate modulus and GAG content as those subjected to High impact. The percent cell death in the Low impact explants increased 3.5 times from week 1 to week 4, while the High impact explant cell death only increased by half that amount. In addition, the patterns of
cell death that occurred in the High week 1 explants were apparent in the Low week 4 explants, including clustering of dead cells in the upper third of the tissue. Thus, the cascade of degeneration that occurred in the Low impact explants was not substantially different from the High impact explants and would likely lead to the same end result, tissue failure and post-traumatic osteoarthritis.

These findings lead to several promising avenues of inquiry. Studying interventions that involve bioactive molecules or mechanical stimulation may shed light on how to reverse or prevent degeneration in the Low impact explants. These interventions may also be able to prevent the culture control explants from changing in culture. Tetracycline and its derivatives, insulin-like growth factor I, matrix metalloproteinase inhibitors, and P-188 (a non-ionic surfactant) have all shown some potential as ameliorative agents of the negative effects of impact, and their potential could be quantified with the current model of articular cartilage and mechanical impact. Cyclical compression or hydrostatic pressure or other mechanical loading regimes may also help block degradation.

By approaching the pathology and physiology of articular cartilage on both the cellular and the tissue level, this body of work has generated knowledge which will aid researchers and clinicians as they work to better understand, and thereby predict, prevent and manage articular cartilage injury. While no current therapy exists to prevent or slow the progression of osteoarthritis, this body of work takes one step closer to effective therapeutic intervention.
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APPENDIX A: LEARNING HOW TO TEACH
CONTINUUM BIOMECHANICS: SEE ONE, DO
ONE, TEACH ONE*

ABSTRACT

Continuum Biomechanics is a graduate level course taught in the Department of Bioengineering at Rice University, Houston, Texas. The course is primarily a theoretical one, based on advanced mathematical concepts. An important element of this course is the development of a lecture by each graduate student. The project involves the entire process of developing a board-based, graduate-level lecture, including conception, presentation, and post-lecture support. This project allows graduate students to develop and improve important teaching skills, including developing a lecture, managing a board legibly, and understanding how much material can be covered effectively in an allotted amount of time.

INTRODUCTION

The Continuum Biomechanics course at Rice University has been developed to teach the basics of continuum mechanics with biomedical applications such that graduate bioengineering students can understand and apply theoretical models in the literature. Biomechanical analytical models are usually complex and require more than a basic solid or fluid mechanics course to understand. The course begins with an overview of continuum mechanics, including vectors and tensors, stress and strain tensors, constitutive equations, and an introduction to elasticity. Viscoelasticity and mixture theory models are also introduced. The course usually attracts 8-10, second-year, Ph.D. students from the Department of Bioengineering. In addition to learning basic continuum mechanics as applied to biomedical engineering, one of the major goals of this course is for students to engage in a comprehensive teaching experience.

To this end, a major component of this course is a teaching project, which requires every student to develop a lecture based upon a continuum mechanical model of biological materials, such as muscle, cartilage, bone, or cells. The teaching project is used to help graduate students develop a style of board lecture loosely based on the education philosophy often seen in medicine of, "see one, do one, teach one." In medicine, teaching in the clinics occurs by the students watching a procedure or history or physical exam, then actually performing the procedure in front of a doctor, and finally teaching it to someone else. In this way much of the clinical knowledge in medicine is passed down. Effectively, a portion of the course is taught by the students themselves on topics of interest to them, selected from a list of general topics. This part of the course
includes creation of a homework problem graded by the presenting student. The lecture is to be completely performed by using only a whiteboard with colored markers, which is an important skill when teaching mathematical models. Using a whiteboard or a chalkboard to teach mathematical models is beneficial because it sets a reasonable pace for the class and is conducive to answering questions and promoting other interactions. As part of this project, faculty from Rice University's Cain Project in Engineering and Professional Communication assist with analyzing, assessing, and improving the board lectures.

The Cain Project in Engineering and Professional Communication was established through a gift from the Gordon and Mary Cain Foundation in 1998. The Project’s mission is to prepare Rice University's science and engineering students to lead through excellence in communication. Instead of teaching stand-alone courses in technical communication, Cain Project instructors collaborate with faculty to integrate written, oral, and visual communication into existing science and engineering courses at the undergraduate and graduate levels. They support the Continuum Biomechanics teaching project by assisting with assignment design, communication instruction, individual coaching, and student assessment for the teaching project.

Similar types of projects with pedagogical experience have been reported in the literature, but most discuss using technology or the internet to assist with teaching.\textsuperscript{329-335} One project reported by Pollock\textsuperscript{336} discusses teaching basic, non-continuum mechanics through “learning by teaching.” This project required students to learn an undergraduate-level topic in dynamics and then teach it to their classmates. Other articles discuss using
the computer or a networked classroom to aid in teaching soil mechanics,\textsuperscript{324} basic mechanics,\textsuperscript{321,335} and mathematics.\textsuperscript{329} As the popularity and interest of presentation software\textsuperscript{337} and internet-\textsuperscript{338-340} and television-based\textsuperscript{341} courses increase, this project allows graduate students to develop basic teaching skills not otherwise covered in the curriculum. The importance of this project is that graduate students, who will soon be teaching in some form in either academics or industry, get the chance to develop a lecture from complex literature, as well as work on skills needed in all aspects of engineering didactics, such as being able to explain difficult concepts clearly and succinctly using only a whiteboard (or pen and paper) to colleagues. Bishop \textit{et al.}\textsuperscript{342} describe a similar experience where a group of students designed and taught a course in Civil Engineering, which could be extended to this project by having the students in the course go on to design and teach the course.

This teaching project allows graduate students to gain the experience of developing a lecture from conception to delivery with constructive evaluation from both peers and faculty. The class members choose, research, and develop their lecture topic within the area of continuum biomechanical engineering. This project is one of few where students experience the entire process of teaching a mathematically complex, graduate-level topic without using presentation software. The board lecture format has many challenges not as acutely experienced when using overheads or software, including the ability to simultaneously write, talk, think, and observe audience reaction in a complex cognitive talk. Presentation software can provide the instructor with cues as to what to discuss next and more opportunities for using visual media or animations. Other challenges of a board lecture are writing legibly, effectively covering a reasonable
amount of material, and requiring a substantial knowledge of the material. While using presentation technology can be effective and captivating, the same technology can lend itself to trying to cover too much information in the allotted time or doing less preparatory work than is optimal.\textsuperscript{343,344} Tufte\textsuperscript{345} has written that presentation software can place the audience in a more passive role or reduce complex information down too much in bullet points. While a board lecture can have many of these flaws, the preparation required to cover a new topic on a chalkboard can be more substantial. Also, many situations and courses exist in academics, industry, and general professional interaction that require or are well-suited to a using board lecture format, from a formal class on continuum mechanics to explaining an idea or an experiment to a colleague in the hallway. Lastly, developing and giving a lecture on a complex topic with supportive homework is not as common to graduate student experience as giving a seminar presentation or grading papers.

**OBJECTIVES**

The overall objective for the course project is to develop and teach a board-based lecture in continuum biomechanics. Within this objective, the students also are tasked with finding the journal articles and books that define their lecture topics and gauging how much material could be covered in their fifty-minute lectures. The goal of each lecture is to teach one or more continuum models of their chosen biological material, as well as to prepare the class to complete a homework assignment of the lecturer's conception on that topic. The homework assignment reinforces or demonstrates the utility of the model. The specific goals for the actual lecture include:
1. For the class to understand the mechanical model

2. For the class to know how the model is used and its significance

3. For the class to know how the model was derived (not have every step of the complete derivation, but to know what equations were used to derive the model and how the equations were manipulated to obtain the model)

4. For the class to be able to complete the homework assignment solely from the notes they take during the lecture in a reasonable period of time

5. For the lecturer to further develop a clear, understandable, and organized lecture style.

These lecture aims define the scope and depth of the topic covered. To understand the significance of the mechanical model, some knowledge of the tissue physiology is necessary. Comprehending the mechanical model does not necessitate every single derivation, but a judicious approach is required to know what portion of the derivation to talk through and what parts to fully derive. The homework assignment has been in several past cases a derivation that was not completed during the lecture, a derivation using different assumptions from those given in the lecture, or an application of the model to a short problem. Developing a lecture style involves practicing with and without an observer, watching the lectures of other students, and noting the style of professors from courses they take. While noting the teaching style of the professors, the students hopefully also were able to select what they liked and disliked of the teaching styles.
ASSIGNMENT

The students are initially tasked with searching through the literature and textbooks for their lecture material. Due to the specificity of the mechanical modeling community, researching mechanical models is not an insignificant assignment. The research process involves multiple databases, as well as library time. An outline of the topic with a summary of the models chosen for presentation and all major references is due three weeks before presentations are scheduled to begin. The outline provides an opportunity to ensure the material being covered is appropriate in breadth and depth. Office hours are available weekly for assistance with researching the literature or learning how to use certain databases. Several students make use of the office hours to learn how to search through different databases, as well as learn how to follow up an initial article by using the references to find a more basic mathematical model. Students who do not already have substantial experience with literature searches need more specific direction.

The lectures, which are videotaped for subsequent viewing, are given using only a whiteboard and markers. A referenced, written report of the mathematical model with derivations and verbiage, and copies of the main literature articles and text chapters utilized for preparation are handed to the course instructor for evaluation and grading. The written report is due before the lecture is given.

The lectures are required to include all elements of a good presentation, including an introduction that establishes the significance of the biological material, the uses of mechanical models to understand the particular tissue or cell, well-organized sections with titles, good flow from section to section, and a conclusion that sums up the key
points and reiterates the significance. Table 16 illustrates the weighting of the different parts of the teaching project, which is discussed in more detail later. An integral part of the board lecture is handling and responding to questions. The students’ ability to answer questions on their topic is directly related to both their understanding of the model and their effectiveness as an instructor. In addition, effectively answering questions in the middle of the lecture and continuing with the flow of the lecture is also a goal. Questions on the material are to be answered by rephrasing the initial information or giving an example, not just repeating lecture material.

**EVALUATION**

The teaching project is worth 33% of the overall course grade. Within the teaching project the grading is broken down into the outline, the rehearsal, the written report and the actual lecture. The grading of the lecture is further broken down into content, delivery, organization, ability to answer questions, the homework assignment, and time management, as seen in Table 16. The students are evaluated after a practice lecture and the actual lecture with the assistance of the Cain Project. A minimum of three days before their lecture, the students present the material to a Cain Project faculty to allow identification of areas that need improvement. The areas found needing the most improvement are different for different students, but usually include delivery style, content, organization, ability to answer questions, and time management. While basics, such as legibility, clear speech, organization, and effectively answering questions were encouraged, each student developed a different lecture style that varied according to pace, demeanor, choice of supporting material, and use of the board.
The outline and practice session are not graded on a scale, while the report and lecture are. The typed reports are usually much more detailed than the lecture.

**Practice Sessions**

The practice sessions reveal significant problems in four areas, including the introduction and conclusion, explanation of the models, board technique, and time management. The practice sessions are held at least three days before the actual lecture with the same lecture environment and materials available as on the day of the lecture.

*Introduction and Conclusion*

Students often make no effort to motivate interest in their model or to describe the significance or applications in the practice lecture. For example, one student began with “Today I’m going to talk about muscle. Let’s begin with Hill’s Equation.” Similarly, as students near the end of their practice lectures, some simply finish the last equation and ask if anyone has questions instead of summarizing the key points and reinforcing the significance.

*Explanation of the Model*

During the practice sessions some students simply copy the equations from their prepared written notes onto the board without thoroughly explaining what they are doing and why they are doing it. Some do not include enough explanation on the board in full sentences to follow the derivations. In some cases, the gaps in students’ explanations reflect an incomplete grasp of the model. Many need to be reminded to set up the main
objective, define the variables, identify governing equations and assumptions, and to account for the relationships between variables, equations, and biological material physiology. In particular, they need help constructing high-level summaries of significant steps in the derivations associated with a model.

**Board Technique**

The gaps and imprecision in students’ verbal explanations translate into clutter on the board in the practice sessions. Students usually begin their practice lectures well – dividing the board into segments, using headings, numbering equations, writing large and legibly. However, as they become flustered or pressed for time, they forget to number equations and label drawings, or crowd equations into one another. Their handwriting, especially the variables and partial differentials, becomes small and illegible. In addition to confronting the challenge of managing the board, students also discover how long it takes to write out equations. Some students have to work on making their handwriting more legible from the very beginning.

**Time Management**

The outlines prepared prior to their practice lectures reveal that several students want to cover material that would take multiple class periods, if not half a semester, to teach. So one challenge is convincing students what constitutes a reasonable amount of material that can be taught in an hour. Even after revising their ambitious plans, some students’ practice lectures last almost two hours, which prompts them to prune more
content and to improve their fluency so that they can articulate their explanations clearly and succinctly.

LECTURE IMPROVEMENT

The in-class lectures are significantly better than the practice sessions. All students make progress in terms of their ability to organize and explain their models. Delivery markedly improves, both in terms of legibility, as well as organization. The lecturers interact with the class, speaking directly to them as opposed to mumbling to the board or staring at their notes as many do in practice. Time management for the actual lecture is good, and while some students have to wrap up faster than desired, no one ends up being cut off.

STUDENT FEEDBACK ON THE TEACHING PROJECT

Evaluations of the teaching project are collected at the end of the course to assess the value of the assignment and coaching. Various students taking the course have described the project as “valuable,” “incredible” and “perfect.” Of the ten respondents in 2002, six described the practice session as “Very useful” and four found it “Useful.” One student commented that the assignment “provided some insight into what teaching in academia would be like.” Another commented, “The preparation for this talk required us to learn the concepts taught in this course.” When several students were asked about the project over a year later, their response was still positive. One student felt the project, “taught (him) how much work can go into planning a good lecture,” as well as felt that the project, “extremely valuable to any student thinking they want to go into academia.”
Another student felt that as he progressed through his graduate education, he continued to draw from this experience and also remembered, "how much effort goes into the preparation of a lecture and the process of educating others."

The overall consensus is that the teaching project worked well. However, two proposed changes to the assignment may result in better lectures:

- Assign each lecturer a presentation partner from the class or have one of the teaching assistants attend the practice session to help identify problems and inaccuracies in the student’s understanding of the model.

- Ask more clarification and elaboration questions during the practice session to test the speaker’s ability to respond and to see how well he or she can adjust the original lecture plan to suit the allotted time.

**DISCUSSION**

Many graduate students have multiple opportunities to give formal, PowerPoint talks on their research or a class project. However, there is a higher degree of complexity and accountability involved in teaching a board-based lecture because in addition to displaying an effective communication approach, the instructor needs to manage the board, respond to questions, and prepare others to use new information immediately to solve problems. This teaching project helps the students understand and overcome the difficulties of teaching analytical models and of developing a lecture at the graduate level based upon the literature. Further, the experience is one of few in graduate school that
explores the entire process of developing and teaching a challenging, graduate level bioengineering topic.

The students improve their style from the practice to the actual event. Many of them discover the difficulty in covering all of the information they thought necessary and learn to make sensible choices about what to include.
Table 16. Evaluation parameters for the teaching project.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outline three weeks before presentations begin</td>
<td>5%</td>
</tr>
<tr>
<td>Meet with CAIN Project faculty with lecture prepared</td>
<td>10%</td>
</tr>
<tr>
<td>Typed Report</td>
<td>25%</td>
</tr>
<tr>
<td>Lecture</td>
<td>60%</td>
</tr>
<tr>
<td>Content (relevance, model explanation)</td>
<td>15%</td>
</tr>
<tr>
<td>Delivery Style (volume, board, etc)</td>
<td>15%</td>
</tr>
<tr>
<td>Organization (intro, flow, conclusion)</td>
<td>10%</td>
</tr>
<tr>
<td>Answering Questions</td>
<td>5%</td>
</tr>
<tr>
<td>Homework Problem and Solution</td>
<td>10%</td>
</tr>
<tr>
<td>Time Management</td>
<td>5%</td>
</tr>
</tbody>
</table>