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EFFECTS OF MECHANICAL IMPACT ON A BOVINE ARTICULAR CARTILAGE EXPLANT SYSTEM

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EFFECTS OF MECHANICAL IMPACT ON A BOVINE ARTICULAR CARTILAGE EXPLANT SYSTEM

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ABSTRACT

EFFECTS OF MECHANICAL IMPACT ON A BOVINE ARTICULAR CARTILAGE EXPLANT SYSTEM

By

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Traumatic injury has been shown to cause osteoarthritis. A mature bovine articular cartilage explant system was developed to evaluate the effects of traumatic (30 or 40 MPa) and physiological (15 MPa) loads compared to cytokine treatment. Proteoglycan (PG) and nitric oxide (NO) release, as well as cell death, were evaluated. In all of the experiments, impacted explants were found to have minimal NO release compared to controls, while in cytokine treatments, interleukin-1β (IL-1) and lippopolysaccharide (LPS), were significantly elevated. Proteoglycan release was minimal in cytokine treatments but varied with load and rate for impacted treatments. Proteoglycan release in the 30 MPa slow (1 sec) load was found to be the greatest, while the lower and faster rate of 15 MPa and 50 msec did not produce the same significant response. Glucosamine-sulfate was used to pre-treat the impacted samples, and found to have no significant effect on cell death, NO or PG release, which is contrary to its ability to inhibit cytokine-induced cartilage degradation. Cell death was increased for all impacted treatments with significant cell death at 30 MPa slow and a trend for greater cell death with higher loads and longer rates of loading. Cytokine treatments gave minimal cell death. Analysis for apoptosis following acute traumatic impact, revealed no significant increase in apoptosis compared to controls. Acute 30 MPa load at slow rate of impact caused necrosis of the chondrocytes and degradation of the matrix, while cytokine treatment caused increased NO production.

I would like to dedicate my thesis to my parents, Emil and Carol Dvoracek and my husband Sean Driknsa. Without their support in my education, this work would not have been possible.

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KEY TO ABBREVIATIONS

CDC.....Centers for Disease Control and Prevention

CS.....chondroitin sulfate

DMEM.....Dulbecco's Modified Eagles Medium

DMMB/DMB.....dimethylmethylene blue

ECM.....extracellular matrix

ER.....endoplasmic reticulum

GAG.....glycosaminoglycan

GS.....glucosamine-SO_4

HA.....hyaluronic acid

Hz.....Hertz

IL-1.....interleukin-1β

KS.....keratan sulfate

LPS.....lipopolysaccharide

MMP.....matrix metalloproteinase

MPa.....mega Pascal

OA.....osteoarthritis

PG.....proteoglycan

INTRODUCTION

According to the Centers for Disease Control and Prevention (CDC), approximately 43 million Americans are affected by arthritis. The CDC estimates that lost productivity and treatment of arthritis is costing the United States over 65 billion dollars annually (CDC, 1998). Cartilage has a slow but natural turnover that, when balanced, maintains the cartilage integrity. In osteoarthritis (OA), the degradation of old or damaged components is greater than the synthesis of new components. Therefore, possible triggers of deterioration due to the imbalance are being investigated. Since OA tends to affect people over the age of 45, it may be a result of long term "wear and tear" on the afflicted joints. However, blunt trauma may initiate cartilage deterioration (Buckwalter, 1983).

Blunt trauma results from a single load applied at a constant rate over the entire surface of an object. Causes of blunt trauma include automobile collisions, when the driver's knee is forced into the dashboard; athletes colliding with each other, equipment, or the ground; and domestic injuries involving falls. Forces experienced in the joint during impact can be four times greater than those of normal physiological loads. Although traumatic injury potentially initiates the development of OA, the biological response of cartilage to applied forces has not been extensively studied. To study the direct effects of mechanical trauma on cartilage deterioration, an equine cartilage explant system, previously used in our lab (Fenton et al., 2000a; Fenton et al., 2000b), has been adapted for bovine tissue.

My thesis research was designed to evaluate the catabolic response of articular cartilage to a single blunt load. Objectives include:

1) develop a standard method to culture and impact bovine articular cartilage explants with detection of catabolic responses;
2) determine if different levels and lengths of loading change the catabolic response of bovine articular cartilage;
3) determine if treatment with glucosamine inhibits the catabolic response seen in bovine articular cartilage following impact;
4) determine if cell death seen at high force impact is due to apoptosis.

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CHAPTER 1

LITERATURE REVIEW

1. Articular Cartilage

Cartilage acts as a resilient covering allowing nearly frictionless movement between bone surfaces. The joint is lubricated by synovial fluid. The articular cartilage, also referred to as hyaline cartilage, is an avascular, aneural, and alymphatic tissue, covering the ends of long bones (Martin, 1994; Johnston, 1997; Buckwalter and Mankin, 1998). The combination of collagen fibrils, proteoglycans (PG), noncollagenous proteins, and water form a matrix that effectively distributes force over the underlying subchondral bone (Martin, 1994; Johnston, 1997).

Articular cartilage consists primarily of two components, the extracellular matrix (ECM) and the chondrocytes (Buckwalter, 1983). Chondrocytes are the sole cell type present in healthy articular cartilage. Chondrocytes, derived from mesenchymal stem cells, are few in number and make up less than 5% of the tissue's volume (Johnston, 1997). However, they synthesize all of the ECM matrix components, as well as those essential to the maintenance of articular cartilage (Buckwalter, 1983; Buckwalter and Mankin,1998).

2. Collagen

By transmitting and distributing loads over the underlying subchondral bone, articular cartilage is an effective shock absorber (Johnston, 1997). The transmission of the forces in the ECM occurs via a meshwork of collagen fibrils, which hold ECM components in place. These fibrils consist of a combination of collagen types. Type II collagen is the primary component but other collagen types are also present in smaller

amounts, including types VI, IX, X, XI (Table 1) (Eyre et al., 1991; Von der Mark et al., 1992; Johnston, 1997). Type VI collagen is found in the pericellular region and binds the cells to the matrix collagen and nearby PG (Roth and Mow, 1980; Poole et al., 1988; Johnston, 1997). Type IX can be found on the surface of type II fibrils (Johnston, 1997), type X is found in hypertrophic growth plate cartilage, the deep calcified zone in adult cartilage, and in OA cartilage (Eyre et al., 1991; Von der Mark et al., 1992; Johnston, 1997).

Table 1: Collagen types pertaining to articular cartilage

Collagen Type	Function
II	fibril forming, provides tensile strength to articular cartilage
VI	fibril associating, in pericellular matrix, binds chondrocyte to matrix collagen and PG
IX	fibril associating, may link type II fibrils with other ECM components
X	fibril associating, may help regulate calcification at the tidemark
XI	fibril forming, interspersed in collagen type II fibrils; regulates fibril diameter

3. Proteoglycan

Approximately 22% to 38% of the dry weight of adult cartilage is composed of PGs (Roth and Mow, 1980; Johnston, 1997; Buckwalter and Mankin, 1998). A PG consists of glycosaminoglycans attached to a core protein. Aggrecan is the primary PG in articular cartilage. Keratan sulfate (KS) and chondroitin sulfate (CS) are glycosaminoglycans, which bind to core protein to form an aggrecan monomer (Buckwalter, 1983; Buckwalter et al., 1984; Johnston, 1997). The ability of monomers to absorb compression is a property of the CS and is due to the sulfated N-

acetylgalactosamine subunits, which attract water and ions in the matrix (Buckwalter, 1983).

A third glycosaminoglycan in the ECM of articular cartilage, hyaluronic acid (HA), is the backbone to which the aggrecan monomers attach (Heinegard and Hascall, 1974; Buckwalter, 1983). The components of aggrecan along with link protein are synthesized intracellularly and excreted into the ECM, with the exception of hyaluronic acid, which is synthesized at the cell membrane and excreted into the extracellular matrix (Buckwalter et al., 1984). Once in the ECM, the components spontaneously assemble into aggregates (Hascall and Heinegard, 1974; Buckwalter, 1983). The aggregates assemble when the N-terminus of the PG core protein, with the aid of a link protein, binds HA (Buckwalter, 1983; Buckwalter et al., 1984). The large amount of CS bound in the aggregated molecules optimizes the ability of the ECM to absorb forces (Roth and Mow, 1980; Buckwalter, 1983).

Although the collagen fibrils throughout the ECM provide tensile strength to hold the structure together (Roth and Mow, 1980), without hydrated negatively charged proteoglycan complexes, the articular cartilage would collapse under compression (McDevitt and Muir, 1976; Roth and Mow, 1980; Poole et al., 1988; Johnston, 1997). When the collagen fibrils and proteoglycan complexes are integrated, the result is a dynamic tissue able to tolerate high compressive and shear loads without damage (Buckwalter, 1983). These properties allow transmission of forces to the underlying bone (Johnston, 1997).

4. The Four Zones of Articular Cartilage

Articular cartilage consists of four zones, each differing in cell density and shape as well as arrangement of components of the ECM. The four zones consist of the superficial zone, transitional zone, deep zone, and the calcified cartilage zone. Structural and metabolic differences between zones enable the articular cartilage to withstand the loading it experiences (Buckwalter, 1983; Johnston, 1997; Buckwalter and Mankin, 1998; Newman, 1998).

4.1 Superficial Layer

The superficial layer is exposed to the joint synovium and contains a thin layer of elliptical cells running parallel to the surface. Cells are held in place by type II collagen fibrils. The fibrils in this layer run parallel to the surface, and provide tensile strength to withstand the shear forces experienced on the surface of the cartilage (Roth and Mow, 1980; Johnston, 1997). The small amounts of rough endoplasmic reticulum, Golgi apparatus, and mitochondria per cell suggest less production of proteins for export to the ECM (Buckwalter, 1983; Buckwalter and Mankin, 1998). The concentration of glycosaminoglycans in this area is lower than in deeper layers, suggesting that it may not have a major role in absorbing compressive forces (Buckwalter, 1983; Buckwalter and Mankin, 1998).

4.2 Transitional Layer

The transitional layer contains cells with a shape that are slightly more spherical but exhibit random spacing and direction, while collagen fibrils in this area are randomly oriented (Roth and Mow, 1980; Buckwalter, 1983; Johnston, 1997; Buckwalter and Mankin, 1998). The cytoplasm of the chondrocytes is filled with rough ER, Golgi, and

mitochondria, indicating an increase of protein production by the chondrocytes (Buckwalter, 1983; Buckwalter and Mankin, 1998). Association of PG with the collagen fibrils begins to increase in this layer (Johnston, 1997), but the degree of contact between the PG and collagen is not as extensive as in the deep layer (Buckwalter, 1983; Buckwalter and Mankin, 1998).

4.3 Deep Layer

The deep layer has the least cell density but is the thickest of the three articular cartilage layers. The collagen fibrils are organized perpendicular to the surface, and the chondrocytes are nearly spherical with a tendency to organize in columns, also perpendicular to the surface (Buckwalter, 1983; Johnston, 1997; Buckwalter and Mankin, 1998). The complex network of collagen fibers holds the high concentration of PG in place within the extracellular matrix (Buckwalter, 1983; Buckwalter and Mankin, 1998).

4.4 Calcified Cartilage Layer

The zone of calcified cartilage separates the articular cartilage from the underlying subchondral bone (Buckwalter, 1983; Johnston, 1997; Buckwalter and Mankin, 1998). The collagen fibrils of the deep zone continue into the calcified layer anchoring the articular cartilage to the calcified bone (Roth and Mow, 1980; Buckwalter, 1983; Johnston, 1997; Buckwalter and Mankin, 1998). Cells in this region are small, have very little cytoplasm, and no ER is present (Buckwalter, 1983; Buckwalter and Mankin, 1998).

5. Pericellular, Territorial, and Interterritorial Regions

Three regions radiate out from the chondrocyte or cluster of chondrocytes; the pericellular, territorial, and interterritorial region (Buckwalter and Mankin,1998).

The innermost region, the pericellular, is rich in PG as well as collagen VI (Poole et al., 1988; Buckwalter and Mankin, 1998). The higher concentration of PG allows this region to absorb and distribute the loads applied to the joint (Poole et al., 1988). The PG attract large amounts of water, that when compressed absorb, the energy and flow to areas of less constriction (Roth and Mow, 1980). The chondrocyte is in direct contact with the matrix in this region, and extends cytoplasmic projections through the pericellular to the adjacent territorial region (Buckwalter and Mankin, 1998). On the chondrocyte surface, several integrins and CD44, allow direct interaction of chondrocytes with the surrounding matrix (Helfrich and Horton, 1999). In vitro studies have identified Integrin $\alpha 1\beta 1$ as binding chondrocyte to type VI in the matrix while both $\alpha 2\beta 1$ and $\alpha 3\beta 1$ have been found to bind chondrocytes to type II collagen (Holmvall et al., 1995; Loeser, 1997). CD44, a cell surface proteoglycan, interacts with hyaluronate, directly linking the cell to the surrounding pericellular matrix (Helfrich and Horton, 1999).

The territorial region surrounds the pericellular region. The collagen fibrils of the territorial region closest to the cell are thin and adhere to the pericellular region (Buckwalter and Mankin, 1998). As the fibrils radiate out from the cell, they form a mesh-like structure around the cell or group of cells. This mesh-like structure is believed to provide protection from mechanical forces during loading and tissue deformation (Buckwalter and Mankin, 1998). As fibrils extend out from the basket formation, their diameter dramatically increases. The fibril arrangement becomes parallel, similar to that in the outer interterritorial region (Buckwalter and Mankin, 1998).

Unlike the pericellular and territorial regions which function primarily to support the chondrocytes, the interterritorial region (the area between cells) provides the articular

cartilage with its mechanical properties (Buckwalter and Mankin, 1998). The fibrils in this region have the largest diameters, and run perpendicular to the surface (Buckwalter and Mankin, 1998).

6. Osteoarthritis

Characterized by pain, deformity, limitation on range of motion, and joint swelling, OA usually results in slow progressive disability (Sweet et al., 1977; Bland, 1983; McDermott and Freyne, 1983; Martin, 1994). Similar observations of OA can be found as far back as 1743 by Hunter (Bland, 1983; Newman, 1998). In 1942, Bennett et al. was the first to clinically describe in detail development of OA in the knee (Bland, 1983). The etiology of OA is still unknown, but most explanations of its development fall into one of two categories: "normal forces acting on abnormal cartilage (with inadequate healing response), or excessive forces acting on normal cartilage" (Sweet et al., 1977; Martin, 1994). In either situation, the end-stage OA results in articular cartilage loss, with the possibility of bone-on-bone weight-bearing and severe disability and pain (Martin, 1994).

Two types of OA have been defined. Primary OA is a rare inherited form characterized by its severity and is intensely inflammatory. Secondary OA is used to describe the disorder when direct causes are known, such as joint instability (Bland, 1983).

The pathophysiology of OA includes focal fissuring, pitting, and erosive lesions on the articular surface (Sweet et al., 1977; Poole et al., 1988). Initially, mitosis and formation of chondrocyte clones with increased rates of synthesis of PG and collagen are detected (Mankin et al., 1971). Increased water content in the ECM has also been noted

in the initial onset of OA (McDevitt and Muir, 1976; Sweet et al., 1977; Bland, 1983; Newman, 1998). Proteoglycan content of the articular cartilage decreases (McDevitt and Muir, 1976; Sweet et al., 1977; Bland, 1983; Johnston, 1997; Newman, 1998), due to an inability of chondrocytes to repair the progressing damage (Mankin et al., 1971; Furukawa et al., 1980; Martin, 1994). Thus, the ability of cartilage to absorb compression applied to the weight-bearing regions decrease (Johnston, 1997). As articular cartilage is lost, sclerosis of the subchondral bone begins (Mankin et al., 1971; McDevitt and Muir, 1976). Remodeling of the entire end of the bone will take place by end-stage OA. Marginal osteophyte formation and subchondral bone cysts are also common with OA development (Mankin et al., 1971; McDevitt and Muir, 1976; Martin, 1994).

7. Research Models of Osteoarthritis

7.1 In vivo models

In vivo models use several different species. Both the Pond-Nuki canine model and the Moskowitz lapine model were the earliest in 1973 (Lozada and Altman, 1999). The Pond-Nuki model directly destabilizes the stifle joint by transecting the anterior cruciate ligament to study how destabilization affects articular cartilage (Lozada and Altman, 1999). The Moskowitz model mimics a meniscal tear by surgically slicing the medial meniscus from the anterior pole to the mid-medial collateral ligament parallel to the underlying bone (Lozada and Altman, 1999). The partially free meniscus can then act as a loose body changing the forces across the surface and possibly destabilizing the knee (Lozada and Altman, 1999). Destabilization of a joint can be induced in multiple forms

and applied to many species. Meniscetomy models, like Moskowitz's rabbit model, have also been applied in guinea pigs and sheep (Lozada and Altman, 1999).

Chemical treatment is another technique to induce OA symptoms. Williams et al. (1997) and Uebelhart et al. (1998) injected 2.0 mg chymopapain directly into the knee joint of adolescent rabbits to chemically induce OA symptoms. Williams et al. (1997) showed that concentrations of keratan sulfate in serum rapidly returned to baseline following chymopapain treatment, while bone-specific collagen cross-links in serum increased once matrix PG were depleted following the treatment. Uebelhart et al. (1998) injected chymopapain directly into the knee joint of rabbits to induce severe PG loss from the articular cartilage. Fibronectin fragments have also been injected into knee joints of rabbit models. Homandberg et al. (1993) has shown such injections have reduced PG content by 50 % in the first seven days in healthy cartilage. Injection of high molecular weight hyaluronan before administration of the fragments in the same model was shown to decrease the PG loss previously seen by almost 30 % (Williams et al., 1997).

Certain species spontaneously develop OA. Dunkin Hartley guinea pigs gradually lose collagen and PG concentrations from the ECM as early as 3 mo. By 22 mo, radiographs reveal osteophytes of the tibia and femur, sclerosis of the subchondral bone, femoral condyle cysts as well as collateral ligaments. Lozada et al. (1999) studied the spontaneous onset of OA in Rhesus monkeys at the Caribbean Primate Research Center in Cayo Santigo, Puerto Rico. Most notably is the similarity of onset to human OA with respect to age, sex, joint histology, and cartilage composition. Osteoarthritis was seen to increase with age and, in females, with parity. In addition, a significantly

higher incidence of OA was seen in free-ranging monkeys to those in cages (Kessler et al., 1986).

Saamanen et al. (2000) developed a transgenic Del 1 (+/-) mouse line, which carries several copies of a transgene, featuring a deletion mutation causing truncated type II collagen. Analysis by histology, northern hybridization, RNase protection assay, and immunohistochemistry revealed severe cartilage degradation, significant reduction in endogenous and transgene type II collagen mRNA with subchondral bone exposure in the end stage. This model was developed to study early pathogenic mechanisms in articular cartilage degradation (Saamanen et al., 1986).

Immobilization of joints in both canine and lapine models have shown that lack of applied force to normally weight bearing regions in the joint decreases PG synthesis. New PG no longer formed aggregates, as revealed by loss of histological staining; additionally a decrease in cartilage thickness was seen. After remobilization, most symptoms were completely reversed (Palmoski et al., 1997).

Trauma models also have been used to study the development of OA. These models examine how blunt trauma to a joint influences the biomechanical aspects of articular cartilage and subchondral bone. Ewers et al. (2000a, 2000b) describes softening of articular cartilage without thickening of the subchondral bone following a blunt insult to the patello-femoral joint. Newberry et al. (1998a) used the same model, a single blunt impact followed by regular exercise, to induce degradation of the joint tissue similar to early stage osteoarthritis. They observed thickening of the subchondral bone and fissuring on the articular cartilage surface (Newberry et al., 1998a). Newberry et al. (1998b) also used the single insult rabbit model to identify changes in cartilage and

subchondral bone following impact of different intensities. At high intensity, thickening of the subchondral bone and softening of the retro-patellar cartilage was observed along with histological evidence of lesions and fissures. Low intensity impacts did not cause the same progressive changes. This study was designed to identify "safe" and "unsafe" ranges of acute tissue stress, with the intention of applying the design to an injury prevention model (Newberry et al., 1998b).

7.2 In vitro models

Hauselmann and Hedbom (1999) categorized in vitro models of cartilage metabolism into three groups: 1) three-dimensional gel, 2) high density monolayer and 3) tissue/explant systems. All of these models have been used to better understand how the tissue, and specifically the chondrocytes, react to their environment. As with the in vivo models, each of these three in vitro models have advantages and disadvantages depending on the information sought. Cartilage metabolism is the primary focus of the tissue/explant in vitro model, with both cellular and matrix reaction to stimuli being sought. The tissue explant model usually consists of freshly excised tissue under aseptic conditions cultured in media.

Hauselmann et al. (1999) describes gel suspension and monolayer culture systems. They describe gel suspension and monolayer systems as having advantages in preservation of chondrocyte phenotype (in alginate or agarose gel), good homogeneity, easy detection of gene expression, quantification of specific gene and isolation of macromolecules. The study of the matrix constituents and supramolecular organization and persistence of matrix-dependent control of chondrocyte behavior are limited with these systems.

Robbins et al. (2000) compared a monolayer and alginate systems on transformed tsT/AC62 chondrocytes to find which system, when challenged with IL-1β, would respond with mRNA known to be activated with IL-1β treatment. Levels of COL2A1, aggrecan, MMP 1,3 and 13, as well as phospholipase A2 type IIA were all found to increase as expected in the alginate system. Inducible nitric oxide synthase mRNA was not found to increase. In the monolayer system, increases in the mRNA levels were not as characteristic of chondrocytes challenged with IL-1β.

Hauselmann et al. (1999) list many positive characteristics of the tissue/explant system including preservation of chondrocyte phenotype, persistence of the differences naturally seen between chondrocyte sub-populations, persistence of matrix-dependent control of chondrocyte behavior, steady state metabolic activity with respect to matrix macromolecules similar to in vivo, and no excessive cell proliferation, as is seen in other in vitro models. Unfortunately, technical aspects including handling, lack of homogeneity between samples and difficulty of study with regard to specific gene products, cellular receptors, and isolation of matrix macromolecules are disadvantages to the tissue/explant system (Hauselmann et al., 1999).

In the case of Dumont et al. (1999), full-thickness articular cartilage explants from steers were studied for stability in culture under serum-free conditions. Cell viability was found to be stable as well as PG metabolism, determined by radio-labeled sodium sulfate and assay by DMMB. Collagen metabolism also remained stable as measured by C-propeptide of type II procollagen. The stiffness of the disks was then tested to evaluate if possible breakdown of the matrix had occurred. Tissue cultured in serum-free media did not show a difference from fresh tissue.

8. Effects of loading on cartilage explants

To study the effects of dynamic compression, Parkkimen et al. (1992), repeatedly loaded bovine articular cartilage. Radio-labeling indicated stimulation of PG synthesis was limited depending on length of interval between compression of 2 to 4 sec, longer intervals of 20 and 60 sec did not have the same effects (Parkkimen et al., 1992; Burton-Wurster et al., 1993). Less force was needed to stimulate PG synthesis in the superficial layer relative to the middle layer (Parkkimen et al., 1992).

Farquhar et al. (1996) evaluated if cyclical impact could induce damage to cultured cartilage. A 50 MPa load was applied every 5 sec for 30 min. Radio-labeling of sulfate incorporation indicated incorporation into PG synthesis at 0 to 4 h after impact but not between 20 to 24 h. Visible damage to the explant was seen at 20 to 50 MPa, while subtle damage could be seen from 5 or 10 MPa insult. Swelling of the explants in 0.01 M NaCl, indicated possible matrix damage 10 d post impact. Water content and fibronectin content were increased in loaded explants.

Loening et al. (2000) published results describing the effects of injurious compression on newborn bovine articular cartilage explants. A significant decrease in stiffness of the cartilage was demonstrated at 24 MPa (confined compression). Chondrocyte apoptosis occurred at loads as low as 4.5 MPa and increased in a dose-dependent manner as load increased. Maximal apoptosis was seen by 24 h post-impact. Unconfined compression at loads of 7 to 12 MPa degradation of the collagen fibril network began to appear, significant degradation at 13 MPa was related to tissue swelling and a dose-dependent response to damage. Glycosaminoglycan release was dose-dependent with significance at 6 to 13 MPa peak stresses. Nitrite release was significant

with 20 MPa peak stress. Loening et al. (2000) reported that cellular death due to apoptosis took place at lower stresses than those required to stimulate matrix degradation and biomechanical changes, suggesting apoptosis may be an early response to tissue injury.

Torzilli et al. (1998) used cyclic loading on live versus killed chondrocyte explants to evaluate if the decrease of PG release immediately following loading was due to a cellular response. In a previous study (Torzilli and Grigiene, 1997), a significant decrease in PG synthesis was observed following static or dynamic impact independent of the length of time applied. Proteoglycan synthesis was studied with radiolabeling, before, during and after continuous 1 Hz, 1 MPa loading for 24 h. Proteoglycan release in live loaded explants decreased by 50 % compared to unloaded explants. Both live and killed chondrocyte explants release 5 to 10 times more PG than non-impacted explants. After 24 h post-impact, PG release of both impacted explant groups returned to levels similar to non-impacted groups. Ewers et al. (2001) impacted (40 MPa) full thickness bovine articular cartilage explants to evaluate two rates of loading, fast (~900 MPa/s) and slow (40 MPa/s), on the distribution of cell death four days post impact. Rate of loading was found to have a significant affect on the degree of matrix damage and the distribution of cell death. Matrix damage, evaluated by measuring total length and depth of surface lesions, was found to be greater in the fast rate of loading compared to the slow rate of loading. Fast rate of loading also resulted in cell death primarily adjacent to lesions, while slow rate of loading resulted in a more diffuse distribution of cell death throughout the tissue.

9. Therapy-Glucosamine

Glucosamine in many different forms has been used to treat the symptoms of OA. Glucosamine, a ubiquitous amino monosaccharide, is a primary building block of PG. Glucosamine is used to synthesize the glycosaminoglycans keratan sulfate and chondroitin sulfate, which in turn form side chains off the core protein of aggrecan (McNamara et al., 1997). Glucosamine also is a component in biglycan, decorin, and hyaluronic acid, all of which are found in articular cartilage (McNamara et al., 1997). Clinical trials using glucosamine sulfate (GS) have been found to modify symptoms of OA, including decreased pain and increased joint flexibility (Houpt et al., 1999). The Osteoarthritis Research Society has classified glucosamine as a symptom-modifying drug (Piperno et al., 2000). Setnikar et al. (1986) studied the pharmacokinetics of glucosamine in both canines and humans, and have shown both oral and I.V. administration provide "free" glucosamine in plasma within 15 min of introduction. In the "free" state, glucosamine was found to be able to diffuse through the biological membranes, thereby allowing a majority of the administrated glucosamine to be absorbed. Upon tissue analysis, Setnikar et al. (1986) showed that glucosamine concentrated in the liver, kidney and articular cartilage, with primary excretion through urine. Additional studies involving GS treatment have shown reduced progression of joint space narrowing in patients with knee OA (Reginster et al., 1999). For this reason, GS is being investigated as a possible disease modifying agent (Piperno et al., 2000).

Possible mechanisms by which glucosamine may bring about these changes include stimulation of the chondrocytes to synthesize glycosaminoglycans and collagen through undefined mechanisms (McNamara et al., 1997). Piperno et al. (2000) showed

treatment with glucosamine significantly increased protein kinase C activity in OA chondrocytes. This might inhibit down regulation of PG synthesis induced by IL-1 (an inflammatory cytokine seen in OA). By allowing PG synthesis to continue, glucosamine may prevent or alleviate the problems associated with PG degradation as seen in OA.

Fenton et al. (2000 a, b) described the effects of Glucosamine-HCl, Glucosamine-SO_4 (GS), and N-acetyl glucosamine to prevent degradation due to LPS or IL-1β treatment. Nitric oxide, PG and MMP released into the media were studied to see if degradation occurred. A dose of Glucosamine-HCl at 25 mg/ml was found to significantly reduce the release into media of NO, PG and MMP. Using similar molar concentrations, Fenton et al. (2000b) then studied GS and N-acetyl glucosamine. In this study, the release NO and PG, as well as total tissue PG content, were analyzed. GS inhibited NO and PG release similar to that of Glucosamine-HCl, while N-acetyl glucosamine responses were highly variable. These studies revealed the potential to limit or inhibit cartilage degradation due to LPS or IL-1B.

10. Focus of my thesis research

Traumatic injury to a joint may lead to post-traumatic OA. Therefore, a better understanding of OA development may be obtained by comparing loads of 15 MPa to 40 MPa and force (50 msec or 1 sec) to cytokine treated explants to examine the initial and direct effects on articular cartilage. Specifically, I studied how blunt trauma affects PG and NO metabolism as well as cell death and apoptosis. With increasing load and length of time applied, increases in cell death, apoptosis, and PG release were expected. An in vitro model allows measurable responses of metabolic release and this, in turn, may allow a clearer understanding of how blunt trauma affects articular cartilage.

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Chapter 2

Acute loads with varying rate of load on a mature bovine articular cartilage explant system

Abstract

Mature bovine articular cartilage explants were used to compare physiological (15 MPa) and traumatic (30 MPa) loaded explants, at fast and slow rates of load (50 msec, 1 sec), to a cytokine treatment (interleukin-1beta or lippopolysaccharide) and a non-impacted control group. Nitric oxide (NO) release was found to be negligible in all impacted treatments, while cytokine treatments produced significant NO concentrations. Activity for matrix metalloproteinases-2 was seen in both loads and rates of impact. Proteoglycan (PG) release into media was greatest from traumatic slow rate of impact. The addition of glucosamine-sulfate (GS) did alter PG release in the treatments. Glucosamine-sulfate 15 MPa slow had less PG release than 15 MPa slow, while GS 30 MPa slow had greater PG release than 30 MPa slow. GS appears to have little or no effect on cell viability due to loading. Traumatic levels of loading at a slow rate caused significant cell death compared to controls and cytokine treatments, and a slow rate of impact in general, gave higher levels of cell death than a fast rate of impacts. Rate and load of impact were found to affect cartilage explants differently than cytokine treatment, therefore care should be taken when designing future experiments as the damage appears to be rate dependent on rate of load as well as the level of load.

1. Introduction

With arthritis affecting more than 43 million Americans (CDC), a better understanding of the initial development of osteoarthritis (OA) is being sought. Secondary osteoarthritis is believed to develop from one of two scenarios, either normal

forces acting on abnormal cartilage (with inadequate healing response), or excessive (injurious) forces acting on normal cartilage.

Swelling, decreased tensile strength, visible degradation of the articular cartilage, and cell death are all characteristics of OA. Mechanical injury of articular cartilage induces similar damage, suggesting mechanical injury can initiate OA development. In vivo, animal models have been used to study how traumatic injury can affect the joint, and in general, articular cartilage. The Pond-Nuki model, which destabilizes the joint by transecting the anterior cruciate ligament and the Moskowitz model, which mimics a meniscal tear by surgically slicing medial meniscus, are two of the earliest traumatic injury models. These models are useful for monitoring damage to the articular surface. Trauma models such as the ones by Ewers et al. (2000a, 2000b) and Newberry et al. (1998a, 1998b) examined single blunt trauma in rabbits. Fissures and changes in subchondral bone were analyzed, but changes in extracellular matrix components were not examined.

Explant loading models have been used to observe the effects of applied forces on articular cartilage; specifically changes in PG and collagen content and synthesis. Repeatedly loaded (0.5 to 1.0 MPa) articular cartilage explants stimulated PG synthesis in the superficial layer to a higher degree than the middle layer (Parkkinen et al., 1992). Explants cyclically impacted at 20 or 50 MPa induced visible damage of the collagen matrix of the cartilage at both levels of load, while only subtle damage was seen at those impacted at 5 or 10 MPa (Farquhar et al., 1996). Swelling in osteoarthritic cartilage is due to the breakdown of the collagen network (Bank et al., 2000). Mechanical injury decreases the tensile strength of the collagen network, allowing the tissue to expand as

fluid is drawn into the negatively charged spaces between proteoglycan aggregates. Breaks in the collagen fibrils were found in the type II fibrils, not at the crosslinks between fibrils (Bank et al., 2000). Swelling was found to be proportional to the amount of degraded collagen in all three zones, and since the collagen provides cartilage with its tensile strength, the decrease in stiffness is due to collagen degradation and not PG loss. Additionally, Quinn et al. (1998) have shown that strains of 50% can significantly decrease strength of the collagen network and thereby decrease the tissue's load bearing ability. Loening et al. (2000) have shown that loads as low as 4.5 MPa induce apoptosis in impacted explants. Previously, Ewers et al. (2001) have shown fast versus slow rates of loading can affect placement and the amount of cell death following impact at 40 MPa of unconfined compression. The fast rate of loading resulted in cell death adjacent to fissures. Compared to the fast rate of loading, the slow rate of loading resulted in greater amounts of cell death with a more diffuse distribution away from the fissures (Ewers et al., 2001).

Cytokine treatment of explants is another model used to study in vitro cartilage degradation. Fenton et al. (2000a) have shown that stimulation by cytokine (LPS or IL-1β) showed increases in NO production, PG, and matrix metalloproteinase release. The addition of glucosamine hydrochloride and glucosamine sulfate could prevent the experimentally induced degradation (Fenton et al., 2000a; Fenton et al., 2000b). Few studies have directly compared the effect of impact or cytokine treatment on articular cartilage damage. One study found injurious compression in calf articular cartilage explants led to the release of high molecular weight proteoglycan fragments, which were different from proteoglycan fragments released from cartilage following Interleukin-1

stimulation (Quinn et al., 1998). The purpose of our research was to compare acute loading and cytokine treated cartilage degradation in mature bovine articular cartilage explants. Increased load and length of load (rate) were expected to cause an increase in PG release as well as an increase in cell death. Possible increase in MMP-2 activity and a decrease in overall PG content were expected with increased load and rate.

2. Materials and Methods

2.1 Experimental Model

Eleven pair of skeletally mature bovine forelegs (18 to 24 mon of age, (9) Holstein, (1) Angus, (1) Herford, steers) were obtained from a local abattoir within three hours of slaughter. Two pair of Holstein were used for each replicate, with the third pair varying in breed in third replicate. The legs were cleaned with distilled water, the joint was skinned, the hoof bagged, and the joint was rinsed again, with distilled water, before opening the joint. The foreleg joints were opened in a laminar flow hood to expose the second, third and fourth carpals. A 6 mm biopsy punch (Miltex Instrument Company Inc., Bethpage NY) was used to make approximately 20 explants per joint which were separated from the underlying bone using a scalpel. Once separated from the bone, explants were placed in a petri dish containing Dulbecco's Modified Eagles Medium (DMEM): F12 (Gibco, USA # 12500-039).

Once all explants were retrieved, they were washed two additional times (10 min each) in DMEM: F12 medium. Approximately 40 mg of cartilage (2 explants) were randomly placed into wells of 24 well culture plates. Each well contained 1 ml of DMEM: F12 supplemented with 50 μg/ml ascorbic acid, 20% fetal bovine serum (Gibco BRL, USA), 21.9 mg/ml glutamine (Sigma, St Louis MO), additional amino acids

(Sigma), and antibiotics (Antibiotic-Antimycotic, Gibco BRL, USA). Explants were allowed to equilibrate two days with media changes daily, and specimens were kept at 37° C, and approximately 7% carbon dioxide, in a humidified NuAire incubator (Plymouth, MN).

After equilibration, explants were assigned to different treatment groups depending on the experiment. An Instron (model 1331, Canton MA) was used to deliver a peak load of the assigned level to the specified groups. Briefly, specimens were placed between two highly polished stainless steel plates of the servo-hydraulic machine, where peak load, time to peak, and maximum displacement were recorded in each experiment. After loading, the explants were washed three times in DMEM: F12 medium (10 min each wash) before placing them in new pre-assigned culture plates. Cytokine treatments were stimulated with either 50 ng/ml of human recombinant interleukin 1β (IL-1) or 10 μg/ml of lippopolysaccharide (LPS) for 24 h beginning on day of impact for loaded treatments. One ml of medium, supplemented as mentioned previously, was added to each well. Individual experiments will be described in the results.

Media from each well were collected every 24 h and kept at 4° C for quantitative analysis within 14 d. Four days post treatment; explants from each treatment were randomly divided for cell viability, papain digest, and gross histology.

2.2 Cell Viability

Two 1 mm slices were cut from the center of each explant selected for cell viability analysis, using a specialized cutting tool designed in our laboratory. The sections were stained using a commercial kit containing calcein and ethidium bromide homodimer (Live/Dead Cell Viability/Cytotoxicity, Molecular Probes, Oregon USA).

All specimens were viewed using a fluorescence microscope (Leica DM LB (50-60 Hz) Leica Mikroskopic und Systeme GmgH, Welzar, Germany). Digital images (100x magnification) of the explant thickness were taken at the center of each slice (Spot Digital Camera, Diagnostic Inc.). Cell viability was quantified by manually counting the dead (red) and viable (green) cells for each picture using the image software, Sigma Scan (SPSS Inc., Chicago IL). The totals from the two slices were averaged for each explant.

2.3 Proteoglycan Analysis

Papain digestion of the remaining explants was used to determine remaining PG content. Media samples were analyzed for PG content. The Dimethylmethylene Blue (DMB) assay was used to determine PG concentration in media or digested tissue content. Chontroitin sulfate was used as the standard (Chandrasekhar et al., 1987).

2.4 Nitric Oxide Analysis

Nitric oxide was measured in media by comparing a standard of sodium nitrite in media to the nitrite (a stable metabolite of nitric oxide) in the samples. The Griess reaction was used to produce a quantitative color change measured at 540 nm (Blanco et al., 1995). All biochemical assays are measured using a Molecular Devices Spectra Max Plate Reader (Spectra MAX 300, Sunnyvale, CA).

2.5 Gelatinase Analysis

Following the final media collection, four explants per treatment were randomly selected to analyze gelatinase activity. Following extraction of the tissue, the Pierce protein assay was used by comparing samples to a standard curve of BSA. Eight μg of protein from pooled explants were loaded for each treatment into an 8% PAGE gel for

separation and identification against Bio Rad SDS-Page Molecular weight standard broad range marker.

2.6 Statistical analysis

In all experiments the mean values for each treatment per day $\pm$ standard deviations were calculated. The results were compared using ANOVA Student-Newman-Keuls method on the program SigmaStat. Statistical significance was considered at $P < 0.05$ unless noted.

3. Results

Severe mechanical load on articular cartilage at 40 MPa with loading rates of 50 msec or 1 sec was compared for effect on PG and NO release to a cytokine treated IL-1 group. Nitric oxide analysis on days 1 and 2 post treatment revealed only IL-1 treatment as significantly greater than control or impacted treatment (Figure 1). Interestingly, neither impacted treatment revealed significantly elevated NO release. Proteoglycan release showed significantly elevated levels of release only in the slow impacted treatment (Figure 2). By day 2, all treatments had returned to control levels.

A high physiological load of 15 MPa and a traumatic load of 30 MPa were compared at different rates (50 msec and 1 sec) to the cytokine treated group of IL-1. When comparing fast and slow impact rates at both 15 MPa and 30 MPa unconfined compression to IL-1 treatment, IL-1 cytokine treatment significantly stimulated NO release compared to all other treatments on both days 1 and 2 (Figure 3). Of the impacted treatments, 30 MPa slow NO release was significantly elevated relative to all impacted treatments on both days of analysis. After treatment of either impact or IL-1, only the slow 30 MPa impact treatments revealed significant elevation of PG release to all other

treatments on days 1 and 2 (Figure 4). Papain digest of explants revealed only IL-1 treated explants had significantly greater PG content than impacted treatments of 15 fast and 30 slow (Table 1). MMP-2 appears to have increased activity in the impacted versus control or IL-1 treatment in an 8% gelatin SDS-PAGE gel (Figure 5).

The previous experiment was repeated with the addition of glucosamine sulfate (GS) being added to each treatment two days prior to impact or cytokine treatment. Analysis of PG release on day 1 revealed a significant increase in all treatments compared to control. Glucosamine treatment had no effect on PG release in 15 MPa or 30 MPa fast treatments compared to treatments without GS on both days. The 15 MPa slow glucosamine treatment was significantly lower in PG release than 15 MPa slow without glucosamine, while the severe compression of 30 MPa slow showed significantly higher PG release versus same impact treatment with glucosamine. All impacted treatments were significantly greater than IL-1 treatment or control (days 1, 2) (Table 2).

Cell viability was analyzed following treatment at impact of 30 MPa fast or slow rate, with and without GS pretreatment. Non-impacted controls were compared to 30 MPa fast and slow rate impacted groups to determine if cell viability was affected by impact (Table 3). All impacted groups were found to have significantly more cell death when compared to the non-impacted controls (with and without GS) ($P < 0.05$). The fast rate of loading groups (with and without GS) were found to have significantly less cell death than the slow impacted groups (with and without GS).

Impact at 15 MPa fast or slow rate was compared to LPS treatment with and without pretreatment of GS. LPS was used for cytokine stimulation in this trial and compared to 15 MPa impact at fast and slow rates with and without glucosamine

treatment. NO release of the impacted treatments was similar to those in the previously mentioned trials (Table 4). No increase was seen in comparison to the control group (day 1 and 2). The LPS treatment was elevated with a significant increase on day 1 versus all other treatments. All glucosamine treatments were significantly lower in NO release on day 2 compared to control or fast, slow impact without glucosamine. PG release on day 1 revealed the LPS treatment release elevated and significant from glucosamine LPS, control, fast glucosamine, fast (Table 5). Glucosamine slow was also elevated and significant compared to the lowest PG release of Glucosamine LPS. On day 2, glucosamine slow was elevated compared to control, LPS, fast, fast glucosamine, glucosamine control and glucosamine LPS.

4. Discussion

Nitric oxide analysis of all the experiments revealed many similarities between experiments regardless of impact load, rate or cytokine treatment used. As with previously published works (Stadler et al., 1991; Bird et al., 1997; Hayaski et al., 1997), IL-1 treatment at 50 ng/ml stimulated a significant increase in NO release. Additionally, Fenton et al. (2000a, 2000b) has shown increased NO release after stimulation with 10 μg/ml LPS 24 and 48 h after treatment. With the exception of 30 MPa slow impact in one experiment, all mechanically loaded treatments showed no change in NO release when compared to control treatments. The increase in 30 MPa slow was not at a concentration comparable to cytokine stimulation and is at the low end of acceptable detection levels for this assay. Loening et al. (2000) saw a significant increase in NO release with calf cartilage at loads of 20 MPa 4 d post impact. Cellular metabolism of immature cartilage has been shown to be higher than that of healthy mature cartilage and

may explain the differences with our results. Loening et al. (2000) reported a 1.37 magnitude increase in NO due to the impact at 20 MPa. While our impacted treatments did not release significant amounts of NO, our cytokine treatment stimulated NO release at levels of 21 times that released by non-impacted controls. So, while the NO release of Loening's et al. (2000) impacted explants did report NO release statistically greater than that of their non-impacted explants, the degree of release was not of a great magnitude when compared to the potential NO release stimulated by cytokine treatment (Fenton et al., 2000a; Fenton et al., 2000b). Cytokine treatment is known to stimulate NO release by directly inducing synthesis of nitric oxide synthase production in chondrocytes via the NFκB pathway. The mechanically loaded mature cartilage explants may not stimulate the same cellular pathway.

Few laboratories have compared PG response in cytokine stimulation and acute loading in mature cartilage. Our results agree with Stefanovic-Racic et al. (1991), Fenton et al. (2000a, 2000b), and Bird et al. (1997), where increased, but not significant, PG turnover was shown following cytokine stimulation. The increase in PG release may be due to an increase in catabolism by increased levels of matrix metalloproteinases. MMP activity has been shown to be activated by the increased NO production due to cytokine treatment (Stefanovic-Racic et al., 1991; Fenton et al., 2000a; Fenton et al., 2000b; Hickery et al., 1998). Mechanical loading gave significant PG release with differences based on load and rate of impact. The 30 and 40 MPa load PG results agree with the previous experiments of Loening et al. (2000), where loads of up to 24 MPa were analyzed. The increase in PG release found in the media following extreme impact may be due to turnover from excessive loading of the articular cartilage, not to chemical

breakdown as seen in cytokine treatments. Loening et al. (2000) found that increased PG release corresponded with greater collagen network damage. Increased matrix damage increases swelling and would allow PG to diffuse more easily from the damaged matrix (Quinn et al., 1998). Total PG content of IL-1 was higher compared to impacted groups but not control. IL-1 is known to decrease PG synthesis (Bird et al., 1997; Hickery et al., 1998) making it unlikely that PG synthesis following the removal of IL-1 was able to increase overall PG content in 24 h. The loading of cartilage, both at high physiological or extreme levels, significantly increased PG release, possibly lowering the overall PG content enough to be different from the cytokine treated. A long term study following treatment with cytokine versus impact may be useful in determining if a long term significant decrease in PG content is observed versus cytokine and control treatments. Pap et al. (1998) and Tetlow et al. (2001) have reported increased MMP activity in OA cartilage, MMP 1, 3, 8, and 13 have specifically been seen in the superficial layer and around lesions originating in the superficial layer. Traumatic injury models have also reported MMP activity. Following impact to canine patellae, MMP-3 was found to be elevated (Pickvance et al., 1993). Using transection of the anterior cruciate ligament or medial cruciate ligament, Hellio Le Graverand et al. (2000) and Bluteau et al. (2001) reported increased MMP-13 and MMP-1, 3, 13 respectively. Our analysis has shown that, in culture following impact, MMP-2 was detectable qualitatively. Clearer bands were present in the impacted treatments than control or IL-1 treated. Less prevalent bands in the IL-1 treatment, also seen by Tetlow et al. (2001), indicated cytokine presence may not be necessary to stimulate MMP activity. Increases in MMP activity

following impact may explain the long-term effects of cartilage degradation that can develop into OA over time.

Glucosamine sulfate (GS) was used to pre-treat explants before stimulation with either LPS or impact. The affect of the pre-treatment to prevent direct damage to the articular cartilage was evaluated. Cytokine treatments had significantly greater NO release than control. Our results following pretreatment with GS agreed with Piperno et al. (2000) and Fenton et al. (2000a, 2000b) experiments with cytokine treatments. A significant decrease in NO release was seen in GS treated LPS groups than those without GS treatment. The addition of GS inhibited the inducible nitric oxide synthase, which produces most of the nitric oxide found after cytokine stimulation (Fenton et al., 2000a). Piperno et al. (2000) has shown glucosamine sulfate significantly increases protein synthesis in a dose dependent manner in OA cartilage (where NO is known to stimulate inflammation), while Fenton et al. (2000b) has directly shown glucosamine sulfate to inhibit PG degradation in explants stimulated by IL-1 or LPS. Together they explain the decreased PG release of their results when GS is added. Our results do not show a significant increase in PG release with cytokine treatment, and therefore the effect of decreased NO to stimulate PG release was not applicable. In mechanically impacted treatments, the effect of GS on PG release appears to be minimal between treatments over the two days monitored, even with significance on both days the results were inconsistent within treatments. The addition of GS significantly lowered PG release in both 15 MPa and 30 MPa slow impacted treatments compared to non-glucosamine impact groups. All impacted treatments showed significant increases in cellular death compared to control. In newborn calf cartilage, Loening et al. (2000) found injurious compression caused

chondrocyte apoptosis to occur with a load as low as 4.5 MPa and increased in a dose-dependent manner with load. Cell death due to apoptosis occurred at lower stresses than those required to stimulate matrix degradation and biomechanical changes, suggesting apoptosis may be an early response to tissue injury. Our results indicate GS does not appear to affect the amount of cell death caused by a particular load, but does appear to be rate dependent. Quinn et al. (1998) and Loening et al. (2000) have both reported cell death in relation to trauma, but neither has analyzed the effect of rate of loading on this aspect. Our finding at 30 MPa would agree with Loening et al. (2000), that extreme trauma causes significant cellular death, but it should be noted that the length the impact lasts also plays a significant role in traumatic injury to mature cartilage. Interestingly, the fast rate of loading (with and without GS) had significantly lower cell death compared to slow (with and without GS). This may be due to the simple affect of the longer impact time damaged more cells.

In conclusion, our results indicate that when studying cartilage degradation, the method of initiation and mechanisms activated can cause significantly different results. Mechanical impact of explants tended to result in mild to severe cell death dependent on rate and level of load. PG release was greater in impacted explants than in the cytokine treatments. NO release was minimal in loaded explants while cytokine treatment released significant levels. MMP-2 activity appeared greater in loaded explants than cytokine treated. The addition of GS was found to have no effect on impacted explants, while cytokine treatments were found to have significantly less NO release following pre-treatment with GS. Further research should focus on catabolic pathways initiated during acute load trauma.

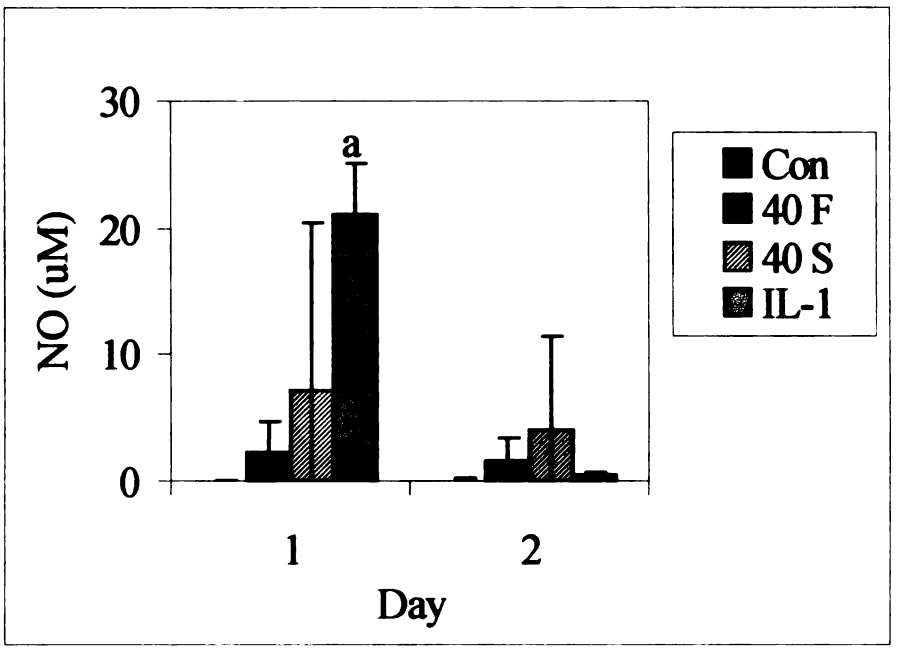

Figure 1: The average amount of nitric oxide (NO) released into the media per well each day post-treatment for control (Con), IL-1 50 ng/ml (IL-1), 40 MPa fast impact (40 F), and 40 MPa slow impact (40 S) (Ave; n=6). The (a) symbol indicated a statistically significant increase in NO release compared to control, fast and slow on that day ($P < 0.05$).

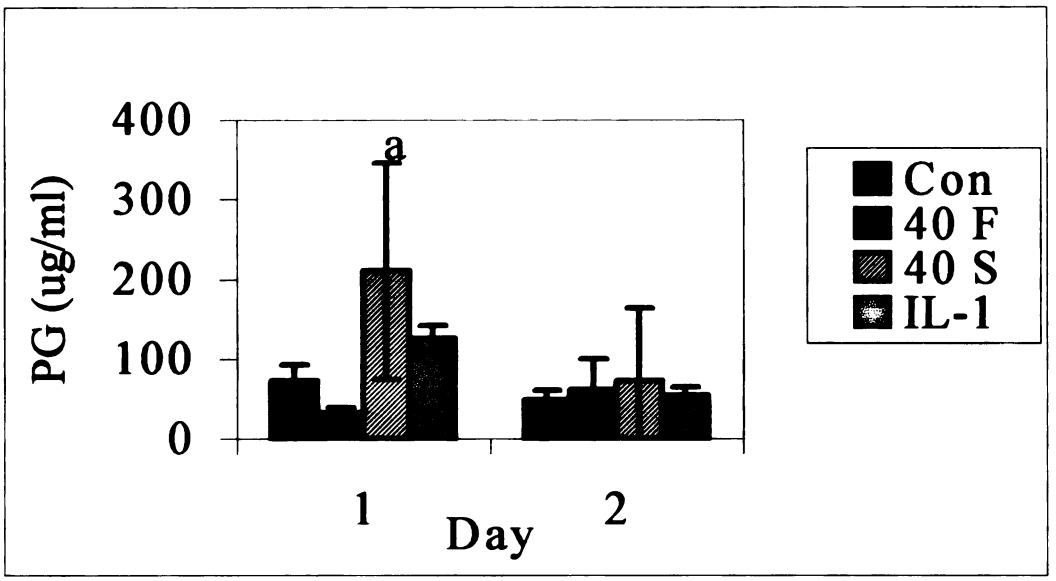

Figure 2: The average proteoglycan released into the media each day post-treatment for control (Con), IL-1 50 ng/ml (IL-1), 40 MPa fast impact (40 F), and 40 MPa slow impact (40 S) (Ave; n=6). The (a) symbol indicates a statistically significant increase in PG release compared to control, fast and IL-1 on that day ($P < 0.05$).

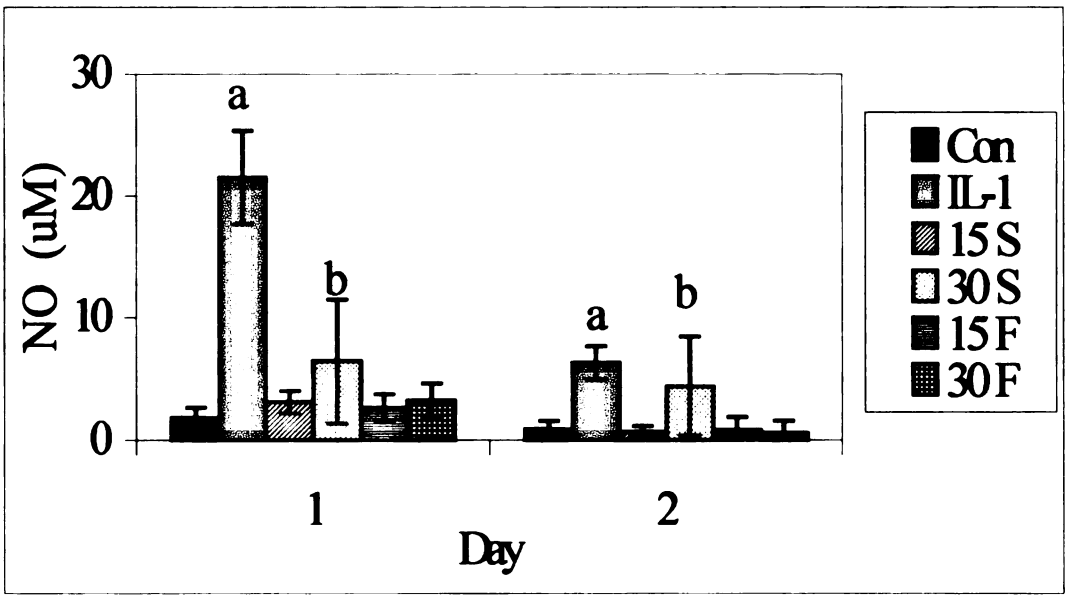

Figure 3: The average amount of nitric oxide (NO) released into the media per well each day post-treatment for control (Con), IL-1 50 ng/ml (IL-1), 15 MPa slow impact (15 S), 30 MPa slow impact (30 S), 15 MPa fast impact (15 F), and 30 MPa fast impact (30 F) (Ave $\pm$ SD; n=8). The (a) symbol indicates a statistically significant increase in NO release compared to control and all impacted treatments on that day ($P < 0.05$). The (b) symbol indicates a statistically significant increase in NO release compared to other impacted treatments on that day ($P < 0.05$).

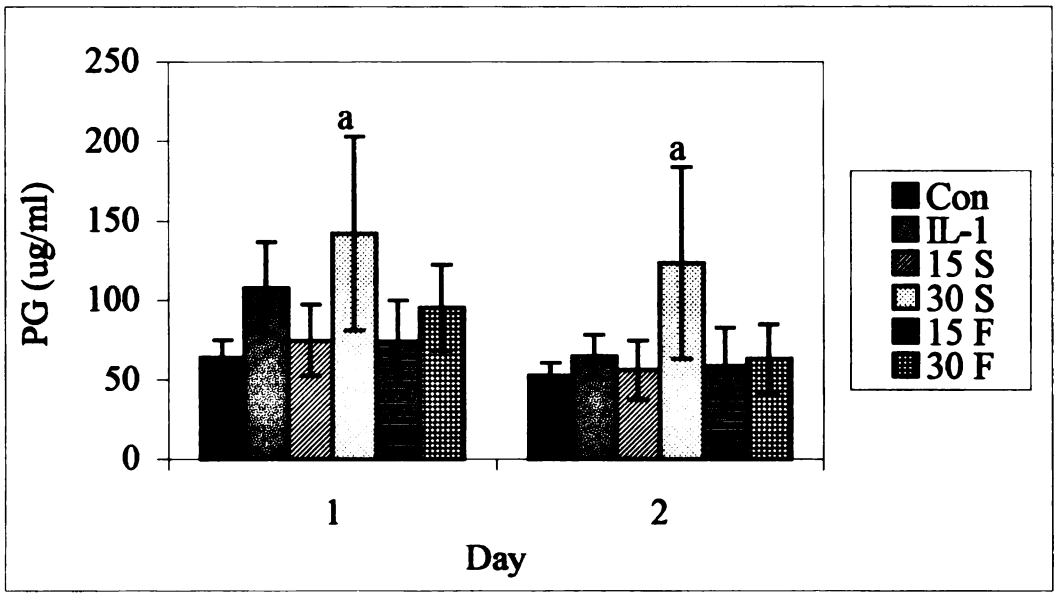

Figure 4: The average proteoglycan released into the media each day post-treatment for control (Con), IL-1 50 ng/ml (IL-1), 15 MPa slow impact (15 S), 30 MPa slow impact (30 S), 15 MPa fast impact (15 F), and 30 MPa fast impact (30 F) (Ave $\pm$ SD; n=8). The (a) symbol indicates a statistically significant increase in PG release compared to control and other treatments on that day ($P < 0.05$).

Table 1 Proteoglycan content of papain digestion

Group	Mean (ug/mg wet weight) ± SD
Con	31.09 ± 0.68
IL-1	34.60 ± 2.15 a
15 MPa S	27.09 ± 1.28
15 MPa F	24.85 ± 5.62
30 MPa S	23.54 ± 5.14
30 MPa F	26.13 ± 2.25

Table 1: The mean μg of proteoglycan (PG) per mg wet weight of explant two days post-treatment for non-impacted control (Con), IL-1 50 ng/ml (IL-1), 15 MPa slow impact (15 S), and 15 MPa fast impact (15 F), 30 MPa slow impact (30 S), and 30 MPa fast impact (30 F) (Ave ± SD; n=3). The (a) symbol indicates statistically significant n PG content compared to all impacted groups ($P < 0.015$).

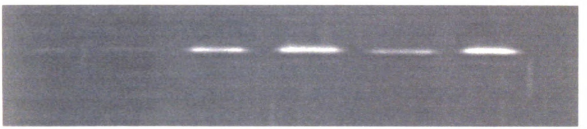

Figure 5: Approximately 8 μg of protein was loaded into a 8% PAGE gel containing gelatin. Lane A, control: Lane B, IL-1β (50 ng/ml); Lane C, 15 MPa (50 msec); Lane D, 30 MPa (50 msec); Lane E, 15 MPa (1 sec); Lane F, 30 MPa (1 sec). This band corresponds to MMP-2 (gelatinase B).

Table 2	Proteoglycan release			
Group	Mean (ug/ml) ± SD			
	Day 1 ($p < 0.001$)		Day 2 ($p < 0.003$)	
Control	53.26 ± 1.43	a	49.98 ± 3.72	a
IL-1	78.12 ± 4.24	b	66.19 ± 5.70	b
15 S	98.84 ± 73.67		90.52 ± 4.72	
30 S	110.31 ± 7.83	d	153.79 ± 7.52	f
15 F	112.81 ± 7.26		112.91 ± 5.57	
30 F	100.69 ± 3.75		92.53 ± 3.56	
15 SGS	89.14 ± 2.78	c	119.07 ± 7.08	
30 SGS	133.02 ± 4.36		108.11 ± 6.32	
15 FGS	105. 64 ± 4.21	e	112.43 ± 4.69	
30 FGS	95.74 ± 5.06		96.61 ± 5.75	

Table 2: The average proteoglycan released into the media each day post-treatment for control (Con), IL-1 50 ng/ml (IL-1), 15 MPa slow impact (15 S), 30 MPa slow impact (30 S), 15 MPa fast impact (15 F), 30 MPa fast impact (30 F), 15 MPa Slow with 2.5 mg/ml glucosamine-SO4 (15 SGS), 30 MPa slow impact with 2.5 mg/ml glucosamine-SO4 (30 SGS), 15 MPa fast impact with 2.5 mg/ml glucosamine-SO4 (15 FGS), and 30 MPa fast impact with 2.5 mg/ml glucosamine-SO4 (30 FGS) (Ave ± SD; n=8). The (a) symbol indicates a significant lower PG release of control to all other treatments. The (b) symbol indicates a significant lower PG release of IL-1 to all impacted treatments. The (c) symbol indicates a significant lower PG release of 15SGS than 15 S. The (d) symbol indicates a significant lower PG release of 30 S than 30 SGS. The (e) symbol indicates a significant lower PG release of 15 FGS than 15 F. The (f) symbol indicates a significant greater than all other groups. The (g) symbol indicates a significant lower PG release in 15 S than 15 SGS ($P < 0.001$).

Table 3 Percentage of cell death

Group	% Mean ± SD
Con	1.19 ± 0.83
Slow	41.28 ± 9.11 a
Fast	25.59 ± 11.78 a b
Con GS	0.12 ± 0.29
Slow GS	40.47 ± 10.20 a
Fast GS	28.75 ± 9.15 a b

Table 3: The mean amount of cell death following final media collection for control without glucosamine (GS) (Con), 30 MPa slow impact without GS (Slow), 30 MPa fast impact without GS (Fast), control with GS (Con GS), 30 MPa slow impact with GS (Slow GS), 30 MPa fast impact with GS (Fast GS) (n = 6). The (a) symbol indicates a statistically significant increase compared to controls with and without GS ($P < 0.05$). The (b) symbol indicates statistically significant decrease compared to slow impacts with and without GS ($P < 0.05$).

Table 4	Nitric oxide release			
Group	Mean (ug/ml) ± SD			
	Day 1 ($p < 0.05$)		Day 2 ($p < 0.05$)	
Control	1.35 ± 0.59		3.41 ± 0.40	
LPS	20.52 ± 6.71	a	7.56 ± 4.06	a
15 S	2.23 ± 1.24		3.12 ± 0.56	
15 F	0.59 ± 0.73		2.52 ± 0.32	
Con GS	0.13 ± 0.38		0 ± 0	b
LPS GS	3.44 ± 0.29		0.60 ± 1.43	b
15 SGS	0.84 ± 0.75		0.61 ± 0.56	b
15 FGS	1.25 ± 1.38		0 ± 0	b

Table 4: The average amount of nitric oxide (NO) released into the media per well each day post-treatment for control (Con), 15 MPa slow impact (15 S), 15 MPa fast impact (15 F), 10 μg/ml LPS (LPS), control with 2.5 mg/ml glucosamine (Con G), 15 MPa slow impact and 2.5 mg/ml glucosamine (15 SGS), 15 MPa fast impact and 2.5 mg/ml glucosamine (15 FGs), and 10 μg/ml LPS with 2.5 mg/ml glucosamine (LPS GS) (Ave ± SD; n=8). The (a) symbol indicates a statistically significant increase in NO release compared to all treatments on that day ($P < 0.05$). The (b) symbol indicates a statistically significant decrease in NO release compared to control and treatments without GS on that day ($P < 0.05$).

Table 5	Proteoglycan release	
Group	Mean (ug/ml) ± SD	
	Day 1 ($p < 0.05$)	Day 2 ($p < 0.05$)
Control	42.48 ± 7.97	37.75 ± 7.40
LPS	67.45 ± 17.23 a	42.84 ± 11.80
15 S	56.62 ± 15.52	49.38 ± 15.69
15 F	44.12 ± 7.79	39.30 ± 5.75
Con GS	48.29 ± 15.54	39.87 ± 11.78
LPS GS	38.76 ± 8.82	34.49 ± 9.13
15 SGS	62.62 ± 20.30 b	60.36 ± 18.75 c
15 FGS	43.81 ± 17.91	37.85 ± 14.56

Table 5: The average proteoglycan released into the media each day post-treatment for control (Con), 15 MPa slow impact (15 S), 15 MPa fast impact (15 F), 10 μg/ml LPS (LPS), control with 2.5 mg/ml glucosamine (Con GS), 15 MPa slow impact and 2.5 mg/ml glucosamine (15 SGS), and 15 MPa fast impact and 2.5 mg/ml glucosamine (15 FGS), and 10 μg/ml LPS with 2.5 mg/ml glucosamine (LPS GS) (Ave ± SD; n=8). The (a) symbol indicates a statistically significant increase in PG release compared to Con, LPS GS, 15 F, 15 FGS, and Con GS on that day. The (b) symbol indicates a statistically significant increase in PG release of 15 SGS compared to LPS GS on that day. The (c) symbol indicates a statistically significant increase in PG release of 15 SGS compared to Con, LPS, LPS GS, 15 F, 15FGS and Con GS ($P < 0.05$).

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Chapter 3

Cell viability over 24 hours following acute traumatic load on mature bovine articular cartilage explants

Abstract

Traumatic injury has been shown to cause osteoarthritis. Two experiments were conducted to study cell death and apoptosis following acute loads of 30 MPa at either fast (50 msec) or slow (1 sec) rates. Cell viability was assessed at 1, 2, 4, 8, and 24 h following loading and compared to a non-impact control group. Apoptosis was compared in non-impact controls to treatments of 30 MPa impact fast and slow. Fast and slow groups were also treated with cycloheximide to reveal if any apoptosis present was protein synthesis dependent. Cell viability analysis revealed increased cell death in impact groups over all time points when compared to controls ($p < 0.001$). Slow impact had increased cell death relative to fast in the middle (24 h) and deep zones in cartilage explants (8 and 24 h) ($p < 0.001$). Apoptosis was not found to be significantly increased in impact treatment versus non-impact. No change was seen in apoptosis due to treatment with cycloheximide. Traumatic injury caused significant cell death, with prolonged impact causing greater cell death at 8 and 24 h in the deep layer. With minimal apoptosis revealed with this procedure, it would appear the damage caused by traumatic impact under our conditions is necrotic in nature.

1. Introduction

With arthritis affecting more than 43 million Americans (CDC), a better understanding of the initial development of OA is being sought. Osteoarthritis is believed to develop from one of two scenarios, either normal forces acting on abnormal

cartilage (with inadequate healing response), or excessive (injurious) forces acting on normal cartilage.

Swelling, decreased tensile strength, visible degradation of the articular cartilage, and cell death are all characteristics of OA. Kim et al. (2000) and Blanco et al. (1998) have both shown OA cartilage to display greater nuclear and cytoplasmic changes consistent with apoptosis, while Aigner et al. (2001) used the same techniques to detect apoptotic cell death and found only singular apoptotic cells in the cartilage of osteoarthritic patients. Mechanical injury of articular cartilage induces similar damage; suggesting mechanical injury can initiate OA development. Stresses similar to those experienced by automobile occupants during knee to dashboard impact (20 to 30 MPa), are sufficient to cause chondrocyte death as well as fissuring of the articular cartilage (Repo et al., 1977). Additionally, Loening et al. (2000) has shown that loads as low as 4.5 MPa induce apoptosis in impacted explants. Previously, our labs have shown fast versus slow rates of loading can affect placement and the amount of cell death following impact at 40 MPa unconfined compression. Fast rate of loading resulted in cell death adjacent to fissures. The slow rate of loading resulted in greater amounts of cell death with a more diffuse distribution away from the fissures (Ewers et al., 2001).

The aim of the current study was to compare the extent and distribution of cell death over a 24-hour period following impact at different rates of loading. In earlier studies, cell death was at a maximum within 24 hours of impact (Ewers et al., 2000; Ewers et al., 2001). Additionally, analysis was used to see if the cell death caused by traumatic impact was apoptotic in nature and what effect rate of impact has on inducing apoptosis. Our hypothesis was the amount of cell death would increase over time with

the slow impact causing the most cell death (as seen in previous experiments). Additionally, apoptosis was expected in all impacted treatments.

2. Materials and Methods

2.1 Experimental Model

Four pair of skeletally mature bovine forelegs (18 to 24 mon Holstein steers) were obtained from a local abattoir, within three h of slaughter. The legs were cleaned with distilled water, the joint was skinned, the hoof bagged, and the joint was rinsed again, with distilled water, before opening the joint. The foreleg joints were opened in a laminar flow hood to expose the second, third and fourth carpals. A 6 mm biopsy punch (Miltex Instrument Company Inc., Bethpage NY) was used to make approximately 20 explants per joint which were separated from the underlying bone using a scalpel. Once separated from the bone, explants were placed in a petri dish containing Dulbecco's Modified Eagles Medium (DMEM): F12 (Gibco, USA # 12500-039).

Once all explants were retrieved, they were washed two additional times (10 min each) in DMEM: F12. Approximately 40 mg of cartilage (2 explants) were randomly placed into wells of 24 well culture plates. Each well contained 1 ml of DMEM: F12 supplemented with 50 μg/ml ascorbic acid, 20% fetal bovine serum (Gibco BRL, USA), 21.9 mg/ml glutamine (Sigma, St Louis MO), additional amino acids (Sigma), and antibiotics (Antibiotic-Antimycotic, Gibco BRL, USA). Explants were allowed to equilibrate two days with media changes daily, and specimens were kept at 37° C, and approximately 7% carbon dioxide, in a humidified NuAire incubator (Plymouth, MN).

After equilibration, explants were assigned to different treatment groups depending on the experiment. An Instron (model 1331, Canton MA) was used to deliver

a peak load of the assigned level to the specified groups. Briefly, specimens were placed between two highly polished stainless steel plates of the servo-hydraulic machine, where peak load, time to peak, and maximum displacement were recorded in each experiment. After loading, the explants were washed three times in DMEM: F12 medium (10 min each wash) before placing them in new pre-assigned culture plates. Individual experiments will be described in the results. Cycloheximide (5 ug/ml) was added per well for 24 h to prevent protein synthesis (Arner et al., 1998). One day post treatment, explants from each treatment were randomly divided for cell viability, or apoptosis.

2.2 Cell Viability

Two 1 mm slices were cut from the center of each explant selected for cell viability analysis at the time designated, using a specialized cutting tool designed in our laboratory. The sections were stained using a commercial kit containing calcein and ethidium bromide homodimer (Live/Dead Cell Viability/Cytotoxicity, Molecular Probes, Oregon USA). All specimens were viewed using a fluorescence microscope (Leica DM LB (50-60 Hz) Leica Mikroskopic und Systeme GmgH, Welzar, Germany). Digital images (100x magnification) of the explant thickness were taken at the center of each slice (Spot Digital Camera, Diagnostic Inc.). Cell viability was quantified by manually counting the dead (red) and viable (green) cells for each picture using the image software, Sigma Scan (SPSS Inc., Chicago IL). The totals from the two slices were averaged for each explant.

2.3 Apoptosis

Explant chosen for apoptosis analysis were snap frozen in liquid nitrogen, and placed vertically in OCT freezing compound. Each pair were then wrapped in aluminum

foil, labeled and stored at - 80° C until they could be sectioned. Using a Leica cryostat, each pair of explants was shaved to approximately the middle of the explant, and several 10 μm sections of each pair were placed on positively charged slides, and stored on dry ice until returned to - 80° C for later analysis using Promega's Apoptosis Detection System, Fluorescein (Madison, WI). The sections were stained using fluorescein and propidium iodide as directed in the apoptosis detection kit for "adherent cells". To assure the apoptosis kit was functioning correctly for each use, a slide was treated with DNase I, which fragmented the DNA present in the chondrocytes, allowing the fluorescein-12-dUTP to attach to free 3 prime ends and give a positive green signal. All specimens were viewed by fluorescence microscope. Digital images (100x magnification) of the explant thickness were taken at the center of each slice (Spot Digital Camera, Diagnostic Inc.). Apoptosis was quantified by manually counting the green (apoptotic) and red (non-apoptotic) cells for each picture using the image software, Sigma Scan (SPSS Inc., Chicago IL). The totals from the slices were averaged for each treatment.

2.4 Statistical analysis

In all experiments the mean values for each treatment per day $\pm$ standard deviations were calculated. The results were compared using ANOVA Student-Newman-Keuls method on the program SigmaStat. Statistical significance was considered at $P < 0.05$ unless noted.

3. Results

In the 24-hour cell viability study, each digital image of the sectioned explants section was divided into superficial (top 20% of sectioned tissue), middle (following 50 % of tissue) and deep layers (bottom 30% of tissue). The live, dead and total number of

cells were tabulated and compared to see how rate may affect the extent and length of time to cause cell death following a 30 MPa blunt impact. Control explants exhibited minimal total cellular death averaging 0.2 $\pm$ 0.7 % over 24 h (not shown in table). A significant increase was seen in both impacted treatments for totals over the 24-hour period (Figure 1). Total fast rate cell death increased significantly from 25 $\pm$7 to 42 $\pm$ 16 % ($P < 0.007$), while the slow rate cell death also significantly increased from 34 $\pm$19 to 66 $\pm$ 13 % ($P < 0.001$) over 24 h. At 24 h post impact, the rate of loading had a significant effect on total cell death. Slow rate of loading had the greatest amount of cell death including significance over the fast rate treatments at 24 h post impact ($P < 0.005$) (Figure 2).

Once the sections were divided into layers, the fast and slow rate treatments were compared at 1, 2, 4, 8, and 24 h for cell death. The superficial layer (top 20 % of thickness) exhibited extensive, yet similar, cell death totals across all time points, with fast averaging 79 $\pm$ 13 % and slow averaging 84 $\pm$ 15 % (Figure 3). The middle layer (50 % of thickness below the superficial layer) exhibited a steady increase in cellular death in both treatments. By 24 h, both fast and slow treatments exhibited significance when compared to earlier time points (Figure 4). Especially noteworthy was greater increase in cell death of the slow treatment versus the fast at the same time point ($p< 0.001$). In the deep layer (bottom 30% of thickness), cell death increased significantly at 8 and 24 h for the slow rate treatment, while the fast rate stayed relatively unchanged across all time points (Figure 5).

A second study, also examining loading at 30 MPa fast and slow rate, examined total cellular viability and apoptosis at 24 h post impact. Cell viability analysis revealed

similar amount of cell death with significant increase in all impacted treatments versus control. Cell viability was not different between rates (Figure 6). The apoptosis analysis revealed the few positive signaling cells (green stained cells) were found in the superficial layer regardless of treatment (Figure 7) and no significant difference was seen between any impact treatments versus control (Figure 8). The positive control DNase I treated slide produces a positive signal from a majority of the cells throughout the thickness of the tissue, indicating the kit was working correctly.

4. Discussion

Blanco et al. (1998) and Kim et al. (2000) have shown OA cartilage displayed increased apoptotic characteristics when compared to healthy cartilage. Kim et al. (2000) specifically compared placement between regions of the increased apoptotic cells. Areas of lesions in osteoarthritic cartilage had the most apoptotic cell confirmed by Tunel, chromatin condensation, and DNA analysis for Bcl-2 and Fas (known indicators of apoptosis). Recently, Aigner et al. (2001) has reported that, when comparing normal and osteoarthritic cartilage from donors of all ages, no major apoptotic or non-apoptotic cell distributions in either type were observed. Instead, only single apoptotic cells were detected in OA articular cartilage, as well as a higher degree of empty lacunae in late-stage OA cartilage versus early-stage and normal tissue.

As blunt trauma has been shown to initiate OA characteristics, studies looking into cell death in loaded explants have been used to look at cell response. Repo et al. (1977) and Jeffery et al. (1995) have shown traumatic loading of 20 to 30 MPa can cause significant cell death, while loads of 15-20 MPa are all that is needed to cause cell death and rupture collagen fibrils at the time of impact (Torzilli et al., 1999). We have shown

that fast traumatic loading caused greater matrix damage as seen in more fissures with greater depths, while slow rates of loading caused more cell death (Ewers et al., 2000). We believe the more matrix damage seen in the fast rate of loading was due to the shorter time span causing greater points of stress on the surface of the explant. With a longer rate of load, the higher level stress is absorbed by deformation of the tissue relieving stress on the matrix. Bank et al. (2000) have reported that traumatic loading causes breaks in the collagen fibrils of the matrix. A sudden high-energy impact may have been too fast to allow deformation and energy transfer, instead causing breaks in the collagen fibrils. A slower time to peak for the same load (or slow rate impact) may have facilitated deformation and less matrix damage, but with more deformation comes more stress on the chondrocytes within the matrix. The stress from the slow rate impact may have been enough to disrupt the cell membrane, causing a slow necrotic process. This may explain why more cell death was seen in the slow rate of impact versus the fast rate of impact.

Apoptosis was only seen in the superficial layer and around lesions for both fast and slow rate impacts. Our studies indicate that the significant cell death related to high impact loading of the articular cartilage does not necessarily bring about significant increases in apoptosis. Instead necrotic cell death was observed, possibly due to stress placed on the cells. These results may explain the results of Aigner et al. (2001) where minimal apoptosis was seen in OA tissue. Traumatic injury may bring about cell death at initiation of OA development, leaving large numbers of empty lacunae in late-stage OA as seen by Aigner et al. With a significant increase in cell death initially, tissue

degradation resulting in OA would be present but without the large increase in cell death at late-stage OA as seen by Blanco et al. (1998) and Kim et al. (2000) in their analysis.

Our results in the apoptotic study involving explants do not agree with Loening et al. (2000). They reported apoptosis at peak loads of 4.5 MPa and increased apoptosis with increased stress in a dose-dependent manner. At a high peak stress of 20 MPa, more than 50% of the cells were found to be apoptotic. The tissue used for their research was newborn calf articular cartilage. Tew et al. (2000) have compared immature (newborn) and mature (18 month) bovine articular cartilage to damage and cell death. Specifically looking for apoptotic response, Tew et al. (2000) found immature tissue had a more pronounced cell death especially in the superficial region. The cell death was determined to be a combination of necrosis and apoptosis. Immature (newborn) articular cartilage differs from mature cartilage in that the chondrocytes of the immature are not yet in the quiescent state that mature chondrocytes reach upon stabilization of development of the tissue. The matrix is less developed in newborn articular cartilage. Collagen fibrils are smaller in diameter and have fewer cross-links to stabilize the structure. This may explain the differences of how the tissue and, specifically, how the cells are affected when loading is applied.

Aigner et al. (2001) used mature human donor tissue and found few apoptotic cells present in the OA cartilage, while other researchers such as Blanco et al. (1998), have reported significantly increased apoptosis in OA cartilage. The differences between these reports have not been explained. As OA development can be initiated by many different causes, i.e. wear and tear versus traumatic injury, the development and resulting cell death may be necrotic or apoptotic depending on how the damage was caused.

In conclusion, our results indicate traumatic injury due to high load impact, caused cell death over 24 h for fast and slow rate in the middle and deep layers. Fast rate impact was found to cause more matrix damage versus slow rate impact, which revealed more cell death. Upon analysis for apoptosis, the traumatic load of 30 MPa only produced apoptotic cells in the superficial layer and around the edges of lesions, revealing most cell death was non-apoptotic in nature for both rates. While the significant damage observed in the fast rate of impact appears immediately following impact, cell death appears to take approximately 24 h to reach the middle and deep layers of tissue. The extended length of time to reach peak cell death suggests future studies should look for means of intervention to prevent the extensive necrosis observed following traumatic loading.

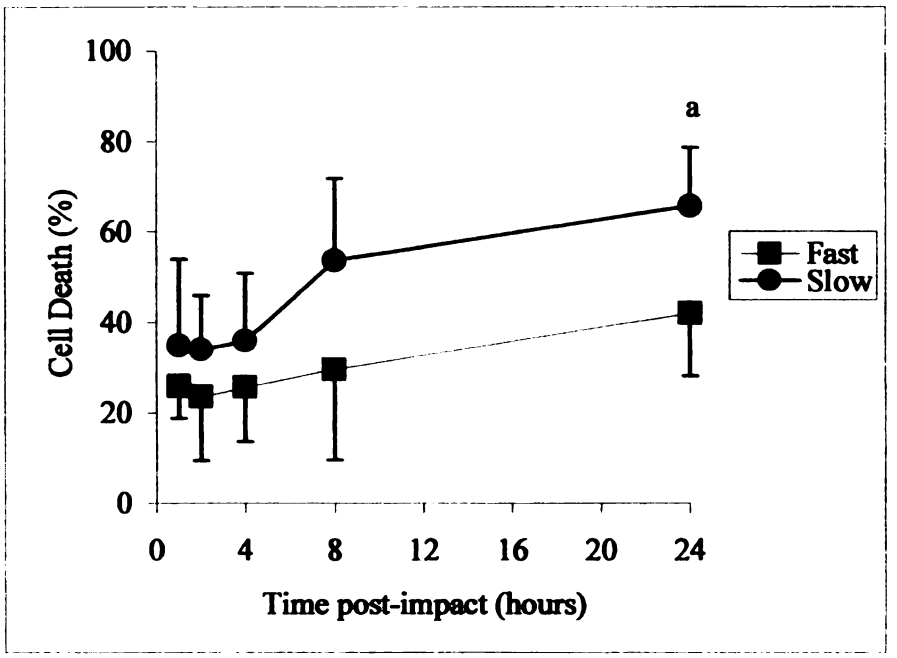

Figure 1: Percentage of total cell death at 1, 2, 4, 8, and 24 hours in Fast and Slow treatments (n=8). Percentage of total cell death for Control was negligible and not include. The (a) symbol indicated a statistically significant increase in total cell death in impacted treatments at 24 hours versus 1 hour ($P < 0.001$ (slow), $P < 0.007$ (fast)).

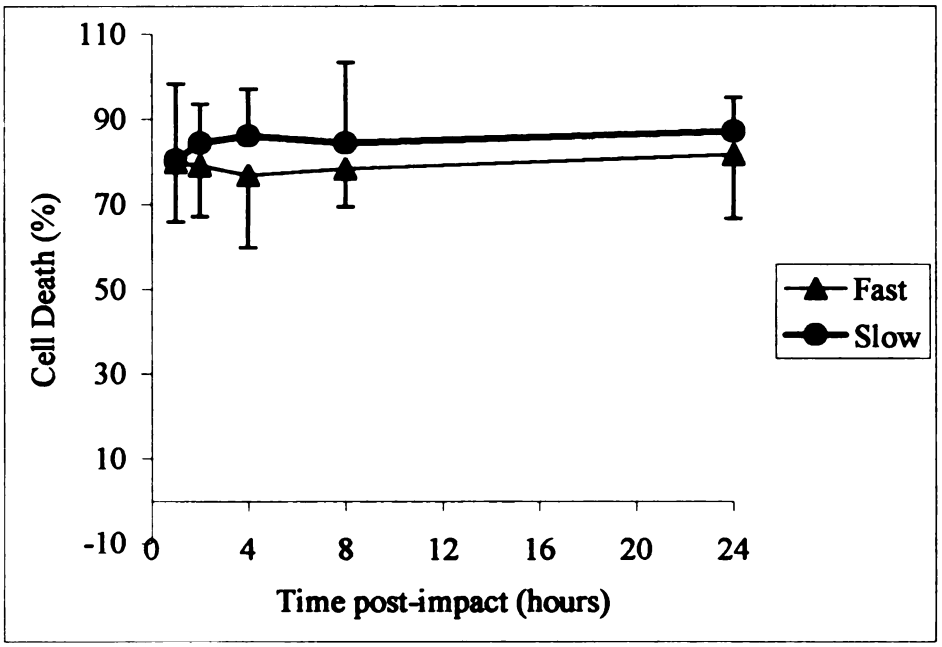

Figure 2: Percentage of total cell death in superficial layer at 1, 2, 4, 8, and 24 h in Fast and Slow treatments (n=8). No significant differences were found between treatments in this layer.

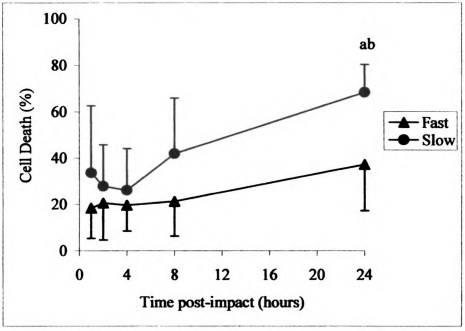

Figure 3: Percentage of total cell death in the middle layer at 1, 2, 4, 8, and 24 hours in Fast and Slow treatments (n=8). The (a) symbol indicated a statistically significant increase in total cell death for the Slow impacted treatment at 24 hours versus 1 hour ($p < 0.001$). The (b) symbol indicated a statistically significant increase in total cell death for the Slow impacted treatment versus the Fast treatment ($p < 0.001$).

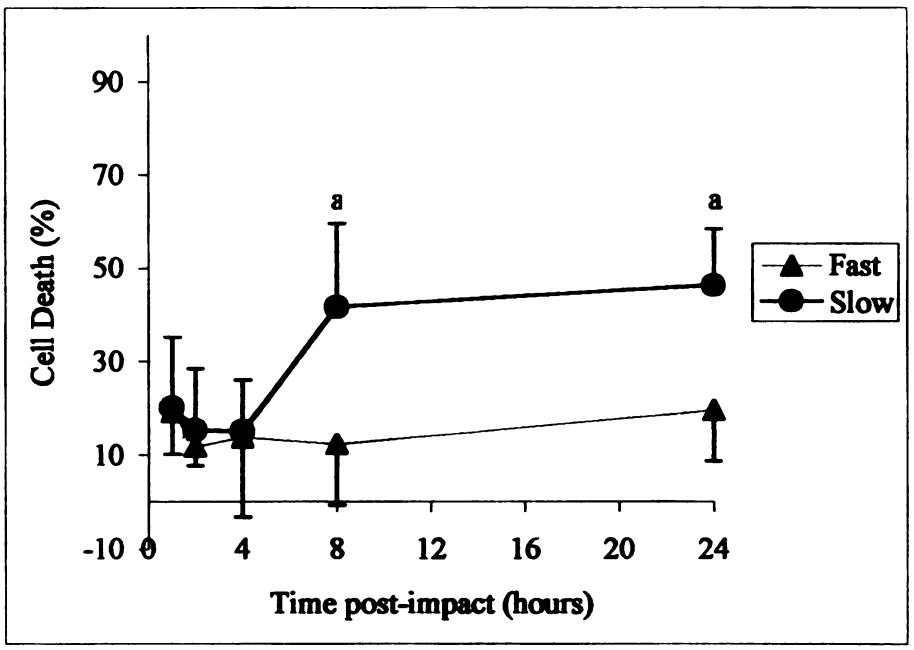

Figure 4: Percentage of total cell death in the deep layer at 1, 2, 4, 8, and 24 hours in Fast and Slow treatments (n=8). The (a) symbol indicated a statistically significant increase in total cell death in the slow impacted treatment at 8 and 24 hours versus 1 hour ($p < 0.007$).

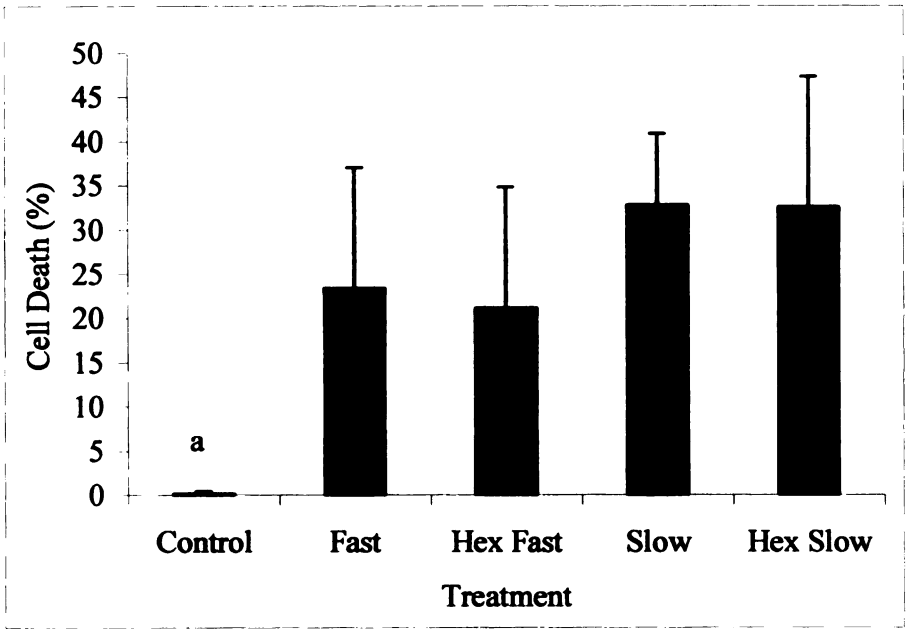

Figure 5: Percentage of total cell death in Control, Fast, Cycloheximide Fast (Hex Fast), Slow, and Cycloheximide Slow (Hex Slow) (n=8). The (a) symbol indicated a statistically significant increase in total cell death in the impacted treatments versus control ($p < 0.001$).

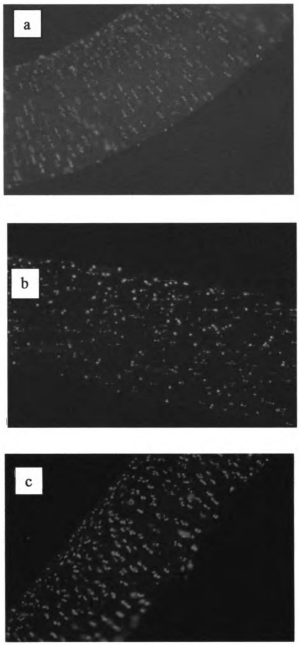

Figure 6: 100x digital photo of cartilage explants treated with the Apoptosis Detection System(10 μm sections) Images in this thesis are presented in color, red cells represent necrotic cells, green cells represent apoptotic cells: a) Control non-impacted section, b) DNase treated positive control, c) Slow impacted (n=8)

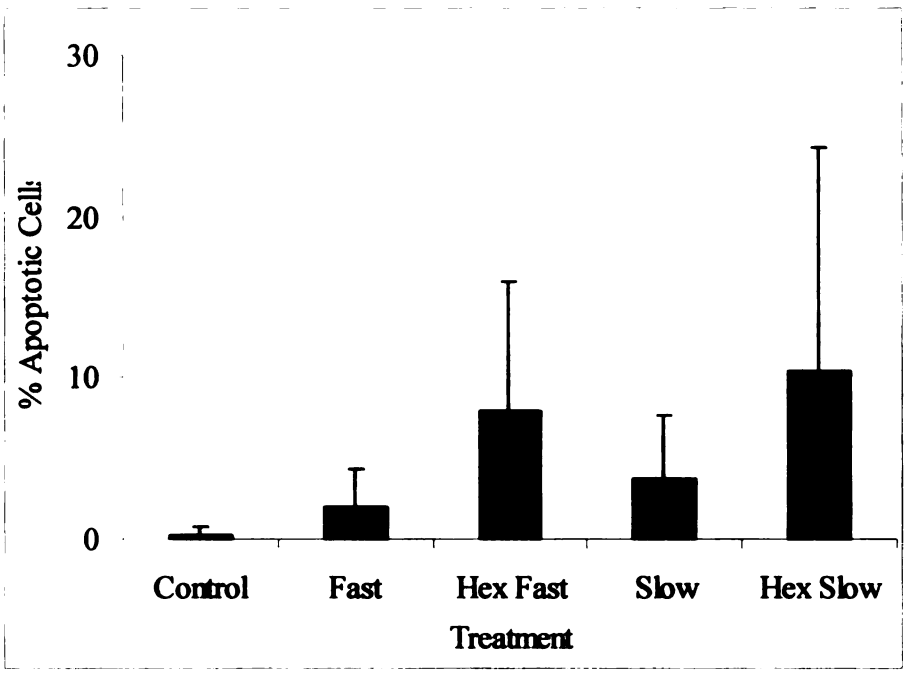

Figure 7: Percentage of total apoptotic cells in Control, Fast, Cycloheximide Fast (Hex Fast), Slow, and Cycloheximide Slow (Hex Slow) (n=8). No significant differences were seen between treatments with this analysis.

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CONCLUSION

The previous chapters described degradation and cell viability resulting from acute traumatic or high physiological impact on mature bovine articular cartilage explants. Using indicators of chondrocyte metabolism, specifically NO production and PG release, we have shown that in vitro acute impact of mature cartilage explants stimulated degradation differently than cytokine treatments. Traumatic load at a slow rate was found to cause the greatest PG release following impact, while cytokine treatment only stimulated a minimal response. Nitric oxide release in cytokine treatments was greatest, while acute impact at all levels released NO comparable to control groups. Cell death was also greatest at traumatic load and slow rate, with a majority of the cells found to be necrotic versus apoptotic. Cytokine treatment did not change cell death percentages from those of control groups, and due to the low cell death, apoptotic response was not analyzed with these treatments. The addition of glucosamine-sulfate (GS) can act as a chondroprotective in explants treated with cytokine. We saw similar results in our IL-1 and LPS treated explants, as NO release was significantly less in those with GS pre-treatment. We have also observed that pre-treatment with GS had no effect on preventing the effects of increased PG release observed in high physiological or traumatic impacted explants. Overall, with greater load and longer rate, more degradation was observed, possibly a combination of breaks in the collagen network allowing greater PG release, and MMP activity. Cell viability was found to have a similar response with greater loads and longer rates, as a traumatic load on an unconfined

explant resulted in a majority of the cells to be necrotic, and high physiological load resulted in less necrosis.

Studies using full-thickness bovine articular cartilage tissue can be a critical tool in understanding how load and rate of impact can affect chondrocytes and the tissue around them. In our experiments, we observed limited effects of loading on articular cartilage. The protective properties of underlying bone are lost when full-thickness cartilage explants are separated from the joint. Although, cartilage attached to bone would be more realistic of the in vivo environment, time and sample size constraints in obtaining cartilage attached to bone made them impractical in these two experiments. Additionally, future studies to detect apoptosis should be confirmed with histological analysis of the cells in at least a small number of the samples.

We had expected to observe increased apoptosis in the traumatic impacted explants due to the increased cell death seen over time. Even though most of the cell death was found to be necrotic, the fact that peak levels of cell death were not reached in the middle and deep layers until 8 to 24 h following impact leaves the potential for intervention and should be a critical area of for future studies. Future studies observing PG synthesis, detection of denatured type II collagen, and quantitative MMP analysis expanding from just MMP-2 to include stromelysin-1 and collagenases activity following impact utilizing this system could further help in understanding loading on articular cartilage. In addition to further biochemical properties, a less destructive load range, based on the high physiological load, and varying rates may give a better idea of how chondrocytes react to acute impact. A full-thickness articular cartilage explant model may not be an ideal scenario for analyzing the effects of loading on degradation, but has

the potential to supply information that may lead to a better understanding of in vivo processes, without the invasiveness of live animal research.