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RESPONSE OF ARTICULAR CARTILAGE TO A BLUNT ACUTE OVERLOAD CAN BE AFFECTED BY INTERMITTENT CYCLIC PRELOAD AND ALTERATION OF PROTEOGLYCAN CONTENTS

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RESPONSE OF ARTICULAR CARTILAGE TO A BLUNT ACUTE OVERLOAD CAN BE AFFECTED BY INTERMITTENT CYCLIC PRELOAD AND ALTERATION OF PROTEOGLYCAN CONTENTS

By

Feng Wei

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ABSTRACT

RESPONSE OF ARTICULAR CARTILAGE TO A BLUNT ACUTE OVERLOAD CAN BE AFFECTED BY INTERMITTENT CYCLIC PRELOAD AND ALTERATION OF PROTEOGLYCAN CONTENTS

By

Feng Wei

Mechanical loading of articular cartilage can influence chondrocyte metabolism and lead to alterations in cartilage matrix composition. Most previous studies have focused on the effect of cyclic loading on cartilage mechanical properties and proteoglycan (PG) synthesis. However, the role of PGs synthesized from cyclic loaded cartilage in response to an acute overloading has not been elucidated. We have therefore conducted studies where low intensity, intermittent cyclic loading was applied to chondral explants prior to an acute unconfined compression on the tissue. Chapter One documented a study by our laboratory showing that 14 days of intermittent cyclic loading on chondral explants has a positive effect on the tissue, by causing mechanical stiffening prior to a blunt force overloading, to limit acute tissue damage. And yet, longer term loading to 21 days results in tissue degradation prior to the acute traumatic event. In Chapter Two a supplement of glucosamine-chondroitin sulfate was used in an experimental setting to alter PG synthesis during cyclic loading of explants and its effect on the response of cartilage to an acute overload was documented. The results showed that experimentally increased tissue PGs during cyclic loading of cartilage help strengthen the cartilage, making them inhibit the degradation of the tissue after long-term cyclic compression and reduce the susceptibility of cartilage to a severe level of mechanical injury. In the long term these types of studies may help understand the role of biologic-based pre-conditioning of articular cartilage for in vitro, or even in vivo studies of blunt force trauma to a joint.

DEDICATION

I would like to thank my parents for their never-ending support and encouragement throughout my life. Without their support I would never have had the drive to accomplish all that I have in my life and academic career.

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Wei F, Haut R: High levels of glucosamine-chondroitin sulfate can alter the cyclic preload and acute overload responses of chondral explants. Journal of Orthopaedic Research, In Review

Peer Reviewed Abstracts

Wei F, Golenberg N, Kepich E, Haut R: In vitro exercise affects the response of articular cartilage to blunt impact loading. Transactions of the 53rd Annual Meeting of the Orthopaedic Research Society, San Diego, California 32:662, 2007

Wei F, Golenberg N, Kepich E, Haut R: Glucosamine and chondroitin sulfate affect the response of exercised articular cartilage to blunt impact loading. Transactions of the 31st Annual Meeting of the American Society of Biomechanics, Stanford, California 31:P4-21, 2007

Wei F, Golenberg N, Kepich E, Haut R: Alteration of proteoglycan synthesis in a cartilage explant during cyclic loading can affect its susceptibility to damage in an acute overload. Transactions of the 54th Annual Meeting of the Orthopaedic Research Society, San Francisco, California, 2008

INTRODUCTION

Articular cartilage is a tough, elastic connective tissue covering the ends of joints. Its purpose is to distribute load and provide a near frictionless surface for the movement of joint surfaces against one another. Cartilage is composed of chondrocytes (cells) surrounded by a matrix of water, collagen (fibrous proteins), and proteoglycans (PG's) (Figure 1). PG's are protein aggregates having polysaccharide side-chain units known as glycosaminoglycans (GAGs). As the joint is subjected to load, the cartilage will deform in order to distribute the load, causing compressive, tensile, and shear stresses throughout the cartilage (Mow and Setton, 1998). The function of the collagen is to provide the cartilage with tensile strength, whereas the PG's are associated more with the stiffness properties of the cartilage in compression (Helminen et al., 1992). The content and structure of PG's and collagen fibers varies throughout the depth of the cartilage. The matrix can be divided into three regions: a superficial tangential zone, a middle zone, and a deep zone (Figure 2).

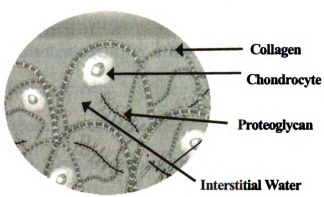

Figure 1. The extracellular matrix of articular cartilage is composed mainly of collagen fibers, proteoglycans, and water.

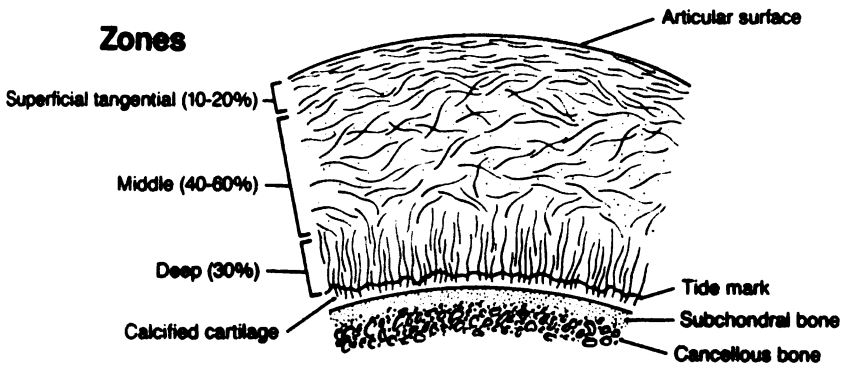

Figure 2. A sketch showing the cross section of cartilage, illustrating the collagen network and the three distinct regions of this tissue.

Osteoarthritis (OA) is a degenerative joint disease associated with multiple factors, genetic, metabolic, biochemical and biomechanical, that act to disturb the equilibrium of anabolic and catabolic events in articular cartilage and adjacent bone (Derfoul et al. 2007). While the mechanisms responsible for this disease are unknown, the risk of OA is increased significantly in joints suffering a major injury (Felson 2000 and 2004). Hereditary defects may predispose to OA, yet other risk factors such as age, excessive joint loading, and joint injury increase the risk for development of this disease (Buckwalter et al., 2004; Helminen et al., 1992; Gelber et al., 2000; Marsh et al., 2002). OA is thought to be initiated by fibrillation (the unbinding of collagen fibrils and surface fraying) and swelling of the cartilage matrix due to the influx of fluid. This increased hydration leads to a softening of the articular cartilage, which increases the pressure on the underlying subchondral bone (Radin et al., 1996). These early stages of OA may initiate an increase in the subchondral bone thickness, and lead to changes such as

osteophyte formations (bony outgrowths) and erosion of the articular cartilage, eventually causing complete loss of this soft tissue (Figure 3).

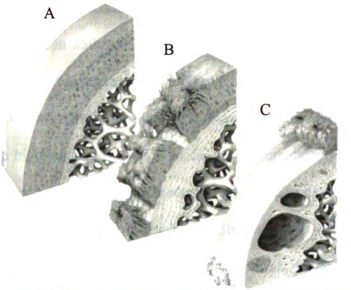

Figure 3. The progressive stages of OA. **A)** Normal articular cartilage and bone. **B)** Cartilage surface becomes fibrillated and the subchondral bone thickens. **C)** Total loss of cartilage with bone cyst formation.

Mechanically injurious loads can generate articular cartilage extracellular matrix damage and chondrocyte death, which have been suggested to facilitate development of OA. We developed a model of post-traumatic OA using the Flemish Giant rabbit (Haut et al. 1995). In a previous in vivo animal study in our lab, a rigid impact mass was dropped with 6 J of energy onto the flexed patello-femoral rabbit joint, resulting in acute surface fissures, progressive degradation of retro-patellar surface cartilage, and thickening of underlying subchondral bone after 3 years (Ewers et al. 2002). In another study, this impact was found to produce patello-femoral contact pressure of approximately 25 MPa (Newberry et al. 1998). A more recent study showed that this level of blunt impact load applied to the patello-femoral joint induces acute chondrocyte necrosis throughout the

retro-patellar cartilage (Rundell et al. 2005). In vitro studies also show that a single blunt impact load can result in surface fissures and cell death in chondral and osteochondral explants. Necrotic cell death was also found in situ using the bovine patella subjected to a single impact of 53 MPa in 250 ms. Cell death occurred largely in the superficial zone adjacent to fissures, but not in impacted areas away from these cracks (Lewis et al. 2003). Studies involving explants removed from bone indicated that the extent of matrix damage would be higher in high versus low rate of loading experiments, while cell death would be greater in low versus high rate experiments (Ewers et al. 2001). A critical threshold stress of 15-20 MPa was found for cell death and collagen matrix damage in unconfined compression using bovine chondral explants (Torzilli et al. 1999). Significant matrix fluid pressurization and surface cracking were observed from unconfined compression of bovine osteochondral explants (Morel and Quinn 2004). Other studies have also associated articular cartilage physiopathology in OA with chondrocyte death (Hashimoto et al. 1998).

The variations in cartilage morphology, mechanical properties (Murray et al. 1998; Murray et al. 1999), and chondrocyte matrix synthesis (Ackermann and Steinmeyer 2005; Buschmann et al. 1999) have been reported and may result from the variations in load. Therefore, exercise would be expected to alter the composition and mechanical behavior of cartilage. Although mechanical stimulation is necessary for cartilage development and homeostasis (Buschmann et al. 1995), abnormal loading has also been shown to initiate cartilage degradation (Farquhar et al. 1996; Jeffrey et al. 1995; Thibault et al. 2002). Mechanical loading conditions ranging from very high strain rate impact loading

conditions, such as traumatic accidents, to very low strain rate loading, such as obesity or joint misalignment, may all contribute to cartilage injury and degenerative joint disease (Morel et al. 2005). In vitro studies of impact loading (Atkinson et al. 1998; Borrelli et al. 1997; Milentijevic and Torzilli 2005; Milentijevic et al. 2003) and high strain rate injurious compression (Quinn et al. 1998b; Ewers et al. 2001; Kurz et al. 2001; Quinn et al. 2001) of cartilage have shown that cell death and matrix damage resulting from fluid pressurization and collagen network tensile failure (Morel and Quinn 2004) could represent initiating events for degenerative processes. Cyclic loading can also lead to chondrocyte death (Levin et al. 2001; Clements et al. 2001; Chen et al. 2003), mechanical weakening of collagen (Thibault et al. 2002), and decrease of collagen synthesis rate (Ackermann and Steinmeyer 2005) in cartilage.

Since prestrain of cartilage explants prior to injurious loading reduced the occurrence of superficial cracks and associated cell death (Morel et al. 2005), the short-term loading history of cartilage can influence its subsequent response to injurious compression due to modifications in tissue structural organization and mechanical properties. The extracellular matrix (ECM) consists mainly of PG's and type II collagen. Based on experimental evidence, PG's are primarily responsible for the compressive stiffness of the cartilage matrix (Mow et al. 1990 and Korhonen et al. 2003). Biosynthesis of PG can be stimulated by intermittently applied cyclic loading (Sah et al. 1989). Investigators have provided many frameworks for identifying both the physical and biological mechanisms by which dynamic compression can modulate chondrocyte biosynthesis. In one study, cultured bovine articular cartilage was subjected to 50 ms, 0.5-1.0 MPa

compressions repeated at intervals of 2-60 s for 1.5 h and simultaneously labeled with $^{35}SO_4$. It was found that explants under a 0.5 MPa load showed significantly increased $^{35}SO_4$ incorporation by compression repeated at 2- and 4-s intervals. Therefore, the stimulation of PG synthesis, as indicated by [^{35}S] sulfate incorporation, is limited to certain loading frequencies and pressures (Parkkinen et al. 1992). In another study, a compressive pressure was introduced for 1, 3 or 6 days using a sinusoidal waveform of 0.5 Hz frequency with a peak stress of 0.1, 0.5 or 1.0 MPa. A maximum PG synthesis was observed at day 3 with 0.5 Hz frequency and 0.5 MPa peak stress (Steinmeyer et al. 1999). However, the role of PG synthesized from intermittently loaded cartilage in response to injurious trauma of articular cartilage has not been fully elucidated.

In Chapter One of the study we hypothesized that physiologically intermittent cyclic loading would stimulate PG synthesis in cartilage explants, resulting in an increased matrix stiffness of the tissue, and therefore reduce its susceptibility to matrix damage and cell death following a single blunt impact loading to approximate 25 MPa. This hypothesis was tested in the present study by systematically applying different durations of intermittent loading to cartilage explants before injurious blunt impact loading.

Understanding the changes in collagen and PG content of cartilage due to mechanical forces is necessary for progress in treating join disorders, such as OA. The use of potentially chondro-protective agents such as glucosamine (glcN) and chondroitin sulfate (CS) has been explored to medicate OA. Previous studies by others have shown that bathing cartilage explants in a supplement of glcN and CS can up-regulate the synthesis

of tissue PG's, and particularly in stressed tissue (Lippiello 2003). However, the role of PG's synthesized from intermittently loaded cartilage in response to an acute compressive overloading of articular cartilage has not been elucidated. Recent studies have also shown that this supplement limits tissue degradation in the presence of inflammatory enzymes in an in vitro setting (Derfoul et al. 2007).

Since previous data showed that PG content directly paralleled with stiffness of cartilage (Chapter 1), we wanted to experimentally increase tissue PG content to stiffen the cartilage, making them inhibit the degradation of the tissue after long term of cyclic compression and reduce the susceptibility of cartilage to a severe level of mechanical injury, such as an acute overload. The objective of Chapter Two of this thesis was to use this supplement in an experimental setting to alter PG synthesis during cyclic loading of chondral explants and document its effect on the response of explants to an acute overload. The hypothesis was that increased levels of tissue PG will significantly limit blunt force trauma to the tissue.

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CHAPTER ONE

EFFECT OF INTERMITTENT CYCLIC PRELOADS ON THE RESPONSE OF ARTICULAR CARTILAGE EXPLANTS UNDER AN EXCESSIVE LEVEL OF UNCONFINED COMPRESSION

ABSTRACT

Mechanical loading of articular cartilage can influence chondrocyte metabolism and lead to alterations in cartilage matrix composition. Most previous studies have focused on the effect of cyclic loading on cartilage mechanical properties and proteoglycan synthesis. However, the role of proteoglycans synthesized from cyclic loaded cartilage in response to an acute overloading has not been elucidated. We have therefore conducted studies where low intensity, intermittent cyclic loading was applied to chondral explants prior to an acute unconfined compression on the tissue. The results of the study showed cyclic preloading increased the production of proteoglycans and mechanically stiffened the explants, making them more resistant to matrix damage and cell death under 25 MPa of acute, unconfined compression up to 14 days. After 21 days of cyclic loading, however, the explants lost compressive stiffness and suffered more extensive damage in the acute, unconfined compression test than controls. This study investigated the role of in vitro cyclic loading on the response of chondral explants to a potentially damaging, acute overload. In the long term these types of studies may help understand the role of biologic-based pre-conditioning of articular cartilage for in vitro, or even in vivo studies of blunt force trauma to a joint.

INTRODUCTION

Joints suffering a major acute injury, such as an osteochondral fracture or ligament rupture, are at risk of developing chronic osteoarthritis (OA) (Felson et al. 2000). In cases of injury to a knee ligament, early detection of bone bruises is seen in over 80% of cases (Vellet et al. 1991; Johnson et al. 1998). These bone lesions have also been observed in clinical cases without acute ligament or osteochondral damage (Snearly et al. 1996). Associated with these bone lesions recent investigators have documented early degeneration of overlying articular cartilage and death of chondrocytes (Fang et al. 2001; Johnson et al. 1998). Damage to articular cartilage overlying MRI detected bone bruises may be due to excessive compressive forces generated in the joint during acute injury, i.e. rupture of the anterior cruciate ligament (ACL) (Fang et al. 2001). These bone lesions and acute cartilage damage may help explain the basis for a post-traumatic OA in the joints of ACL-injured patients, independent of whether they are surgically reconstructed (Daniel et al. 1994; Myklebust & Bahr 2005). Research on animal models has shown that acute trauma to articular cartilage causing matrix damage and cell death can lead to chronic disease in joints (Ewers et al. 2002). A more recent study has also shown that during these acute trauma experiments contact pressure in the rabbit patello-femoral joint averages approximately 25 MPa (Rundell et al. 2005), and it induces acute chondrocyte necrosis throughout the retro-patellar cartilage.

The development of cell death and matrix damage in cartilage is known to vary with the levels of compressive loading and strains (deformations) applied to the tissue (Torzilli et al. 1999; Quinn et al. 2001). In particular, direct relationships have been established

between the levels of tissue deformation and cellular damage (Torzilli et al. 2006). In order to limit or even help mitigate the process that may lead to OA in joints suffering blunt force trauma, it would seem reasonable to examine factors that might affect the mechanical properties of cartilage before the acute traumatic event. For example, increasing the structural stiffness and strength of articular cartilage may alter the long-term consequences of blunt force to the joint. Recently, Quinn et al. have shown that pre-strain of the tissue by a short-term history of compressive loading helps limit the extent of damage in chondral explants to an acute blunt force loading (Morel et al. 2005). The basis for this action may be that these pre-strains cause matrix consolidation and an increase in structural stiffness of the tissue prior to the blunt impact force. Another mechanism that could potentially help limit the extent of joint injury during a traumatic event is to increase the matrix stiffness of articular cartilage in a material sense prior to impact. In vitro and in vivo studies have shown that physiologically intermittent cyclic loading of articular cartilage can up-regulate the synthesis of tissue proteoglycans (PGs) (Sah et al. 1989; Parkkinen et al. 1992; Saadat et al. 2006). However, the role of PG synthesized from intermittently loaded cartilage in response to an acute compressive overloading of articular cartilage has not been elucidated.

The hypothesis of the current study was that low intensity, intermittent cyclic loading of chondral explants will up-regulate the production of tissue PGs, resulting in an increase of the explant mechanical stiffness that will help limit the extent of matrix damage and associated chondrocyte death following a single, severe level of blunt force loading. These data may have a direct bearing on the issue of pre-impact conditioning of chondral

explants in laboratory experiments dealing with the short-term consequences of impact trauma. Additionally, these data may also relate to the role of regular exercise on the long-term consequences of human joints following a traumatic joint injury, such as an acute ligament rupture.

METHODS

DISSECTION AND TISSUE CULTURE

Bovine forelegs from mature animals (18-24 months of age) were obtained from a local abattoir within two hours of slaughter. The legs were skinned and rinsed with water prior to exposing the metacarpal joint under a laminar flow hood. A biopsy punch (Miltex Instrument Company, Bethpage, NY) was used to make twenty-four 6 mm diameter chondral explants from the lower metacarpal surface of the limbs. Each explant was separated from the underlying bone with a scalpel. All specimens were washed three times in Dulbecco's Modified Eagle Media: F12 (DMEM: F12) (Gibco, USA, #12500-039), and then placed in the media supplemented with 10% fetal bovine serum, additional amino acids and antibiotics (penicillin 100 U/ml, streptomycin 1 μg/ml, amphotericin B 0.25 μg/ml) in a 24-well plate. The media was replaced every 2 days throughout the study and harvested and stored frozen at -80°C. The well plate was placed in a mechanical loading device (the "cartilage exerciser" (described below)) inside of a humidity-controlled incubator (37°C, 5% CO_2, 95% humidity). The osmolarity of the media was 300mosM (Osmete 2, Precision Systems), and the pH was 7.4. Physiological and metabolic stability of this explant system has been demonstrated previously (Phillips and Haut 2004; Baars et al. 2006; Wei et al. 2006 and 2007).

INDENTATION TESTING OF EXPLANTS

The 24 explants were randomly assigned to three groups, 7, 14 and 21 days cyclic loading (n=4) versus control (n=4). Prior to and after cyclic loading, each explant was subjected to mechanical characterization using an indentation stress relaxation test. After

allowing a minimum of 30 minutes in media for the explants to equilibrate, mechanical indentation tests were performed before and after 7, 14 and 21 days of cyclic loading. Cartilage explant thickness was measured twice at perpendicular orientations across the center of the explant using a digital vernier caliper (Mitutoyo Corp.: Absolute Digimatic, Model No. CD-6" CS) with a resolution of 0.01 mm (Steinmeyer et al. 1997 and 1999). The two thickness values were averaged. The explants were placed on a flat level surface so that the face of the explant was perpendicular to the indenter tip. A magnet with a 4.3 mm diameter hole was placed on top of the explant to secure the edges and help resist curling of the explants. The explant and fixture were then submerged into a room-temperature phosphate buffered solution (PBS with pH 7.2) (Figure 1). A 2.39 mm diameter spherical, non-porous probe was lowered into the cartilage until a preload of 0.03 N was attained and held for 60 s. The indenter was then pressed into the cartilage 25% of the total thickness in 2 s and maintained for 600 s while resistive loads of relaxation were measured (Data Instruments, Acton, MA: model JP-25, 25 lb capacity), amplified and collected at 1,000 Hz for the first second and 20 Hz thereafter. The stress relaxation curves were obtained and fitted with a fibril-reinforced biphasic FE model (Soulhat et al. 1999). Cartilage matrix modulus (E_m), fiber modulus (E_f) and permeability (κ) were evaluated with a custom-written Gauss-Newton constrained nonlinear least square minimization procedure.

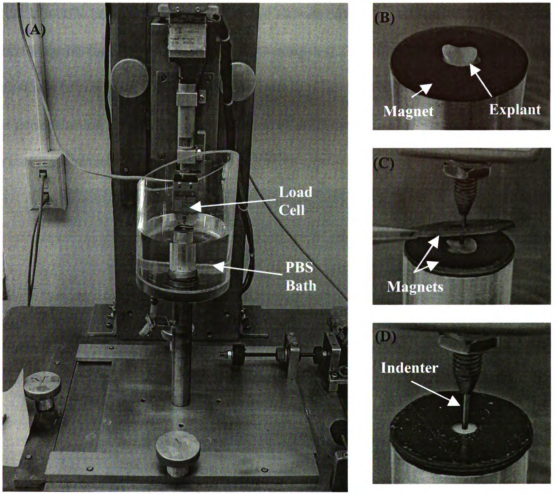

Figure 4. (A) Explant indentation test system and fixture. (B) First, explant is placed in hole of bottom magnet on flat steel surface. (C) Second, a top magnet is lowered over the top of the explant to hold down edges. (D) Finally, the indenter tip is lowered to a preload of 0.03 N.

CYCLIC LOADING OF EXPLANTS

The "cartilage exerciser" consisted of 12 loading chambers simultaneously powered by air (Figure 2). The control explants rested in the same "cartilage exerciser" as the cyclic loaded ones, but without cyclic loading on them. Pneumatic cylinders forced the pistons downward to apply a compressive load to the specimens through 14.6 mm diameter non-porous Teflon® platens. The "cartilage exerciser" was designed to hold a 24-well culture plate, so that 12 cartilage samples would be mechanically loaded and 12 unloaded control

explants would be subjected to an identical culture environment. Intermittently applied, uniaxial cyclic loading was introduced by using a 0.2 Hz sinusoidal waveform with a peak stress of 0.5 MPa. The cyclic loads were applied for 10 cycles followed by a load-free period lasting 3600 s. During the period of unloading the load platen was lifted from the cartilage surface.

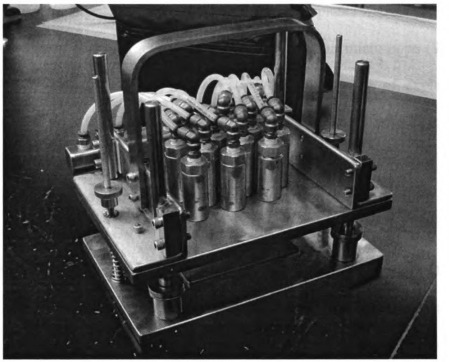

Figure 5. The "cartilage exerciser" mechanical loading device witl 12 cylinders to apply compressive loads to the cartilage explants.

UNCONFINED COMPRESSION TESTS

After cyclic loading, all explants (including the control) were taken to 707 N (~25 MPa), following a 5 N preload, in unconfined compression between two polished stainless steel plates. A 0.5 Hz (1 s time to peak) haversine loading protocol was programmed for application onto the explants in a servo-controlled hydraulic testing machine (Instron, model 1331, retrofitted with 8500 plus electronics, Canton, MA). Peak load, time to peak,

and maximum explant compression were documented in each experiment. Immediately after impact loading, the explants were returned to culture for 24 hours before cell viability tests.

EXPLANT SURFACE FISSURE AND CELL VIABILITY TESTS

For matrix damage analysis, the surfaces of all impacted explants were wiped with India ink and immediately photographed at 25× under a dissection microscope (Wild M5A, Wild Heerbrugg Ltd, Switzerland) to determine the total length of explant surface fissures (Baars et al. 2006). The total fissure length was measured with digital imaging software (Sigma Scan, SPSS Inc., Chicago, IL, USA). One observer (F.W.) digitally recorded the length of the surface fissures in each photograph.

After fissure length measurement, all explants were washed three times in DMEM:F12 and used to determine cell viability. Each explant was cut through its entire thickness with a specialized cutting tool with parallel blades spaced 0.5 mm (Phillips and Haut 2004; Ewers et al. 2001). These thin slices were then stained with calcein AM and ethidium homodimer (EthD-1), according to the manufacturer's specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR, USA). Viable cells were distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of virtually non-fluorescent cell-permeant calcein AM to intensely fluorescent calcein. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence. EthD-1 is excluded by the intact plasma membrane

of viable cells. Three slices were viewed across approximately 2 mm near the center of each explant in a fluorescence microscope (Leica DM LB, Leica Mikroskopie und Systeme GmgH, Wetlzar, Germany) and photographed using a digital camera (Spot Digital Camera, Diagnostic Instruments Inc.). These images were partitioned into the superficial zone (top 20%), intermediate zone (middle 50%) and deep zone (bottom 30%) (Phillips and Haut 2004). An average total cell count was calculated for each zone of the explants, using image analysis software (Sigma Scan, SPSS Inc., Chicago, IL, USA).

DETERMINATION OF PROTEOGLYCAN AND HYDROXYPROLINE CONTENTS

Cartilage explants were digested overnight at 60°C with papain digestion solution at a pH 6.0. Papain digested cartilage explants and culture media were independently DMB assayed for sulfated PGs by the reaction with 1,9-dimethylmethylene blue dye solution in polystyrene 96 well plates and quantitated with spectrophotometry at a wavelength of 530 nm using a Bio Tek microplate reader. Chondroitin sulfate A sodium salt from bovine trachea (Sigma-ALDRICH GmbH, Steinheim, Germany) was used as the standard (Steinmeyer et al. 1999). Some explants were used for hydroxyproline (HYP) measurement. After a guanidine HCl extraction to remove PG, tissue samples were acid-hydrolyzed in a 70°C water bath for 24 hours and microplate assayed to determine the total HYP content in the tissue (Brown et al. 2001; Bank et al. 1997).

STATISTICAL ANALYSIS

All experiments were repeated three times to obtain consistent results (n=12). The data obtained from post-cyclic loaded explants were normalized by the pre-cyclic loaded

values and subsequently analyzed with two-way repeated measures ANOVA and Student-Neuman-Keuls post hoc tests to determine differences from control and within cyclic loading groups. PG and HYP biosynthesis was normalized by tissue wet weight and reported in μg PG and μg HYP per mg wet weight, respectively. Statistical significance was indicated for $P<0.05$. All experimental data were reported as mean ± standard deviation.

RESULTS

INDENTATION TEST

All explants underwent indentation testing for mechanical characterization. Parameters before and after cyclic loading were compared in terms of "% of before cyclic loading" as in Figure 3. Fourteen days of cyclic loading significantly decreased the fiber modulus and permeability, but increased the matrix modulus of the explants. After 21 days, however, all other parameters reversed, except for the fiber modulus. After 7 and 14 days of cyclic loading, the matrix modulus increased approximately 22% and 35%, respectively, while 21 days of cyclic loading decreased the matrix modulus approximately 12%. After 7 days of cyclic loading, the fiber modulus significantly decreased nearly 80% and remained at this low level at 14 and 21 days. After 7 and 14 days of cyclic loading, the explant permeability decreased approximately 30% and 45%, respectively, while 21 days of cyclic loading significantly increased the permeability by nearly 54%.

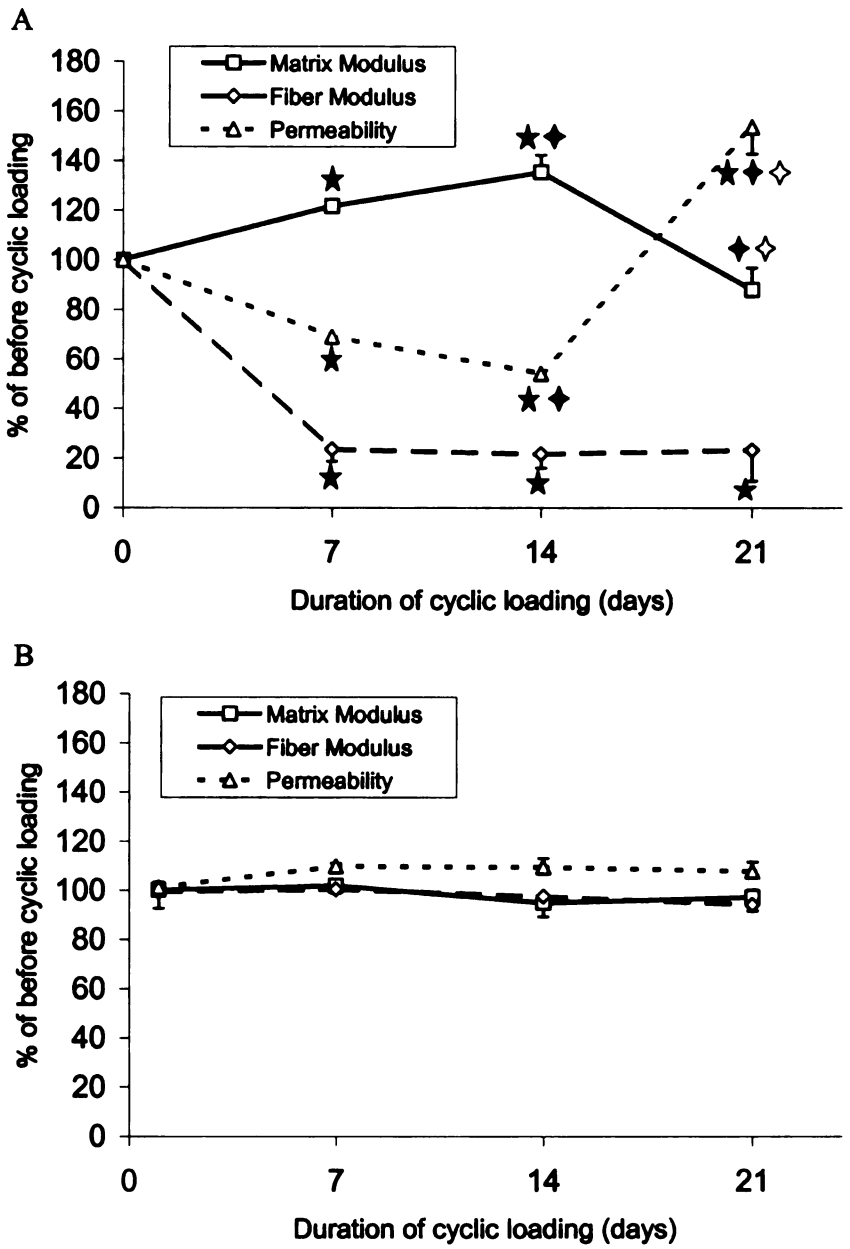

Figure 6. Up to 14 days cyclic loading significantly decreased the fiber modulus and permeability, but increased the cartilage matrix modulus. After 21 days, however, the matrix modulus significantly decreased and tissue permeability increased (A). In all groups, the controls (non-cyclic loading) remained constant for all parameters (B). Statistically significant differences were observed: ★ from control, ✦ from day 7, and ✧ from day 14.

UNCONFINED COMPRESSION TESTS

The stress-strain response of all explants had a nonlinear (toe) region (0-10 MPa) followed by a linear region (10-25 MPa) during unconfined compression in 1 s (Figure 4A). Explants that were regularly compressed up to 14 days exhibited a stiffened response, with the longer durations of loading shifted more to the left. However, after 21 days of loading the explants became less stiff than the control. Statistically significant differences were observed between each test group and the control responses. The slope of the linear region (modulus) between 10 MPa and 25 MPa was increased approximately 18% of control at day 7 (105.5 ± 15.3 MPa to 123.0 ± 16.2 MPa) and approximately 30% of control in day 14 (105.5 ± 15.3 MPa to 133.2 ± 11.9 MPa). After 21 days of cyclic loading, however, the modulus of the explant decreased significantly below the 7 and 14 days to 94.2 ± 15.0 MPa (Figure 4B).

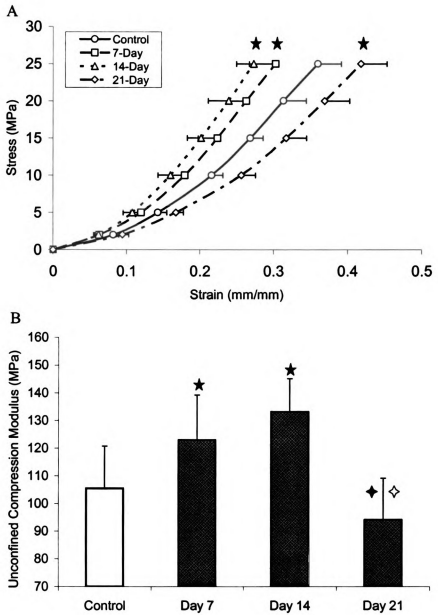

Figure 7. The control shown in this figure is a combination of controls from all groups. Explants cyclic-loaded up to 14 days exhibited a stiffer response than controls, resulting in a shift to the left, with the longer duration of loading shifted more. After 21 days of cyclic loading, however, the explants became less stiff than the controls. ★: Statistically significant differences from control as determined by two-way repeated ANOVA, $P<0.05$.

FISSURE LENGTH

Cyclic loading affected the fissure length of cartilage explants (Figure 5). After 7 days of cyclic loading and unconfined compression the length of surface fissures decreased 57% from control (7.9 ± 1.9 cm to 3.4 ± 1.3 cm). After 14 days of cyclic loading and impaction, the fissure length decreased to 42% of control (3.3 ± 1.5 cm), not different from 7 days but statistically different from control. After 21 days of cyclic loading, however, there was an increase (approximately 170% of 7 and 14 days) of fissure length (9.0 ± 2.0 cm). This value was not statistically different from control, but different than the 7 and 14 day fissure length (Figure 6).

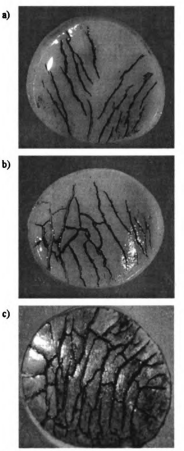

Figure 8. a) 7 days; b) 14 days; c) 21 days cyclic loaded explants after the unconfined compression. Explants in group c) exhibited excessive surface fissuring across the entire surface.

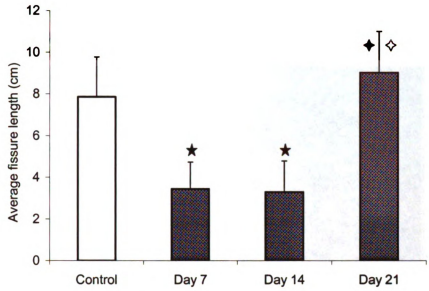

Figure 9. Cyclic loading up to 14 days significantly decreased the explant surface fissure length, while after 21 days of cyclic loading an increase of fissure length from previous groups was observed. Statistically significant differences were observed: ★ from control, ✦ from day 7, and ✧ from day 14.

CELL VIABILITY

Cyclic loading prior to the unconfined compression test affected the cell viability of the explants (Figure 7). Cyclic loading up to 14 days significantly saved cells in the superficial zone. After 14 days, non-cyclic loaded (control) cartilage had only 40% live cells, while cyclic loaded cartilage had 80% (Figure 8). After 21 days of cyclic loading the average number of live cells decreased significantly in both superficial and deep zones following impact loading (28% of live cell in superficial zone and 42% of live cell in deep zone). No significant differences were observed in the middle zone for all groups. Explant maximum strains from the unconfined compression tests were used to calculate the percentage of cell death per unit strain. Cell death per unit strain was significantly decreased versus controls up to 14 days of cyclic loading (70% and 35% of controls at

day 7 and day 14, respectively). In contrast, after 21 days of cyclic loading the cell death per unit strain was not different from control (Figure 9).

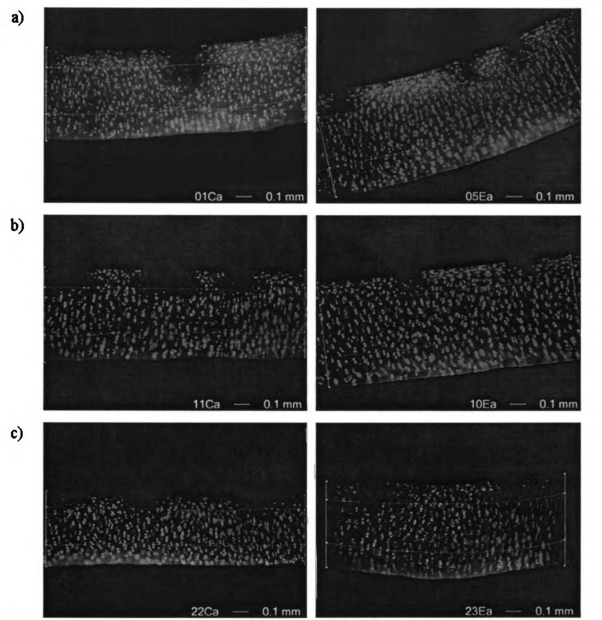

Figure 10. Typical images of cartilage explants stained for cell viability. Live cells stained green and dead cells stained red. The cracks on the surface were due to the unconfined compression on the explants. a) 7 day control (left) and cyclic loaded (right); b) 14 days control (left) and cyclic loaded (right); c) 21 days control (left) and cyclic loaded (right).

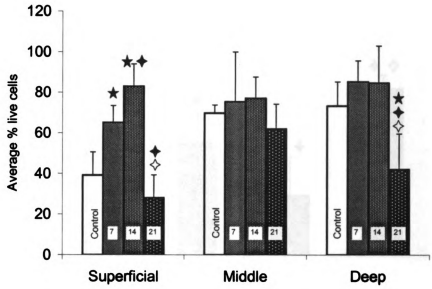

Figure 11. Cell viability in the superficial, middle and deep zones of cartilage explants following unconfined compression. Cyclic loading affected the cell viability mostly in the superficial zone. No significant differences were observed in the middle zones of all groups. Cyclic loading, up to 14 days, significantly saved cells in the superficial zone. After 21 days of cyclic loading, however, the average number of live cells decreased significantly in both the superficial and deep zones. Statistically significant differences were observed: ★ from control, ✦ from day 7, and ✧ from day 14.

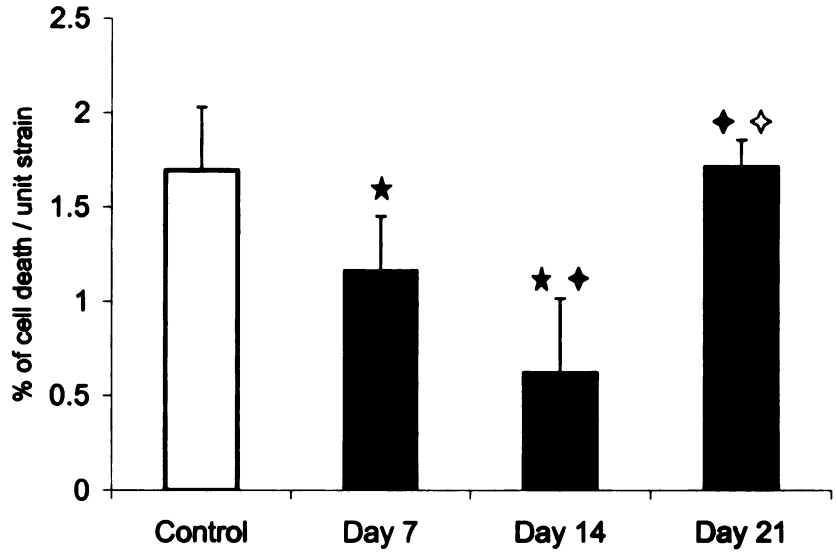

Figure 12. Along with cyclic loading period, cell death per unit strain was significantly decreased for up to 14 days of cyclic loading (70% and 35% of control at day 7 and day 14, respectively), while after 21 days of cyclic loading there was no difference from control. Statistically significant differences were observed: ★ from control, ✦ from day 7, and ✧ from day 14.

PROTEOGLYCAN AND HYDROXYPROLINE CONTENTS

Cyclic loading up to 14 days significantly increased PG content of the cartilage explants, with the largest increase (40%) at 14 days (Figure 10A). After 21 days, however, cyclic loading resulted in a significant loss in PG's. After cyclic loading PGs were released from cartilage explants into the media in all 3 groups (Figure 10B). Cyclic loading also resulted in a significant decrease in tissue HYP content by approximately 20% in all 3 groups (Figure 11).

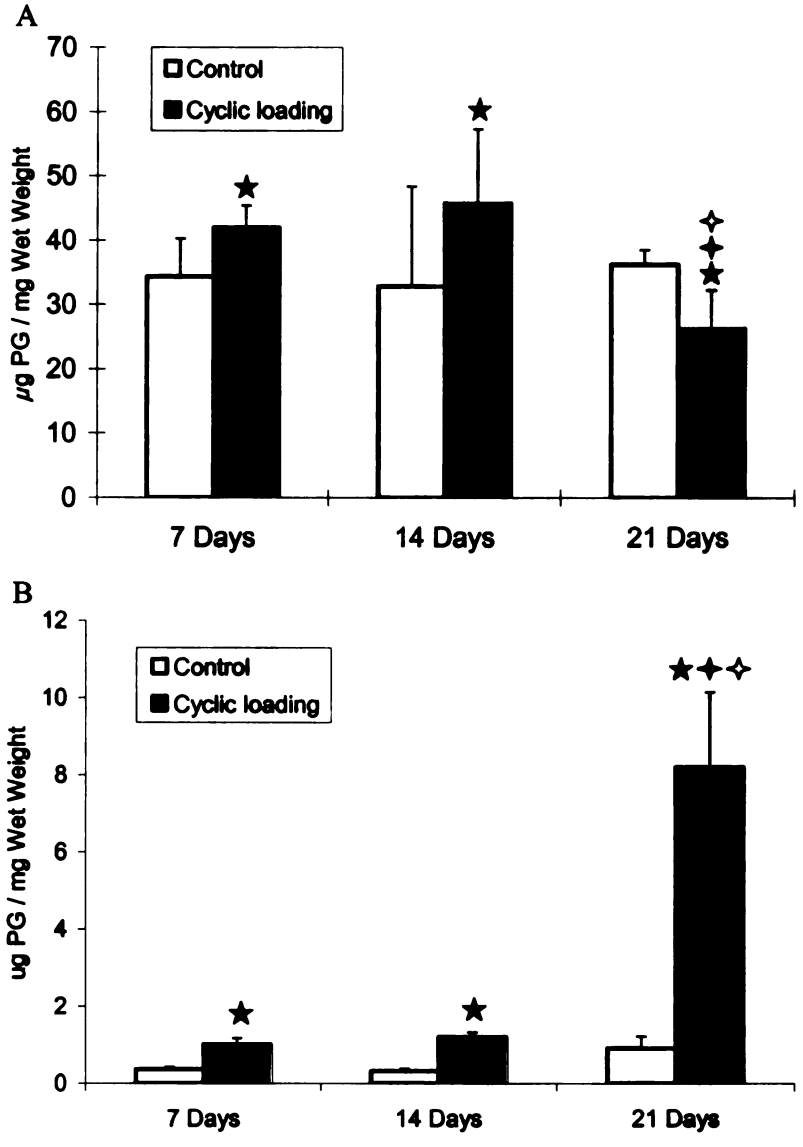

Figure 13. (A) Total tissue proteoglycan (PG) content per mg tissue wet weight. Cyclic loading up to 14 days increased PG content in the tissue sample. After 21 days of cyclic loading, however, cartilage explants significantly lost PG's. (B) Total PG content in media per mg tissue wet weight. After cyclic loading PG's were released from tissue samples into media in all 3 groups. After 21 days of cyclic loading PG content in the media significantly increased 8 times than control. Statistically significant differences were observed: ★ from control, ✦ from day 7, and ✧ from day 14.

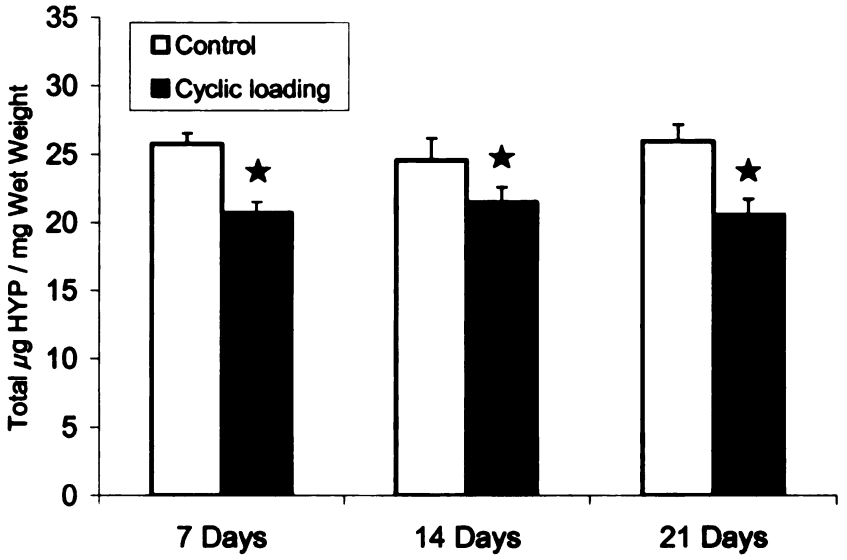

Figure 14. Total hydroxyproline (HYP) content per mg tissue wet weight. Cyclic loading decreased tissue HYP content by approximately 20% in all 3 groups. Statistically significant differences were observed: ★ from control.

DISCUSSION

The hypothesis of the current study was that low intensity, intermittent cyclic loading of chondral explants would up-regulate the production of PGs by matrix chondrocytes to increase the material properties of the specimens, making them more resistant to blunt force overloading. The results of the study showed cyclic preloading did mechanically stiffen the explants, making them more resistant to matrix damage and cell death under 25 MPa of unconfined compression up to 14 days. However, after 21 days of cyclic loading the explants lost stiffness and suffered more extensive damage in the acute, unconfined compression test than controls without cyclic preloading.

Numerous aspects of this study parallel the findings of the previous literature. In one study Sah et al. (1989) reported that multiple 2-hr compressions of calf articular cartilage explants (at 8-hr intervals) increases the incorporation of $^{35}SO_4$ during 1.5-5.5 hr after release. However, the study was unable to show a significant increase in glycosaminoglycan (GAG) content of the explants. More recently, Parkkinen et al. (1992) have shown that a 0.5 MPa load significantly increased $^{35}SO_4$ incorporation by chondrocytes for compressions repeated at 2- and 4-s but not at 20- and 60-s intervals for 1.5 hr. Steinmeyer et al. (1999) show that peak stresses of 0.1, 0.5 or 1.0 MPa applied for 10 s followed by a load-free period of 10, 100 or 1000 s increases the incorporation of $^{35}SO_4$ after 3 days of cyclic loading, but again no significant change in tissue PG content was documented in the study. Similarly, Thibault et al. (2002) cyclically loaded osteochondral explants with 40 ramps of 250 μm amplitude (~2-5 MPa) with a 20 s rest between each ramp loading without documenting a significant increase in GAG content

of the cartilage. In contrast to these previous studies, a significant increase in tissue PG content was noted at 7 and 14 days following 10 cycles of 0.2 Hz sinusoidal loading at 0.5 MPa followed by 3600 s of unloading in the current study. These data suggest that PG synthesis in these in vitro experiments is dependent on the intensity, frequency and duration of intermittent cyclic loading.

Fewer studies have investigated alterations in the collagen network following in vitro cyclic loading of articular cartilage. In Thibault et al. (2002) cyclic loading resulted in a statistically significant rise in the tissue content of denatured collagen immediately after the loading period. This collagen was then released to the culture media during a 10 day post-loading period. In the current study there was a statistically significant decrease in the tissue content of collagen at 7 days, and that remained at the same level for the 21 day duration of cyclic loading.

The above changes in the tissue contents of PG and collagen were found to temporally parallel with the documented alterations in mechanical properties of the explants. More specifically, the increase in tissue PGs at 7 and 14 days paralleled with an increase in matrix modulus, E_m, and a decrease in tissue permeability κ. These results are supported by a previous study, in another laboratory, that showed enzymatic depletion of PGs in bovine cartilage results in a parallel decrease in the matrix modulus and an increase in permeability from a fibril reinforced poroelastic model of cartilage, based on unconfined relaxation tests of the tissue (Korhonen et al. 2003). Additionally, in the Korhonen et al. study enzymatic depletion of collagen generated a corresponding decrease in fibril

modulus, E_f. A significant decrease in E_f and increase in tissue permeability was noted early after cyclic loading in the Thibault et al. study, where they suggested correlation with an increased content of denatured collagen in the loaded explants. In the current study there was also a significant decrease in total collagen content at 7 to 21 days that paralleled with a decrease in tissue E_f.

A dramatic increase in tissue damage to 25 MPa of unconfined compression was noted after 21 days of cyclic loading in the current study. The effect also involved a dramatic decrease in the tissue matrix modulus E_m, an increase in tissue permeability, and a decrease in tissue PG content. This may be explained by an increase in the production of degenerative enzymes, such as matrix metalloproteinase-3 (MMP-3), which is noted to occur following extended cyclic loading of chondral explants (Lin et al. 2004). This referenced study documented a significant increase in MMP-3 after 24 hr of cyclic loading at 1 or 5 MPa. The temporal difference in the previous and current studies could be due to the frequency and magnitude of the in vitro cyclic loading on the tissue explants.

In the current study it was also interesting that while the collagen content and fiber modulus E_f of the explants significantly decreased after 7 days, the unconfined compression modulus of the explants was significantly greater than controls, and it more directly paralleled with the temporal changes in tissue PG content, the matrix modulus E_m and permeability κ. An analysis of the model sensitivity to changes in E_f, E_m and κ (unpublished) suggested that the stiffness of the explant in the current unconfined

compression test should depend on the fiber modulus E_f as much as E_m. A potential explanation for the lack of E_f response in the current study could be the presence of upper surface friction in the unconfined test. This could minimize the role of collagen fibers that are primarily oriented radial in the upper tissue layer that also act to constrain the explant laterally during the unconfined experiment. And yet, previous unconfined compression relaxation experiments, reported by Korhonen et al, displayed sensitivity of their tests to an enzymatic loss of collagen in the chondral explant. Future studies will be required to understand more precisely if the lack of E_f sensitivity in the current experiments was due to the test protocol (0.5Hz sinusoidal loading or relaxation testing), friction or a problem in the validity of the fibril reinforced model for these cartilage explant tests.

As suggested earlier, the long term health of joint cartilage may relate directly to the extent of matrix and cell damage that occurs during an acute overloading of a tissue. The current study indicated that mechanical stiffening of the chondral explant by cyclic preloads increased the tissue content of PG's and resulted in less strain during unconfined compression at 25 MPa. This was correlated with a significant increase in cell survival, as was hypothesized in the study. This result suggested that cell membrane damage may directly relate to the amount of matrix deformation, as proposed earlier by others (Torzilli et al. 2006). Additionally, the percentage of cell damage per unit compressive strain was also significantly decreased for up to 14 days of cyclic loading. One explanation for this result may relate to an increase in PG's surrounding individual cells in the pericellular matrix as a result of cyclic preloading. This effect has been documented previously

following low intensity, cyclic loading (Parkkinen et al. 1992; Quinn et al. 1998). Such deposition of PG's concentrated in or near the pericellular matrix of chondrocytes may significantly alter (increase) the local stiffness around cells to help protect them from damaging distortions during severe levels of unconfined compression, such as that imposed on the chondral explants in the current study (Guilak et al. 1999).

This study, as understood by the authors, is the first to investigate the role of in vitro cyclic loading on the response of a chondral explant to a potentially damaging, acute overload. In the long-term these types of studies may help understand the role of biologic-based pre-conditioning of articular cartilage for in vitro, or even in vivo, studies of blunt force trauma to a joint. The current data would suggest both a positive and a negative aspect to the idea of cyclic pre-conditioning or regular exercise in the prevention of long-term chronic disease in joints suffering an acute overload, such as that proposed during acute ligament injury. This in vitro study may suggest that the frequency, magnitude and duration of the pre-conditioning may play a role in its potential chondral protective effects. Clearly, additional in vitro and in vivo studies are wanted.

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CHAPTER TWO

HIGH LEVELS OF GLUCOSAMINE-CHONDROITIN SULFATE CAN ALTER THE CYCLIC PRELOAD AND ACUTE OVERLOAD RESPONSES OF CHONDRAL EXPLANTS

ABSTRACT

A recent study by our laboratory has shown that 14 days of low intensity, intermittent cyclic preloading of chondral explants has a positive effect on the tissue by elevating the concentration of proteoglycans (PGs) to cause a mechanical stiffening of the explants prior to an acute overload and limit the extent of tissue damage. And yet, longer term loading to 21 days results in tissue degradation prior to the acute traumatic event and excessive damage from an acute overload. Previous studies, by others, have shown that bathing chondral explants in a supplement of glucosamine-chondroitin sulfate (glcN-CS) can up-regulate the synthesis of tissue PGs, particularly in stressed tissue, and the supplement serves as an anti-inflammatory agent. The hypothesis of the current study was the supplementation of culture media with a high concentration of glcN-CS would up-regulate the production of tissue PG and limit or mitigate long term degradation of chondral explants under cyclic preloading and limit tissue damage in an acute overload. The current study showed that, in the presence of supplement, cyclic preloading significantly increased tissue PG content and matrix modulus by approximately 65% and 300%, respectively, at 21 days, resulting in a reduction of matrix damage and cell death during an acute overload event. These experimental data may help explain the biological action of high concentration of this culture supplement and its effect on the mechanical properties of this in vitro tissue model.

INTRODUCTION

Traumatic joint injury by blunt mechanical forces has been demonstrated to be a risk factor for the development of osteoarthritis (OA) (Wilder et al. 2002). In vitro studies of impact loading (Torzilli et al. 1999; Quinn et al. 2001) of cartilage have shown cell death and matrix damage. Cyclic loading of articular cartilage has also shown chondrocyte death (Lin et al. 2004; Sauerland et al. 2003) and mechanical weakening of collagen (Thibault et al. 2002) which are dependent on the intensity and frequency of loading. In contrast, other in vitro and in vivo studies have shown positive aspects of low intensity, intermittent compressive loading of cartilage, such as increased biosynthesis of proteoglycans (PGs) (Sah et al. 1989; Parkkinen et al. 1992; Saadat et al. 2006). Changes in the PG and collagen contents of cartilage have been shown to significantly alter the mechanical properties of this tissue (Korhonen et al. 2003). Thus, it is reasonable to assume that cyclic preloading of cartilage can alter the mechanical properties of the tissue and help determine the extent of matrix damage and cell death that will occur due to the blunt force overloading of a joint.

Recent studies in our laboratory have shown that low intensity, intermittent cyclic preloading of chondral explants prior to an acute overload yields an increase in the synthesis of tissue PGs, resulting in a less severe traumatic insult to an acute overload event. Interestingly, between 14 and 21 days of cyclic preloading the chondral explants experience a significant loss of tissue PGs which leads to a dramatic loss in mechanical properties. An acute overloading of the explanted tissue then yields matrix damage and cell death significantly greater than non-preloaded controls. The study suggests that low

intensity, intermittent cyclic loading can increase the biosynthesis of tissue PG up to a point, and thereafter there seems to be a yet unknown biological event that causes tissue degeneration and compromises the explanted tissue in an acute overload event, such as that which may occur during an acute knee ligament rupture (Fang et al. 2001).

Previous in vitro studies, by others, have documented frequency and intensity dependent responses suggesting that cyclic compression of articular cartilage modulates the metabolism and turnover of PGs and collagen in a complex and multifactorial manner. For example, cyclic intensities of 0.5 MPa in 4 s intervals for 1.5 hrs stimulates PG production, while at 1.0 MPa PG production by tissue cells is down-regulated (Parkkinen et al. 1992). Another study at 0.5 MPa documents an increase in endogenous PGs at low and high frequencies of cyclic loading over 6 days, without significant changes in the total PG content of the tissue (Sauerland et al. 2003). Similarly, an early study at a frequency of 0.5 Hz and either 0.1, 0.5 or 1 MPa indicates maximum PG synthesis at 3 days and a dramatic release of endogenous PGs and excessive cell death at 6 days (Steinmeyer et al. 1999). While a more recent study at 0.5 Hz for intensities of 1 and 5 MPa over 24 hrs shows a significant loss of tissue PG and cell death with production of the matrix metalloproteinase-3 (MMP-3) adjacent to damaged collagen fibers (Lin et al. 2004). Similarly, under 0.5 MPa of 1 Hz cyclic loading significant increases in the synthesis of MMP-2 and -9 have been indicated after 3 hrs (Blain et al. 2001). Furthermore, previous in vivo studies have shown that moderate intensities of cyclic loading can have a positive effect on the mechanical properties of joints by elevating the concentration of PGs in articular cartilage (Helminen et al. 2000). Saadat et al. (2006)

documented that 80 cumulative hours of physiological joint loading led to a 46% increase of PG synthesis. On the other hand, other studies have shown that a more strenuous regimen of exercise can also have a deleterious effect on joint cartilage, resulting in a significant modulus decrease of 13-14% and a reduction of PG content (Helminen et al. 1992 and 2000). Similar effects have been documented for in vitro experiments using explanted cartilage wherein low or moderate levels of intermittent cyclic loading has positive effects on the mechanical properties of explants (Parkkinen et al. 1992, Sah et al. 1989; Steinmeyer et al. 1999), while other studies with more severe levels of cyclic loading have shown detrimental effects on cartilage explants, with an increase of cell death and matrix damage (Thibault et al. 2002).

OA is a manifestation of an imbalanced synthesis of articular cartilage matrix and the associated growth factors. Understanding the changes in collagen and PG content of cartilage due to mechanical forces is necessary for progress in treating join disorders, such as OA. The use of potentially chondro-protective agents such as glucosamine (glcN) and chondroitin sulfate (CS) has been explored to medicate OA. Previous studies by others have shown that bathing cartilage explants in a supplement of glcN and CS can up-regulate the synthesis of tissue PGs, and particularly in stressed tissue (Lippiello 2003). Recent studies have also shown that this supplement limits tissue degradation in the presence of inflammatory enzymes in an in vitro setting (Derfoul et al. 2007). However, the role of PGs synthesized from intermittently loaded cartilage in response to an acute compressive overloading of articular cartilage has not been elucidated. While clinical trials of oral glcN in OA patients support the contention that it is a disease-modifying

agent (Richy et al. 2003), other studies find no clinical effect (Hughes and Carr 2002). In contrast, more consistent results have been documented in more environmentally controlled tissue and cell culture experiments. This combination has, indeed, been shown to function as a "biological response modifier" that can boost the natural protective responses of tissue under adverse environmental conditions, such as mechanical compression (Lippiello 2003). Using human OA articular cartilage, treatments with glcN sulfate have shown increased aggrecan mRNA levels, as well as a decrease in the activity of MMP-3 (Dodge and Jimenez 2003). Other in vitro studies have shown that incubation of chondral explants in glcN after exposure of the tissue to the proinflammatory cytokine interleukin-1 prevents the increase in PG release and MMP activity (Fenton et al. 2000).

Since previously data showed that PG content directly paralleled to stiffness of cartilage, we wanted to experimentally increase tissue PG content to strengthen the cartilage, making them inhibit the degradation of the tissue after long term of cyclic compression and reduce the susceptibility of cartilage to a severe level of mechanical injury, such as an acute overload. The objective of the current study was to use this supplement in an experimental setting to alter PG synthesis during cyclic loading of chondral explants and document its effect on the response of explants to an acute overload. Thus, the hypotheses of the current study were that, during low intensity, intermittent cyclic loading, a high concentration of glcN-CS would up-regulate the production of PGs in an explanted tissue to alter its mechanical properties in relatively short-term experiments. By increasing the mechanical stiffness in this fashion, there would be a reduction in the extent of matrix damage and cell death following an acute overload on the explant.

Secondly, because of the documented inhibition effects of glcN on aggrecanase and MMPs, supplementation of the culture media with this product during cyclic preloading will limit or even mitigate the degeneration effect noted to occur between 14 and 21 days in this in vitro model.

METHODS

DISSECTION, TISSUE CULTURE AND GROUPING OF EXPLANTS

Bovine forelegs from mature animals (18-24 months of age) were obtained from a local abattoir within two hours of slaughter. The legs were skinned and rinsed with water prior to exposing the metacarpal joint under a laminar flow hood. Biopsy punches were used to make one hundred and forty-four 6 mm diameter chondral explants from the lower metacarpal surface of the limbs. Each explant was separated from the underlying bone with a scalpel. All specimens were washed three times in Dulbecco's Modified Eagle Media: F12 (DMEM: F12) (Gibco, USA), and placed in the media added with 10% fetal bovine serum, additional amino acids and antibiotics (penicillin 100 U/ml, streptomycin 1 μg/ml, amphotericin B 0.25 μg/ml) in 24-well plates. The explants were randomly assigned to three groups: control (n=72), cyclic loading (n=36) and cyclic loading with supplement (n=36). Each group was subjected to 7, 14 and 21 days of testing periods (Figure 1). The concentration of supplement in the media with was 500 μg/ml glcN (FCHG49®) and 250 μg/ml CS (TRH122®) to try to maximize the hypothesized effect, based on previous studies (Rundell 2005). The media was replaced every 2 days throughout the study and harvested and stored frozen at -80°C. A mechanical loading device, "cartilage exerciser" (described below), was used to cyclically load the explants inside of a humidity-controlled incubator (37°C, 5% CO_2, 95% humidity). The osmolarity of the media was 300mosM (Osmete 2, Precision Systems), and the pH was 7.4. Physiological and metabolic stability of this explant system has been demonstrated previously (Phillips and Haut 2004; Baars et al. 2006; Wei et al. 2007a and 2007b).

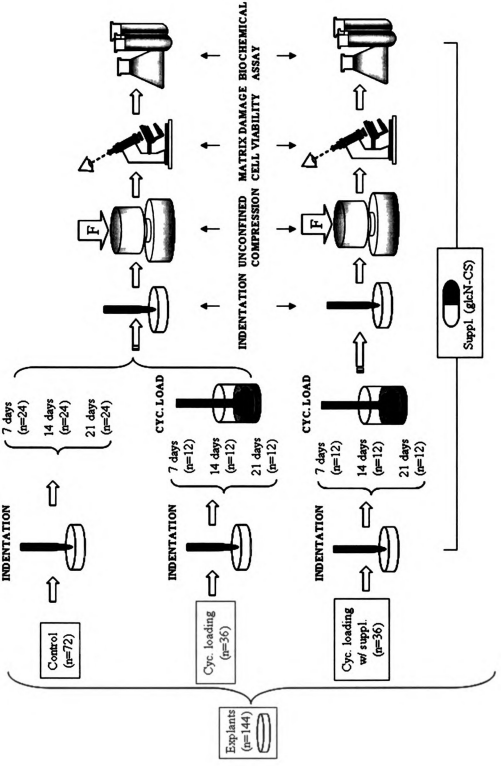

Figure 15. Flowchart showing the experimental designing and grouping, with different schematics representing different testing.

INDENTATION TESTING OF EXPLANTS

Prior to and after cyclic loading, each explant was subjected to mechanical characterization using an indentation stress relaxation test (Figure 1), followed by an equilibration of 30 min in the media. Cartilage explant thickness was measured twice at perpendicular orientations across the center of the explant using a digital vernier caliper with a resolution of 0.01 mm (Steinmeyer et al. 1997 and 1999). The two thickness values were averaged. The explants were placed on a flat level surface so that the face of the explant was perpendicular to the indenter tip. A magnet with a 4.3 mm diameter hole was placed on top of the explant to secure the edges and help resist curling of the explants. The explant and fixture were then submerged into a room-temperature phosphate buffered solution (pH 7.2). A 2.39 mm diameter spherical, non-porous probe was lowered into the cartilage until a preload of 0.03 N was attained and held for 60 s. The indenter was then pressed into the cartilage 25% of the total thickness in 2 s and maintained for 600 s while resistive loads of relaxation were measured, amplified and collected at 1,000 Hz for the first second and 20 Hz thereafter. The stress relaxation curves were obtained and fitted with a fibril-reinforced biphasic FE model (Soulhat et al. 1999) for an assumed Poisson's ratio of 0.25 and void ratio of 5.67. Cartilage matrix modulus (E_m), fiber modulus (E_f) and tissue permeability (κ) were evaluated with a custom-written Gauss-Newton constrained nonlinear least square minimization procedure.

CYCLIC LOADING OF EXPLANTS

The "cartilage exerciser" consisted of 12 loading chambers simultaneously powered by air. The control explants rested in the same "cartilage exerciser" as the cyclic loaded ones, but without cyclic loading on them. Pneumatic cylinders forced the pistons downward to apply a compressive load to the specimens through 14.6 mm diameter non-porous Teflon® platens. The "cartilage exerciser" was designed to hold a 24-well culture plate, so that 12 cartilage samples would be mechanically loaded and 12 unloaded control explants would be subjected to an identical culture environment. Intermittently applied, uniaxial cyclic loading was introduced by using a 0.2 Hz sinusoidal waveform with a peak stress of 0.5 MPa. The cyclic loads were applied for 10 cycles followed by a load-free period lasting 3600 s.

UNCONFINED COMPRESSION TESTING OF EXPLANTS

After cyclic loading, all explants (including the control) were taken to 707 N (~25 MPa), following a 5 N preload, in unconfined compression between two polished stainless steel plates. A 0.5 Hz (1 s time to peak) haversine loading protocol was programmed for application onto the explants in a servo-controlled hydraulic testing machine (Instron, model 1331, Canton, MA). Immediately after this acute loading, the explants were returned to culture for 24 hours before cell viability tests were performed.

MATRIX DAMAGE AND CELL VIABILITY TESTING OF EXPLANTS

For matrix damage analysis, the surfaces of all impacted explants were wiped with India ink and immediately photographed at 25× under a dissection microscope (Wild M5A, Wild Heerbrugg Ltd, Switzerland) to determine the total length of explant surface

fissures (Baars et al. 2006). The total fissure length was measured with digital imaging software (Sigma Scan, SPSS Inc., Chicago, IL, USA). One observer (F.W.) digitally recorded the length of the surface fissures in each photograph.

After the measurement of fissure length, all explants were washed three times in DMEM:F12 and used to determine cell viability. Each explant was cut through its entire thickness with a specialized cutting tool with parallel blades spaced 0.5 mm (Philips and Haut 2004; Ewers et al. 2001). These thin slices were then stained with calcein AM and ethidium homodimer (EthD-1), according to the manufacturer's specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR, USA). Three slices were viewed across approximately 2 mm near the center of each explant in a fluorescence microscope (Leica DM LB, Leica Mikroskopie und Systeme GmgH, Wetlzar, Germany) and photographed using a digital camera (Spot Digital Camera, Diagnostic Instruments Inc.). An average percent live cell count was performed based on the images from each explant, using image analysis software (Sigma Scan, SPSS Inc., Chicago, IL, USA). Explant maximum strains from the unconfined compression tests were used to calculate the percentage of cell death per unit strain.

BIOCHEMICAL ASSAYS

Seventy-two cartilage explants (36 control, 18 cyclic loading and 18 cyclic loading with supplement) were digested overnight at 60°C with papain digestion solution at a pH of 6.0. Papain digested cartilage explants and culture media were independently DMB assayed for sulfated PGs by the reaction with 1,9-dimethylmethylene blue dye solution in polystyrene 96 well plates and quantitated with spectrophotometry at a wavelength of 530

nm using a Bio Tek microplate reader. Chondroitin sulfate A sodium salt from bovine trachea (Sigma-ALDRICH GmbH, Steinheim, Germany) was used as the standard (Steinmeyer et al. 1999). The other seventy-two explants were used for hydroxyproline (HYP) measurement. After a guanidine HCl extraction to remove PG, tissue samples were acid-hydrolyzed in a 70°C water bath for 24 hours and microplate assayed to determine the total HYP content in the tissue (Brown et al. 2001; Bank et al. 1997).

STATISTICAL ANALYSIS

The data obtained from post-cyclic loaded explants were normalized by the pre-cyclic loaded values and subsequently analyzed with two-way repeated measures ANOVA and Student-Neuman-Keuls post hoc tests to determine differences between groups. PG and HYP biosynthesis was normalized by tissue wet weight and reported in μg PG and μg HYP per mg wet weight, respectively. Statistical significance was indicated for $P<0.05$. All experimental data are reported as mean ± standard deviation.

RESULTS

INDENTATION TESTING

All explants underwent indentation testing for mechanical characterization before and after cyclic loading (Figure 1). Matrix modulus, fiber modulus and permeability of the explants were compared in terms of "% of before cyclic loading" (Figure 2). Cyclic loading with the supplement significantly increased the tissue matrix modulus by approximately 300% up to 21 days (260.2 ± 47.0%, 300.9 ± 80.3% and 307.9 ± 40.6% higher after 7, 14 and 21 days cyclic loading, respectively, than before). Cyclic loading without supplement was also different than control, however, the matrix modulus was decreased by 58.4 ± 19.4% after 21 days (Figure 2A). Cyclic loading with and without supplement significantly decreased the tissue fiber modulus by approximately 40% and 60%, respectively, at all time points (Figure 2B). The fiber modulus was significantly higher with versus without the supplement. Up to 14 days of cyclic loading the permeability of the explants without supplement significantly decreased (71.1 ± 8.5% and 31.0 ± 7.7% of before cyclic loading in 7 and 14 days groups, respectively), and then it increased dramatically at 21 days (143.0 ± 9.9% of before cyclic loading). In contrast, cyclic loading with supplement decreased the tissue permeability significantly at all time points (46.7 ± 17.4%, 34.9 ± 16.4% and 35.4 ± 5.2% of before cyclic loading in 7, 14 and 21 days groups, respectively) (Figure 2C). For all three parameters the controls (non-cyclic loaded) remained at the same level (approximately 100%) for all time points.

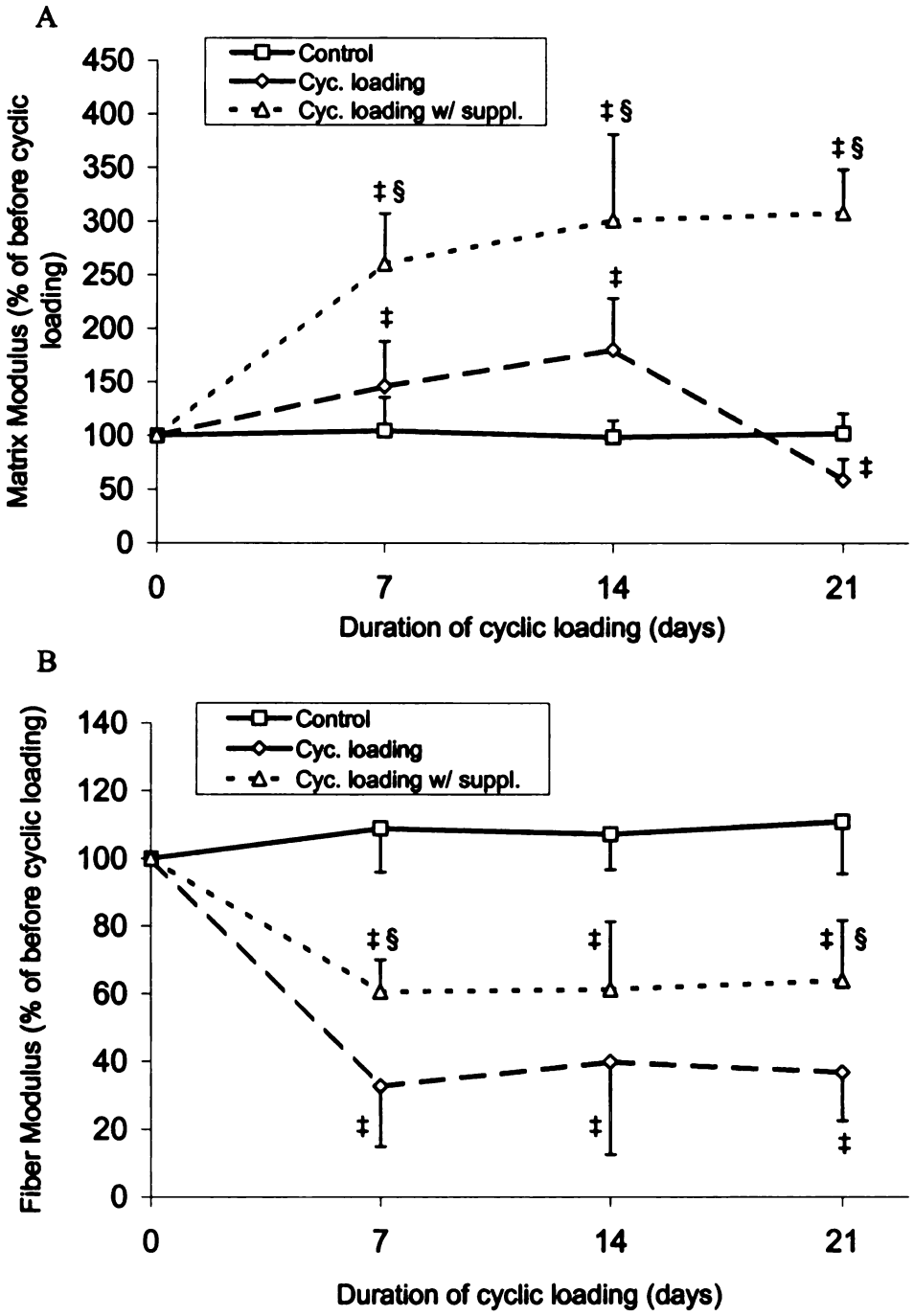

Figure 16. Cyclic loading with supplement strengthened the cartilage matrix modulus by approximately 3 times than control (A), and helped inhibit the degradation of the explants from longer time (21 days) cyclic loading (A and C). Cartilage fiber modulus, however, decreased approximately 40% and 60% by cyclic loading with and without supplement, respectively (B). Statistically significant differences were observed from control (‡), and from cyclic loading (§).

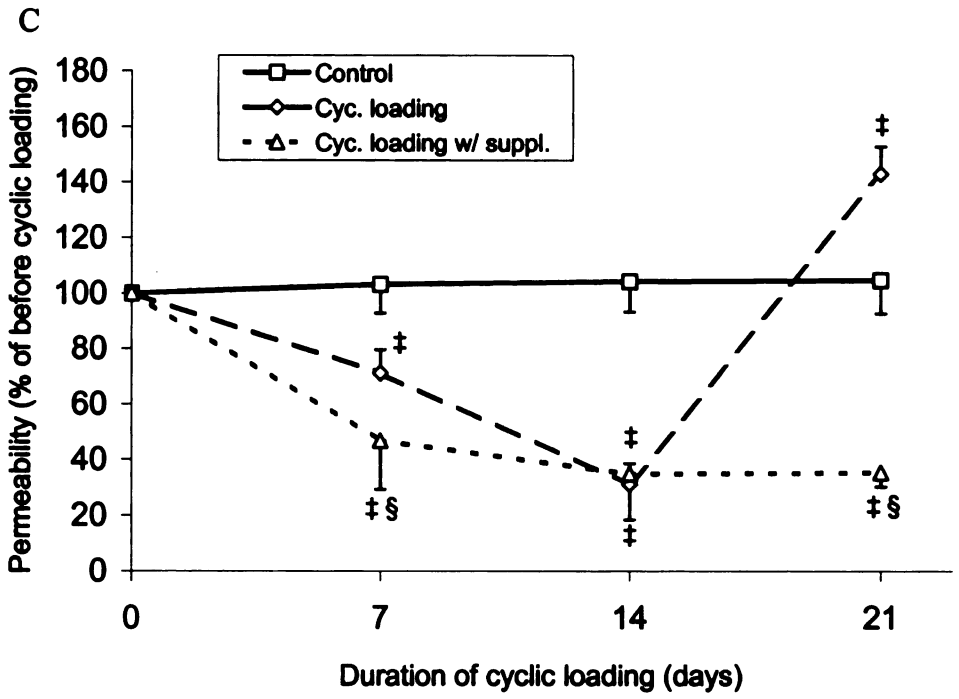

Figure 16. (cont'd)

BIOCHEMICAL ASSAYS

In the presence of supplement, cyclic loading of the explants significantly stimulated tissue PG synthesis, resulting in an increase of tissue PGs by approximately 65% of control (from approximately 30 μg PG/mg tissue wet weight to approximately 50 μg PG/mg tissue wet weight). Cyclic loading without supplement also increased PG content up to the supplemented level by 14 days, however, a significant loss (approximately 30%) in PGs was noted after 21 days (from 34.5 ± 5.3 to 24.3 ± 6.0 μg PG/mg tissue wet weight) (Figure 3A). Cyclic loading without supplement also resulted in an approximately 20% decrease in tissue HYP content (from 25 μg HYP/mg tissue wet weight to 20 μg HYP/mg tissue wet weight, approximately). In the presence of supplement, however, cyclic loading at 14 and 21 days decreased the tissue HYP content

by only approximately 10% (Figure 3B). The tissue content of HYP with supplement was indicated to be significantly higher than without media supplementation.

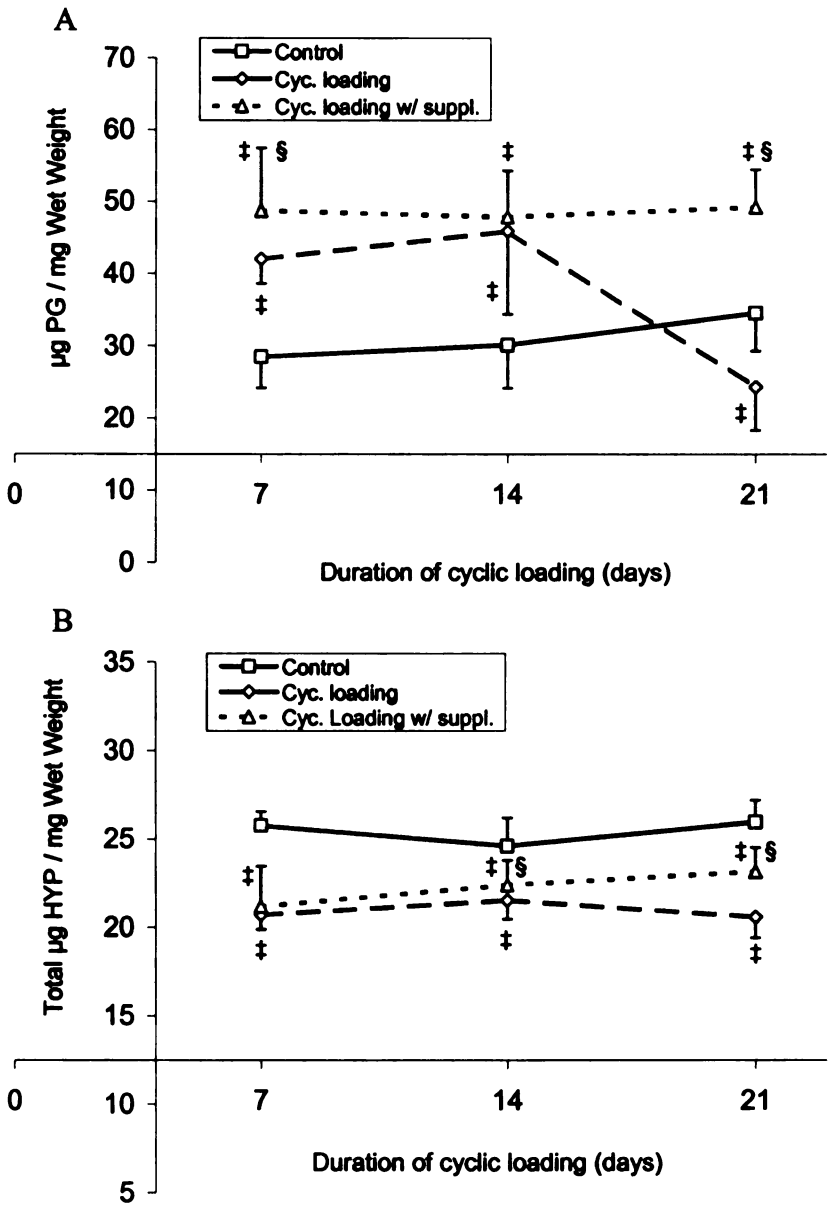

Figure 17. PG synthesis was stimulated by the presence of supplement at all time points, while without the supplement the PG content decreased at 21 days (A). Tissue HYP content decreased approximately 10% and 20% by cyclic loading with and without supplement, respectively (B). Statistically significant differences were observed from control (‡), and from cyclic loading (§).

MATRIX DAMAGE AND CELL VIABILITY

Cyclic loading decreased explant surface fissure length induced by the 25 MPa overload by approximately 37% (from 91.4 ± 25.7 mm to 56.8 ± 18.9 mm) up to 14 days (Figure 5A). Furthermore, cyclic loading saved nearly 22% more cartilage cells than control up to 14 days (Figure 5B). In contrast, with a longer duration of cyclic loading (21 days) superficial fissures and associated cell death (Figure 4C-D) increased significantly compared with controls (fissure length increased by 32% and percentage of live cells decreased by 15%, approximately) (Figure 5A-B). In the presence of supplement, however, cyclic loading resulted in a significant decrease in fissure length and more live cells after overloading than control at all time points (Figure 4E-F and Figure 5A-B). Cell death per unit strain was significantly decreased versus controls up to 14 days of cyclic loading (decreasing by approximately 30% and 65% at day 7 and 14, respectively). In contrast, after 21 days of cyclic loading the cell death per unit strain was not different from control. In the presence of supplement, however, cyclic loading significantly decreased the cell death per unit strain at all time points (decreasing by approximately 72%, 68% and 73% for 7, 14 and 21 days of cyclic loading, respectively) (Figure 5C).

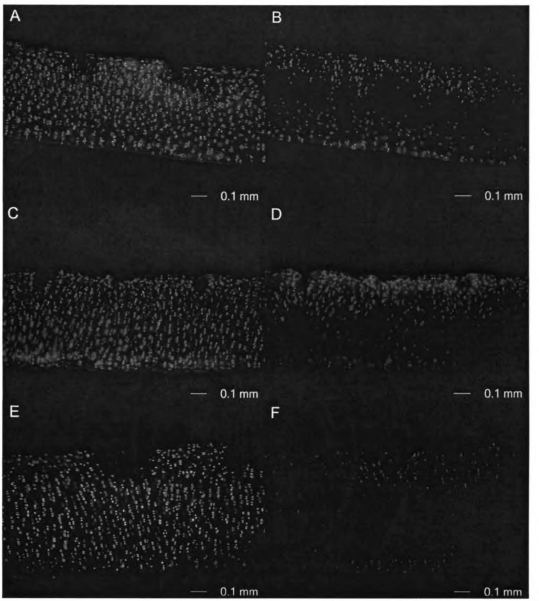

Figure 18. Typical images of cartilage explants stained for cell viability. Live cells stained green and dead cells stained red. Special filter was used to see only the dead cells for counting purpose (BDF). The cracks on the surface were due to the 25 MPa unconfined compression on the explants. All images showed are from explants after 21 days of testing period. AB-control; CD-cyclic loaded; EF-cyclic loaded with supplement.

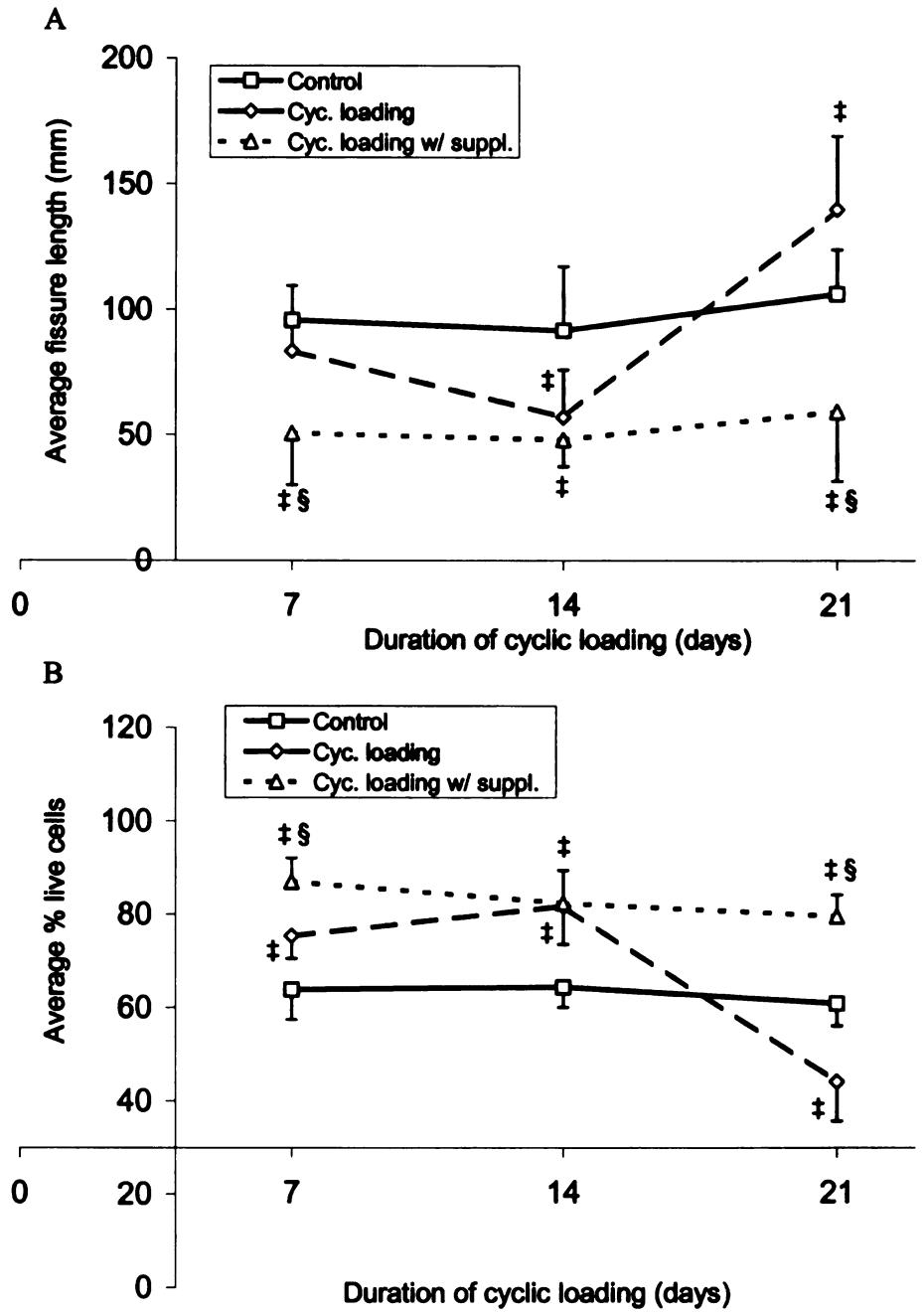

Figure 19. Cyclic loading initially decreased explant surface fissure length from the 25 MPa overload and saved approximately 20% more cartilage cells than control up to 14 days, but with a longer duration (21 days) of cyclic loading superficial fissures and associated cell death increased significantly (AB). In the presence of supplement, however, cyclic loading inhibited the matrix further damage and cell death at 21 days (AB), and significantly decreased the cell death per unit strain at all time points (C). Statistically significant differences were observed from control (‡), and from cyclic loading (§).

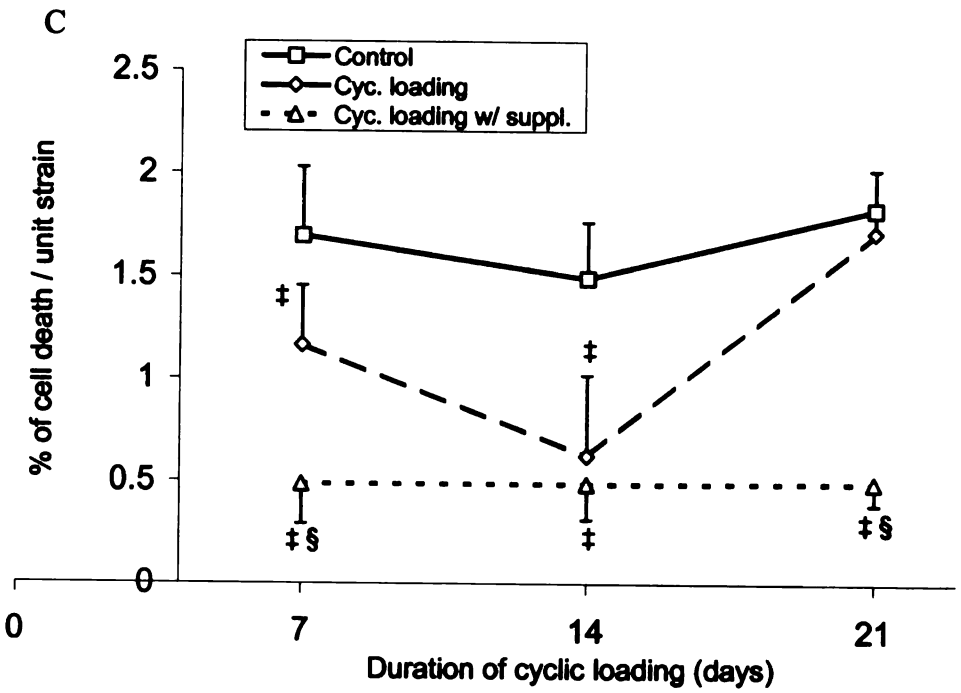

Figure 19. (cont'd)

DISCUSSION

The hypotheses of the current study were that a high concentration of glcN-CS added to culture media during low intensity, intermittent cyclic loading of chondral explants will increase the production of tissue PGs versus cyclic loading in media alone. With the increase in tissue PGs the study further hypothesized that there would be a significant increase in explant stiffness, resulting in less tissue and cell damage following a single acute mechanical overload. Additionally, the current investigation hypothesized that this media supplement would be effective in limiting, or potentially mitigating, a degeneration in mechanical properties of these explants between 14 and 21 days of cyclic loading. Each of the above hypotheses was supported by experimental data presented in the current study.

Mechanical forces in the chondrocyte environment can affect the cellular processes involved in the biosynthesis of extracellular matrix. In turn, the biomechanical properties of cartilage depend on its collagen and PG content (Saadat et al. 2006). The results of the current study showed that supplementation of bovine culture media with glcN-CS did experimentally stimulate tissue PG synthesis during low intensity, intermittent cyclic loading of explants and help stiffen the cartilage to inhibit its degradation between 14 and 21 days, making them more resistant to matrix damage and cell death under 25 MPa of unconfined compression. The study indicated that an alteration (increase) of tissue PG content significantly limited the extent of blunt overload trauma to cartilage and dramatically reduced the cartilage susceptibility to damage in a single, severe level of acute overload.

Various aspects of the study parallel findings of other in vitro research investigations on the biological and mechanical response modifications documented with the supplementation of culture media with glcN or glcN-CS. Most notably the study supported one investigation suggesting that these compounds function as biological response modifiers (BRMs), which can boost responses of cartilage to environmental stresses, such as mechanical compression (Lippiello et al. 2003). This referenced study shows a significant increase in tissue glycosaminoglycans (GAGs) with supplemented (250 μg/ml Cosamin® DS) media versus without, following 24 hrs of unconfined compression at 0.5 MPa using aged bovine cartilage. The notion was presented that stressed chondrocytes utilize the compounds more efficiently than non-stressed cells. This effect was supported in the current study which showed a statistically significant increase in the production of tissue PGs at 7 days in supplemented versus non-supplemented, cyclically loaded explants (Figure 3A). A possible limitation of the current study, however, was that these data were not normalized per viable chondrocyte. However, data from this laboratory (unpublished) indicated that 21 days of cyclic loading does not statistically alter the percentage of live cells in the explants compared to unstressed controls. Additionally, cyclic compression did not statistically alter the specimen thickness, suggesting no change in tissue wet weight over time (Wei et al. 2007a). Interestingly at 14 days the PG content of supplemented versus non-supplemented, cyclic loaded specimens was not statistically different. This may be due to some yet unexplained large variance in the non-supplemented, cyclic loading data at this time point. Furthermore, in the absence of supplement our data indicated that, compared

to the controls, moderate cyclic loading of cartilage explants up to 14 days stimulated PG synthesis in the tissue, resulting in a significant increase of cartilage matrix stiffness, a decrease in tissue permeability, and a limitation of superficial cracks and chondrocyte death resulting from an acute overload. This may be due to less strain (data not shown) observed under 25 MPa of unconfined compression. Correspondingly, an increase of compressive strain explained a significant increase in tissue damage from overloading after 21 days of cyclic loading. In the presence of supplement, however, the high levels of tissue PG were retained after 21 days of cyclic loading, resulting in maintenance of tissue stiffness and helped limit matrix damage following traumatic overload. We think this might be due to the anti-inflammatory properties of the supplement (Derfoul et al. 2007).

Previous studies, by others, have established direct and indirect correlations between the PG content of cartilage and its matrix modulus and permeability, respectively (Korhonen et al. 2003). This may help explain the data in the current study which showed a higher PG content and matrix modulus at 7 and 14 days for supplemented versus non-supplemented and control specimens. Additionally, the current study indicated a dramatic drop in fiber modulus versus unstressed controls at 7 days that persisted at 14 and 21 days. Korhonen et al. show that removal of collagen from cartilage lowers the fiber modulus. This effect paralleled with a decreased content of explant collagen versus unstressed controls at each time point in the current study. Additionally, at 14 and 21 days the collagen content of supplemented explants statistically exceeded that of the non-supplemented, cyclic loaded group. In the current study, while a statistical reduction in fiber modulus versus controls was also documented for supplemented explants, the

modulus was generally higher than non-supplemented, cyclic loaded explants. The effect, however small, may be due to an increase in production of collagen by the chondrocytes in the supplemented, stressed groups (Varghese et al. 2007; Lippiello et al. 2003). More likely, the higher content of collagen may reflect the fact that the supplemented group was stiffened because of a rise in PG content and yielded less matrix damage (Thibault et al. 2002).

While the previous study of Korhonen et al. established correlations between the contents of PG and collagen and alterations in matrix modulus and fiber modulus, as well as permeability, the stiffness of these chondral explants under unconfined compression was mathematically sensitive to a combination of matrix modulus, fiber modulus and tissue permeability (unpublished data). At 7 days, the content of tissue PGs for supplemented explants was greater than that of non-supplemented, cyclic loaded explants, but this lead to not only an increase in matrix modulus and a decreased tissue permeability, but also an increase in fiber modulus of the explant. As a result the unconfined compression resulted in less matrix damage and cell death in supplemented versus non-supplemented explants. At 14 days, in contrast, more collagen remained in supplemented than non-supplemented specimens. While the PG content between the 2 groups was not different, the matrix modulus of supplemented specimens was statistically greater and the fiber modulus was slightly higher (not statistically) than in the non-supplemented group. The results from the current study clearly showed that high levels of glcN-CS supplementation between 14 and 21 days of cyclic preload helped inhibit cartilage degradation and mitigate matrix damage and cell death in an acute overload of the tissue. At 14 days, however, no

statistically significant differences were observed between supplemented and non-supplemented, cyclically loaded explants in terms of fiber modulus, tissue permeability, PG content, fissure length, percent of live cells and cell death per unit strain (Figure 2-4). Therefore, we conclude that while the effect of glcN-CS supplementation during cyclic preload was obvious at 7 days, it is very low at 14 days time point. However, when the cyclic preload continued long enough to damage the cartilage (somewhere between 14 and 21 days), the supplement, functioning as BRM, affected the tissue responses to the cyclic preload and the later acute overload. In another word, cyclic preloading of cartilage had positive effect on the tissue up to a certain point, and before that point, supplementation of glcN-CS would intensify the effect and strengthen the cartilage in response to a traumatic event. When cyclic preload started to degrade the cartilage, however, the high levels of supplement would protect the cartilage from being damaged and alter the response of the tissue to the cyclic preload and the acute overload in terms of inhibiting the degeneration of cartilage. These data show that a one-to-one correlation between PG and collagen content of the explant and matrix modulus and fiber modulus, respectively, may not exactly exist. It would seem reasonable that these tissue constitution-mechanical property correlations might be more complex.

The most dramatic effect of media supplementation in the current study was the reduction in matrix damage and cell death that occurred between 14 and 21 days with supplementation. The hypothesis of a previous study (Wei et al. paper in review) was that the documented degenerative effect in mechanical properties was likely due to the production of MMPs (possibly MMP-3) as a result of intermittent cyclic compression at

0.5 MPa (Lin et al. 2004). The hypothesis of the current study was that media supplementation would limit or mitigate the effect. The study showed not only a significant increase in explant PG content with supplementation, but more collagen in the explants. Again we hypothesize that the increase in tissue collagen likely may be the result of less damage to this structure during cyclic loading due to a stiffened matrix, rather that a statistically significant increase in the biosynthesis of new, functional collagen. However, the mechanism by which supplementation of the culture media with glcN-CS retained more collagen and PG than cyclic loaded specimens without supplement is yet unknown and will require further study. And, as suggested in studies by others that examine the effects of selective and non-selective metalloproteinase inhibitors on interleukin-1-induced cartilage degradation, the effects of media supplementation on altering the material properties of cartilage may only be delayed in time (Wilson et al. 2007). Future studies will be needed to determine if the same effects will be seen with altered frequency, amplitude and with an extended duration of cyclic loading of the supplemented explants.

The current in vitro study does not directly have utility in answering the question of whether this supplement will have efficacy in helping to mitigate long term joint disease, such as post-traumatic OA, resulting from an acute traumatic overload, such as rupture of a knee ligament (Fang et al. 2001). The major problems with extending these results to a clinical setting are the short duration of in vitro studies, as well as the elevated concentration of the supplement. Studies have shown that high doses of glcN-HCl have detrimental effects on bovine articular cartilage explants cultured in vitro (De Mattei et

al. 2002). At concentration of 25 mg/ml glcN a decrease of over 90% in cell viability was observed in their study. While in our short-term in vitro experiments the dose of supplement (500 μg/ml glcN and 250 μg/ml CS) was much lower than that documented above, it is still significantly higher than clinical levels (Persiani et al. 2005). The concentrations of glcN in serum after oral and intravenous administration range from 1 to 20 μg/ml, while CS concentrations are in the 5-200 μg/ml range (Adebowale et al. 2002; Du et al. 2004; Volpi 2002). Yet, unpublished studies from this laboratory do show (Appendix A) some reductions in the cyclic load-induced degradation of a chondral explant for even a reduced (by 50%) concentration of supplement. Our experimental data may suggest some long-term utility of the supplement to provide a chondro-protective effect for joint cartilage suffering an acute overload, such as that which might occur during knee ligament rupture (Lahm et al. 1998). And yet, it also seems inviting to propose in vivo studies at clinical concentrations to see if this supplement may have incremental, positive effects on the already documented effects of physiological levels of cyclic loading on changes in the mechanical properties of joint cartilage (Saadat et al. 2006). Another potential utility for these types of in vitro studies may be in the area of tissue-engineered cartilage (Waldman et al. 2004; 2006). While recent studies show the utility of cyclic mechanical loading on engineered cartilage stiffness, supplementation of the culture media with high concentrations of glcN-CS may show utility in producing better products (Varghese et al. 2007). As the current study may imply, however, the intensity, frequency and duration of intermittent cyclic loading may play important roles in the response of these engineered tissues to media supplementation (Wei et al. paper in review-b).

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CHAPTER THREE

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

CONCLUSIONS

In the previous Chapters we have conducted studies where low intensity, intermittent cyclic preloading was applied to chondral explants prior to an acute unconfined compression on the tissue. Based on the results of Chapter One, we used a high level of nutraceutical (glucosamine-chondroitin sulfate, glcN-CS) in an experimental setting to inhibit the degeneration of cartilage after long duration of cyclic preloading (21 days) and limit the blunt force trauma to the explanted tissue. The effects of cyclic preload and high levels of glcN-CS on chondral explants responses to acute overload were documented.

The hypothesis of Chapter One was that low intensity, intermittent cyclic preloading of chondral explants will up-regulate the production of tissue proteoglycans (PGs), resulting in an increase of the explant mechanical stiffness that will help limit the extent of matrix damage and associated chondrocyte death following a single, severe level of blunt force overloading. The results of Chapter One showed cyclic preloading did mechanically stiffen the explants, making them more resistant to matrix damage and cell death under 25 MPa of unconfined compression up to 14 days. After 21 days of cyclic preloading, however, the explants lost stiffness and suffered more extensive damage in the acute, unconfined compression test than controls without cyclic preloading. These data may have a direct bearing on the issue of pre-impact conditioning of chondral explants in laboratory experiments dealing with the short-term consequences of impact trauma (Wei

et al. paper in review-a). Additionally, these data may also relate to the role of regular exercise on the long-term consequences of human joints following a traumatic joint injury, such as an acute ligament rupture (Lahm et al. 1998).

The objective of Chapter Two was to use a high concentration of glcN-CS (500 μg/ml glcN and 250 μg/ml CS) in an experimental setting to alter PG synthesis during cyclic preloading of chondral explants and document its effect on the response of explants to an acute overload. Thus, the hypotheses of Chapter Two were that a high concentration of supplement would up-regulate the production of PGs in an explanted tissue to alter its mechanical properties in relatively short-term experiments. By increasing the mechanical stiffness in this fashion, there would be a reduction in the extent of matrix damage and cell death following an acute overload on the explant. Secondly, because of the documented inhibition effects of glcN on aggrecanase and matrix metalloproteinases (MMPs), supplementation of the culture media with the product during cyclic preloading will limit or even mitigate the degeneration effect noted to occur between 14 and 21 days of the in vitro model in Chapter One. The results of Chapter Two showed that the supplement of glcN-CS did experimentally stimulate tissue PG synthesis during moderate cyclic preloading of chondral explants and help stiffen the cartilage to inhibit its degradation at 21 days, making them more resistant to matrix damage and cell death under 25 MPa of unconfined compression. The study indicated that an alteration (increase) of tissue PG content significantly limited the extent of blunt overload trauma to cartilage and dramatically reduced the cartilage susceptibility to damage in a single, severe level of acute overload.

Since in the short-term in vitro experiments the dose of supplement used in Chapter Two was much higher than clinical levels (Persiani et al. 2005), more recently, we used a lower concentration of supplement in the bovine media (250 μg/ml glcN and 125 μg/ml CS). The studies showed that with long duration of cyclic preloading, a lower concentration of supplement still inhibited the degeneration of cartilage explants between 14 and 21 days as the high level of supplement did in Chapter Two (Appendix A). These experimental data, including the data from Chapter Two, may suggest some long-term utility of the supplement to provide a chondro-protective effect for athletes suffering an acute overload, such as that which might occur during knee ligament rupture (Felson et al. 2000).

RECOMMENDATIONS FOR FUTURE WORK

Chapter One was the first study to investigate the role of in vitro cyclic preloading on the response of a chondral explant to a potentially damaging, acute overload. In the long-term these types of studies may help understand the role of biologic-based pre-conditioning of articular cartilage for in vitro, or even in vivo, studies of blunt force trauma to a joint. The current data in Chapter One would suggest both a positive and a negative aspect to the idea of cyclic pre-conditioning or regular exercise in the prevention of long-term chronic disease in joints suffering an acute overload, such as that proposed during acute ligament injury. This in vitro study may suggest that the frequency, magnitude and duration of the pre-conditioning may play a role in its potential chondral protective effects. Clearly, additional in vitro and in vivo studies are wanted.

In Chapter One a dramatic increase in tissue damage to 25 MPa of unconfined compression was noted after 21 days of cyclic loading. The effect also involved a dramatic decrease in the tissue matrix modulus, an increase in tissue permeability, and a decrease in tissue PG content. This may be explained by an increase in the production of degenerative enzymes, such as matrix metalloproteinase-3 (MMP-3), which is noted to occur following extended cyclic loading of chondral explants (Lin et al. 2004). MMP-3 is a connective tissue matrix-degrading enzyme. It was formerly known as proteoglycanase and is generally considered to be one of the major proteoglycan degrading enzymes in cartilage. MMP-3 is implicated in cartilage destruction in osteoarthritis and may also be involved in tissue remodeling in the physis (Armstrong et al. 2002). Lin et al. documented a significant increase in MMP-3 after 24 hours of cyclic loading at 1 or 5

MPa. Therefore, we hypothesize that the MMP-3 production might dramatically increase between 14 and 21 days of cyclic loading in current study, and more experiments are needed.

Chapter One also documented that while the collagen content and fiber modulus of the explants significantly decreased after 7 days, the unconfined compression modulus of the explants was significantly greater than controls, and it more directly paralleled with the temporal changes in tissue PG content, the matrix modulus and permeability. An analysis of the model sensitivity to changes in tissue matrix modulus, fiber modulus and permeability suggested that the stiffness of the explant in the current unconfined compression test should depend on the fiber modulus as much as matrix modulus. Previous unconfined compression relaxation experiments, reported by Korhonen et al, displayed sensitivity of their tests to an enzymatic loss of collagen in the chondral explant. Future studies will be required to understand more precisely if the lack of fiber modulus sensitivity in the current experiments was due to the test protocol (0.5 Hz sinusoidal loading or relaxation testing), friction on the explant upper surface, or a problem in the validity of the fibril reinforced model for these cartilage explant tests.

The long-term health of joint cartilage may relate directly to the extent of matrix and cell damage that occurs during an acute overloading of a tissue. The studies in Chapter One indicated that mechanical stiffening of the chondral explant by cyclic preloads increased the tissue content of PG and resulted in less strain during unconfined compression at 25 MPa. This was correlated with a significant increase in cell survival. The percentage of

cell damage per unit compressive strain was significantly decreased for up to 14 days of cyclic preloading. One explanation for this result may relate to an increase in PGs surrounding individual cells in the pericellular matrix as a result of cyclic preloading. This effect has been documented previously following low intensity, cyclic loading (Parkkinen et al. 1992; Quinn et al. 1998). Such deposition of PGs concentrated in or near the pericellular matrix of chondrocytes may significantly alter the local stiffness around cells to help protect them from damaging distortions during severe levels of unconfined compression (Guilak et al. 1999). Therefore, future studies will be needed to test PG grain density around individual chondrocytes under the current cyclic loading protocol.

As discussed in Chapter Two, the increase of tissue collagen in supplemented explants versus non-supplemented, cyclic loaded explants likely may be due to less damage to the explant structure during cyclic loading from a stiffened matrix, rather than a statistically significant increase in the biosynthesis of new, functional collagen. However, the mechanism by which supplementation of the culture media with glcN-CS retained more collagen and PG than non-supplemented, cyclic loaded specimens is yet unknown and will require further study. And, as suggested in studies by others that examine the effects of selective and non-selective metalloproteinase inhibitors on interleukin-1-induced cartilage degradation, the effects of media supplementation on altering the material properties of cartilage may only be delayed in time (Wilson et al. 2007). Future studies will be needed to determine if the same effects will be seen with an extended duration (for example, 28 days) of cyclic explant loading in the presence of supplement.

The current in vitro study does not directly have utility in answering the question of whether this supplement will have efficacy in helping to mitigate long term joint disease, such as post-traumatic OA, resulting from an acute traumatic overload, such as rupture of a knee ligament (Fang et al. 2001). The major problems with extending these results to a clinical setting are the short duration of in vitro studies, as well as the elevated concentration of the supplement. In our short-term in vitro experiments the dose of supplement (500 μg/ml glcN and 250 μg/ml CS) was higher than clinical levels. The concentrations of glcN in serum after oral and intravenous administration range from 1 to 20 μg/ml, while CS concentrations are in the 5-200 μg/ml range (Adebowale et al. 2002; Du et al. 2004; Volpi 2002). Yet, studies from this laboratory do show (Appendix A) some reductions in the cyclic load-induced degradation of a chondral explant for even a reduced (by 50%) concentration of supplement. Therefore, more experiments in use of the current in vitro model are needed in the future to investigate the possible lowest (as close to the clinical levels as possible) concentrations that will have the same in vitro effects on chondral explants. And yet, it also seems inviting to propose in vivo studies at clinical concentrations to see if this supplement may have incremental, positive effects on the already documented effects of physiological levels of cyclic loading on changes in the mechanical properties of joint cartilage (Saadat et al. 2006). Another potential utility for these types of in vitro studies may be in the area of tissue-engineered cartilage (Waldman et al. 2004; 2006). While recent studies show the utility of cyclic mechanical loading on engineered cartilage stiffness, supplementation of the culture media with high concentrations of glcN-CS may show utility in producing better products (Varghese et al. 2007). Finally, as the current study may imply, the intensity, frequency and duration of

intermittent cyclic loading may play important roles in the response of these engineered tissue to media supplementation (Wei et al. paper in review-b).

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Appendix A: Low Concentration of Supplement

Since in the short-term in vitro experiments the dose of supplement used in Chapter Two was much higher than clinical levels, we recently used a lower concentration of supplement in the bovine media (250 μg/ml glcN and 125 μg/ml CS). Twenty-four explants were used in the test, with 12 controls and 12 cyclic loading with low concentration of supplement (n=4 in each of 7, 14 and 21 days groups). Data of cyclic loaded explants without supplement were combined from Chapter Two. The experimental design and procedure were the same as described in Chapter Two.

Matrix modulus, fiber modulus and permeability of the explants were compared in terms of "% of before cyclic loading". Cyclic loading significantly increased the tissue matrix modulus by approximately 145% and 180% at 7 and 14 days, respectively. No differences were observed between cyclic loading with and without low concentration of supplement. With longer duration of cyclic loading at 21 days, however, cyclic loading without supplement decreased the matrix modulus by 58.4 ± 19.4%, while cyclic loading with low concentration of supplement significantly increased the matrix modulus by 184.5 ± 64.9% (Figure 1A). Cyclic loading significantly decreased the tissue fiber modulus by approximately 65% at all time points (32.6 ± 17.8%, 39.8 ± 27.4% and 36.8 ± 14.3% of before cyclic loading at 7, 14 and 21 days, respectively). No significant differences were observed between cyclic loading with and without low concentration of supplement (Figure 1B). Up to 14 days of cyclic loading the permeability of the explants without supplement significantly decreased (71.1 ± 8.5% and 31.0 ± 7.7% of before cyclic loading in 7 and 14 days groups, respectively), and then it increased dramatically

at 21 days (143.0 ± 9.9% of before cyclic loading). In contrast, cyclic loading with low concentration of supplement decreased the tissue permeability significantly at all time points (61.4 ± 19.3%, 35.4 ± 28.3% and 26.4 ± 16.0% of before cyclic loading in 7, 14 and 21 days groups, respectively). No significant differences were observed between cyclic loading with and without low concentration of supplement up to 14 days (Figure 1C). For all the three parameters the control (non-cyclic loaded) remained at the same level (approximately 100%) for all time points.

To conclude, up to 14 days of cyclic loading with low concentration of supplement, tissue matrix modulus significantly increased, while both tissue fiber modulus and permeability significantly decreased, but no significant differences were observed between cyclic loading with and without low concentration of supplement. With longer duration of cyclic loading, however, low concentration of supplement still inhibited the degeneration of cartilage explants between 14 and 21 days as high level of supplement did in Chapter Two.

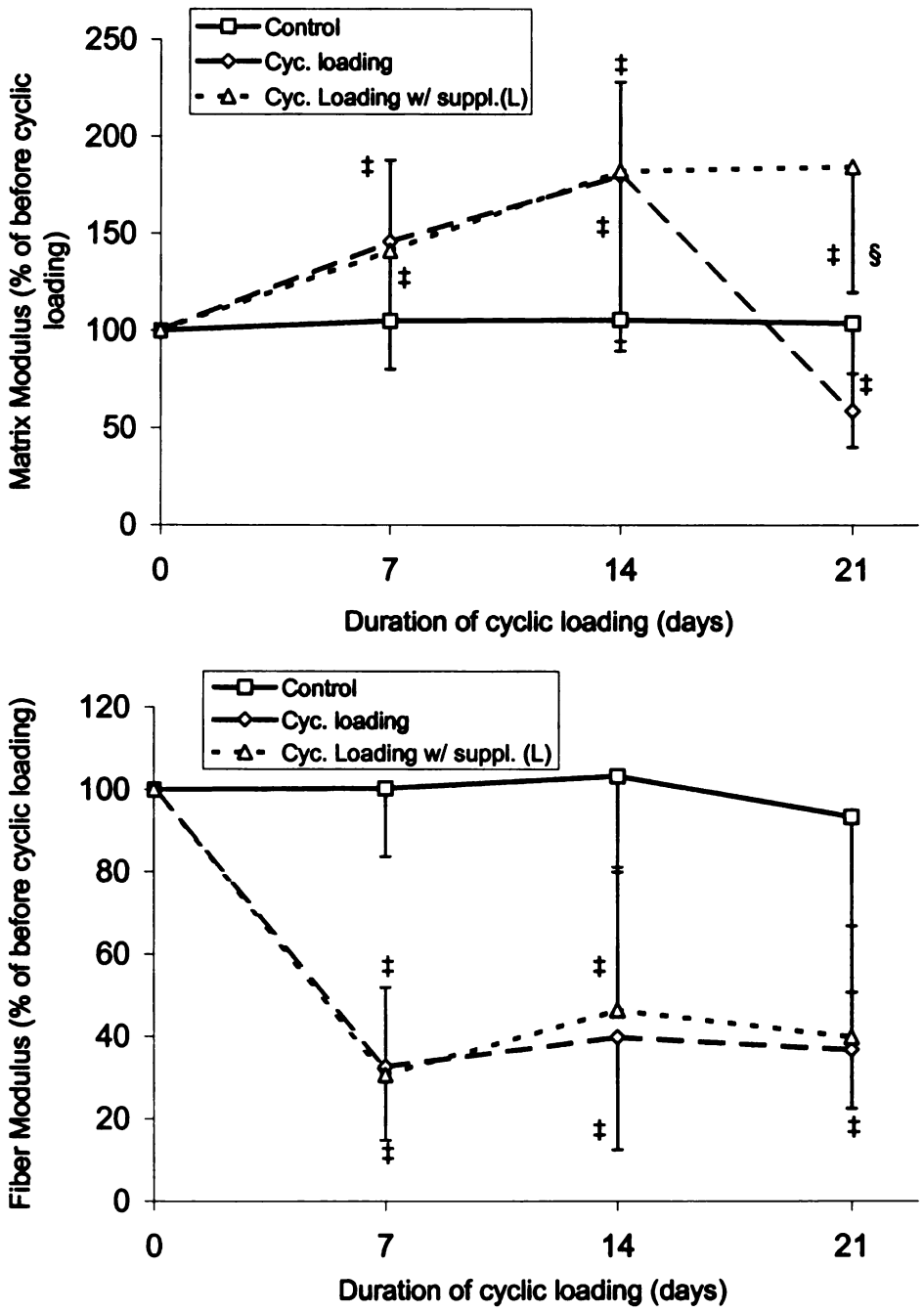

Figure 20. Cyclic loading with low concentration of supplement strengthened the cartilage matrix modulus by approximately 200% of the control (A), and helped inhibit the degradation of the explants from longer time (21 days) cyclic loading (A and C). Cartilage fiber modulus, however, decreased approximately 60% by cyclic loading, independent of supplement (B). No significant differences were observed between cyclic loading with and without low concentration of supplement up to 14 days in all the three parameters. Statistically significant differences were observed from control (‡), and from cyclic loading (§).

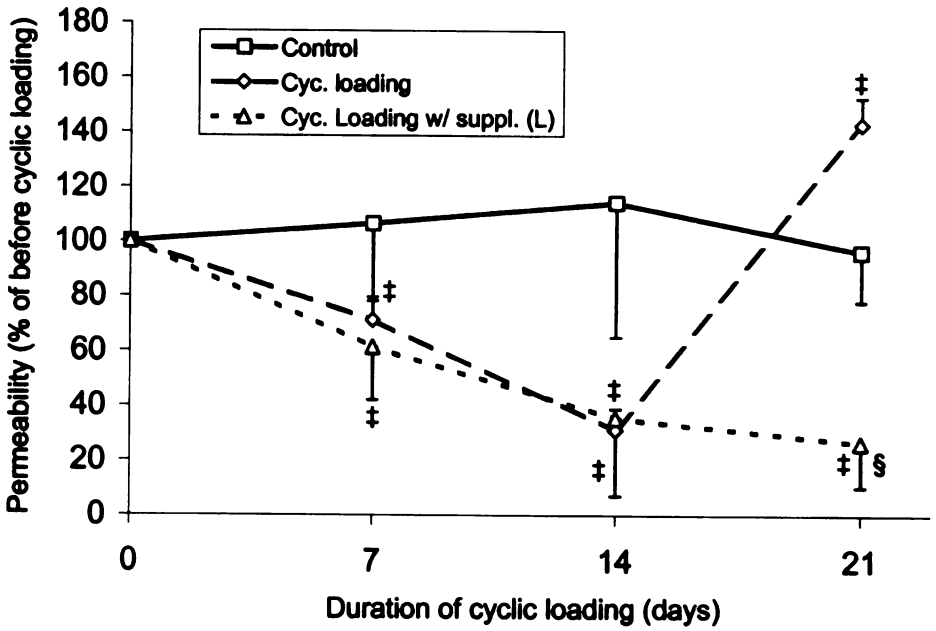

Figure 20. (cont'd)

Appendix B: PROCEDURE FOR OBTAINING CARTILAGE EXPLANTS FROM BOVINE METACARPAL JOINTS

1. Call Bellinger's meat packaging/slaughter house Monday's and ask for 2 pairs of Bovine Knees (1-800-778-4577). Pick up the knees on Tuesday's.
2. Prepare all media and confirm availability of supplies.
3. After obtaining the knees, wash the outside of the leg in a sink to remove mud. Use a scalpel to remove skin from leg down to hoof. Remove hoof. Place waste in biohazard bag. Wash to remove any contaminants.
4. Set up the Laminar flow hood. Lay down laboratory paper in hood, place scalpel, biopsy punches, tweezers, and a petri dish with media (no FBS) in hood.
5. Take leg into hood. Dissect open the metacarpal joint (lower of two joints at top of removed leg) using a scalpel.
6. Using a biopsy punch, punch several explants in the lower (articular) surface of cartilage of the joint. Use the scalpel to remove the explants by slowly cutting under the punched area along the bone surface. Use the tweezers to place the explant in the petri dish of media. Repeat until you have more than enough explants.
7. In hood, perform three washes by using tweezers to place the explants in new petri dishes with media. Swirl around in each dish. Place an explant into each well of a 24 well plate with 1 ml of media (with 10% FBS) in each well. If explants are cultured for more than 2 days, change media every two days.

Appendix C: BOVINE MEDIA STOCK RECIPE

1. Measure 1L of ddH_2O. Pour 600mL of ddH_2O into 2L Erlenmeyer flask with stir bar.
2. Add 1 package of powdered media DMEM:F12 (Gibco #12500-062). 1 package makes 1L of media.
3. Add 20mL AA solution (Gibco #11130-051).
4. Add 3.89g Sodium Bicarbonate ($NaHCO_3$) (for final concentration of 44mM).
5. Add 2mg lactalbumin hydrolysate (for final concentration of 2μg/mL).
6. Add 1μL of diluted sodium selenite stock solution to the media (for final concentration of 1μg/mL).
7. Add 10μL of ascorbic acid stock solution.
8. Add 10μL dexamethasone stock solution (for final concentration of 100μg/mL).
9. Add 10μL of manganese sulfate stock solution to the media.
10. Bring media up to 900mL with ddH_2O and pH to between 7.3 and 7.4, then bring volume to 1L and sterile filter.
11. Before using bovine media for cell culture, add 10mL antibiotics (Biochem stores #15240-062) to the 250mL sterile filtered jar.

Stock Solution Concentrations

Sodium selenite (1mg/mL) - dilute the stock solution 1/1000 for 1μg/mL.

Ascorbic acid (5mg/mL)

Dexamethasone (10mg/mL)

Manganese sulfate (16.9mg/mL)

APPENDIX D: BOVINE MEDIA HANDLING SOP

1. Remove FBS and antibiotics (if needed) from freezer and thaw by placing tube in warm water.
2. Set up laminar flow hood by turning on the fan and lights. Sterilize by wiping down the inside surfaces with 70% ethanol. Sterilize 101-1000μL pipetter, marker, 24-well plate, and anything else placed in hood with 70% ethanol.
3. Remove two 250mL jars of bovine media from refrigerator and place in hood with thawed FBS and antibiotics.
4. If needed, pour 10mL antibiotic into 250mL of bovine media. Gently mix by swirling. This should protect media from contamination for two weeks.
5. For a standard 24-well plate, 1mL of culture media is needed per well. Pipette 2.5mL FBS into a conical tube. Bring to 25mL by adding antibiotic-treated media (final concentration of 10% FBS by volume media). Vortex to mix.
6. Pipette 1mL of 10% FBS antibiotic-treated bovine media into each well of the 24-well plate. Cover.
7. Set up three petri dishes with bovine media from the untreated jar. Rinse explants in each dish before placing in the fresh 24-well plate.
8. Put remaining FBS in freezer. Sterilize hood again before shutting off.
9. Change media in well plate every 2 days throughout an experiment.

APPENDIX E: BOVINE EXPLANT INDENTATION SOP

Calibration and Program set up:

1. Turn on both the program selector box, and the Validyne strain gage amplifier.
2. Attach the small hook into the bottom of the load cell.
3. Open the "J&Jpreload3.vi" program by double clicking on its shortcut, labeled as "J&Jpreload.vi" on the desktop.
4. Using the "Preview" setting on the LabVIEW screen, which displays the current force in Newtons, zero the load cell to **0 ± 0.001** using the small screwdriver. The zeroing control is located in the center of the DANA box.
5. Hang the small 100 gram weight on the hook attached to the load cell. This should read **-0.9807 ± 0.002** on the Preview display if the load cell is calibrated correctly. This value corresponds to -0.4000 on the voltmeter. If the value is off more than ± 0.002 then use the gain control (just below the zero control) to adjust the value. Then take off the weight and repeat steps 4 and 5. If the problem persists contact Cliff Becket.
6. Repeat step 5 using the 200 gram weight, the "Preview" read out should be **–1.961 ± 0.002** N, which corresponds to a value of -0.8000 on the voltmeter.
7. In the J&Jpreload3.vi program, verify that the following settings are accurate:

   Block 1: # samples = 3000, rate = 10

   Block 2: # samples = 600, rate = 100

   Block 3: # samples = 9600, rate = 10

   Preload N = 0.02

Channel calibration

Units/volt 0 = 1.0000

Units/volt 1 = 2.4517

Units/volt 2 = 1.0000

Analog Trigger = Disabled

PreloadHold Time in Seconds = 300

These settings control the collection of data. The data will be displayed in Newtons.

8. Run the "Reset MinIndenter" program, located on the desktop, by double clicking. (This must be done any time the program selector box is turned on, before you can reprogram the Mini Indenter).
9. Open up the "Miniprogram" on the desktop of the computer. This is a HyperTerminal program. The indenter program is set up to be 30, 1 on the program selector box.
10. In the Miniprogram, the indenter program is # 1300. Type "q1300" then hit the carriage return 8 times to display the program. It should read the following:

1300 H 1

1302 I ***a***

1305 V ***a***

1308 K 0

1310 O 0

1314 R ***b***

1318

Where ***a*** = thickness of specimen x 100

b = 2 x ***a***

So for example, if the specimen being tested is 0.43 mm thick, program 1300 will read as the following:

1300 H 1

1302 I 43

1305 V 43

1308 K 0

1310 O 0

1314 R 86

1318

Refer to the mini indent manual for a complete description of these commands. If a line is incorrect, change it by typing "p" then the line number and the appropriate command letter and number. Press Esc to exit edit mode, and type p on the last blank line to specify the end the program.

11. Double check the program by running a simulated test. Place the program selector switches on 30, 1 for the indent program.
12. Rotate the mini indenter jogging knob, located on the top of the indenter, to 0.
13. Push the red start button on the program selector box. Make sure the knob rotates from 0 to 2, which translates to 0.1 mm of downward travel. If this is incorrect, recheck the program in the HyperTerminal and make corrections as necessary.

Equipment set-up

1. Check to make sure that the black cable labeled "Mini Indenter Trigger Out" (with the BNC connecter) is **NOT** connected to the A2D interface box. The program will not run correctly unless this cable is unattached.

*However, the trigger signal is still functional and could be used on another A2D interface box hooked up to another computer if so desired.

2. Loosen all the claps on the mounting plate, 6 total. These are the round knobs located around the edge of the fixture.
3. Attach the steel rod with a small washer glued on top to the center of the horizontal mounting plate. Note that the small reservoir and clamp should be attached to the rod, if not do so now.
4. Fill the reservoir with PBS, making sure not over fill. Be very careful not to splash any PBS up onto the load cell, or to spill any PBS near the circuit card located outside of the program selector box.

Test procedure:

1. Explant desired number of samples from bovine joint. Record weights of each off the joint.
2. Dab the cartilage surface with India ink to highlight any surface fissures, wipe off excess, and photograph for records.
3. Place samples in numbered well plate filled with bovine media solution. Let explants swell for a minimum of 15 minutes prior to indentation testing.
4. Measure thicknesses of each specimen using a micrometer, and record. These values will be used in the Miniprogram, q 1300, as explained in program setup # 10.
5. Place explant on top of the rod, in the center of the washer.
6. Verify that the load cell is attached in the correct orientation. The end where the gray cable is attached must be located at the top half of the load cell.

7. Screw in the flat 1mm indenter into the load cell. Jog the XY plate so the indenter is located directly above the center of the explant. This is the desired testing site. Once the location is roughly where desired, there is a round knob available in order to manually adjust the plate in both the X and Y directions in a much smaller, more accurate amount.
8. Lower the indenter as close as possible with out touching the explant. Once in place, tighten all the clamps.
9. Raise the reservoir containing the PBS so the explant is submerged.
10. Zero the load cell with the indenter tip in the PBS.

    *Note: Make sure the entire indenter tip is not, or will not be submerged. Lowering the top, larger portion of the indenter into the PBS will cause the zero to be off.
11. Lower the indenter head so that it is very close, but not touching the surface of the explant, (0.5-0.1 mm away).

    *Note: Use the Fast and Slow actuator jog buttons (located on the selector box) to move the indenter up and down while the Z-axis clamps are tightened down.
12. Verify that the program switches are on 30, 1 and that the "J&Jpreload3.vi" program is in the run mode, (the run arrow in the top left is black). Then click the green start button on the LabVIEW screen and let it run.

    **(As soon as the green START button is triggered the indenter will IMMEDIATELY begin running.).
13. Start a stopwatch once the preload is reached.
14. Be sure not to move or bump either the Mini Indenter or the desk it sits on in order to avoid unnecessary jolts or noise in the graph.

15. The program will start acquiring data once the preload of 0.02 N is reached. 5 min after that, it will automatically indent 10% of the thickness. 10 min after the indent, press the red stop button on the "J&Jpreload3.vi" screen. Use the fast actuator jog up to get the indenter off of the explant. Loosen the Z-clamps, and raise the indenter with the Z up button if more room is desired.
16. Save the data file just collected in its appropriate location, and repeat steps for next sample explant.

APPENDIX F: IMPACTING BOVINE EXPLANTS (INSTRON INSTRUCTIONS)

1) Turn on main power (red switch).

2) Check load cell (2000lbs).

3) Warm up hydraulic system:

   Move cylinder up so it has room to drop (actuator jog up).

   Position → Waveform: 1Hz 25mm sine wave

   Position → Waveform: Start (bottom left button). Run ~ 200 cycles.

   Press finish. (Not stop!)

4) Assemble Instron setup for bovine explants.

5) Plug in load cell. Calibrate: Set up→ Cal. →Auto→Go.

   Green light will flash faster, then stops when done.

6) Lower cylinder ~ ½ in from sample platform. Loosen clamp→ lower→ clamp.

7) Check PIDL values:

Position→Setup→loop	13	1	0	1
Load→	10	1	2	1

8) Place sample on platform. Lower cylinder. (Actuator on low or high.) Touch cylinder to sample w/ out applying force.

9) Create folder- My Docs- Flaps 5.

10) Open Flaps 5 Program- Follow program instructions to Run. Press Remote. Yes, use control limits.

11) After all samples have been impacted:

   - Unclamp→ raise cylinder→clamp.
   - Turn hydraulics off.

- Main switch off.
- Dis-assemble completely.
- Shut down computer.
- Fill out log book.

APPENDIX G: LIVE/DEAD VIABILITY STAINING FOR ARTICULAR CARTILAGE EXPLANTS (Manufacturer and Part #: Molecular Probes L-3224)

Allow kit reagents to warm to room temperature before use

1. Remove media from explants and save it for analysis if desired.

   *This does not have to be done in a sterile environment.

2. Replace media with DPBS (PBS made with distilled water) – wash tissue 3 times with 1mL DPBS. This removes dilute serum esterase activity which may increase fluorescence by hydrolyzing Calcein AM. After third wash leave ~1mL PBS (or enough to cover tissue) in each well to keep tissue moist.

3. Cut cartilage explants into thin strips using the mechanical slicer (which will cut 4 sections, each 0.5mm thick). Place tissue back in appropriate well.

4. Turn out the lights – work with as little light as possible from now on; stain loses fluorescence if exposed to light.

5. Add enough stain to cover all tissue (usually ~0.5mL per well for a 24 well plate).

   Prepare stain daily – Keep out of direct light.

   Add 10μL of supplied 2mM EthD-1 to 5mL DPBS. Vortex to mix. Combine 2.5μL of supplied 4mM Calcein AM with the 5mL of EthD-1 solution. Vortex to ensure mixing. Simply use multiples of these numbers if more stain is needed. Refer to Excel doc viability stain amounts.

6. Incubate with stain ~15min at room temperature. Keep covered – light decreases fluorescence intensity.

7. Remove stain and wash 3 times with PBS, after third wash leave ~1mL (or enough to cover tissue) in each well to keep tissue moist.

8. Place on a glass slide. View under fluorescence microscope immediately. If you are viewing the cross-section, you should see many "columns" of cells with some scattered. If you are accidentally viewing the top or bottom, they will appear totally scattered. Keep covered – light decreases fluorescence intensity.

Polyanionic calcein produces a green fluorescence in live cells and EthD-1 produces a red fluorescence in dead cells.

DPBS: KCl (200mg/L), KH_2PO_4 (200mg/L), NaCl (8000mg/L), Na_2HPO_4 (1150 mg/L)

APPENDIX H: PROTEOGLYCAN ASSAY

Papain Tissue Digest Protocol (for 24 samples)

1) Take papain from fridge and heat to 37C.
2) Heat water bath to 60C.
3) Prepare papain solution just before use.
    - 14 ml PBS
    - 26 mg EDTA
    - 24.5 mg cysteine
    - 39.5 μl papain
4) Add 500μl to each sample.
5) Add 500μl to ~4.5 mg Chondroitin Sulfate A to create standard solution (record exact value of Chondroitin used)
6) Incubate at 60C (water bath) overnight (until tissue is gone).
7) Boil samples (in four 50 ml beakers with ~20 ml water) on hot plate for 5 mins to inactivate the papain.
8) Place vials in freezer until ready to complete assay.
9) Vortex samples for a few seconds.

DMB Tissue Sample Assay

Chondroitin Dilution: standards

*** Vortex between each step!!!

1) Mix 2μl of the chondroitin standard solution + 398μl of dilution buffer in vial 1.
2) Add 200 μl dilution buffer to vials 2-8.

3) Take 200μl from 1st vial and add to 2nd.

4) Take 200μl from 2nd vial and add to 3rd. Continue for all remaining vials.

5) Toss 1st dilution.

Dilute samples

*** Vortex between each step!!!

1) Mix 4μl of sample + 396μl of dilution buffer in vial 1. (use 6μl + 394μl if only part of sample was digested for PG assays).

2) Add 200 μl dilution buffer to vials 2-5.

3) Take 200μl from 1st vial and add to 2nd.

4) Take 200μl from 2nd vial and add to 3rd. Continue for all remaining vials.

5) For each sample, repeat step 1–4.

Prepare Microplate & Run Plate Reader

1) Add 50μl of standards to wells A11-G11 & A12-G12 (columns 11 & 12 are duplicates).

2) Each sample will occupy a single row. Add 50μl of each concentration of diluted samples to two wells (duplicates). Ex: add the 1st dilution to A1 & A2, the 2nd dilution to A3 & A4, and so on.

3) Turn on plate reader (switch on back).

4) Add 200μl DMB dye to every well.

5) Place microplate in reader (line up A1). The assay is time-sensitive so achieve a reading immediately following the addition of the DMB.

6) Plot known standard concentration against standard absorption and fit with a linear regression. Want R^2 for control > .98 (.996-.999 obtainable).

7) Use the regression equation to solve for the unknown sample concentrations (x) by plugging in their absorptions (y).

DMB Media Sample Assay

Base Standard Media Creation

1) Measure out ~5mg Chondroitin Sulfate A.
2) Dilute with 1ml Bovine Media to every 5mg chondroitin.
3) Add 44μl of the resulting solution to 1156μl Bovine Media to create chondroitin standard. Toss 1st solution.

Chondroitin Media Standard Dilution

*** Vortex between each step!!!

1) Mix 100μl of the chondroitin media standard solution with 300μl of dilution buffer in vial 1.
2) Add 200μl dilution buffer to vials 2-8.
3) Take 200μl from 1st vial and add to 2nd.
4) Take 200μl from 2nd vial and add to 3rd. Continue for all remaining vials.

Dilute samples

*** Vortex between each step!!!

1) Mix 100μl of sample + 300μl of dilution buffer in vial 1.
2) Add 200 μl dilution buffer to vials 2-5.
3) Take 200μl from 1st vial and add to 2nd.
4) Take 200μl from 2nd vial and add to 3rd. Continue for all remaining vials.
5) For each sample, repeat step 1–4.

6) From here, follow the above procedure under "Prepare Microplate & Run Plate Reader".

Stock Solutions:

Dilution buffer (500ml): Mix 2.05g sodium acetate + 250 μl Tween 20 + water. pH 5.3.

DMB Reagent (0.5 L):

1) Weigh 0.008g 1,9-dimethylene blue. Using a 50ml tube, dissolve dye in 2.5ml of 100% ethanol.
2) In a separate tube, mix 1 ml formic acid and 1 g sodium formate in 20 ml water. (Work with formic acid in hood!).
3) Add mixture to 50ml tube of ethanol-DMB mixture.
4) Pour DMB mixture into a 1L graduated cylinder (rinse 50ml tube 3 X to transfer all chemicals) and add water to 500ml.
5) Transfer to a 0.5L bottle and label with date.

***Shelf life 1 month in the dark.

Sample Calculations:

From the spreadsheet in OBL's z-drive we can get x μg/ml PG (unadjusted) by solving the straight line equation, this sample calculation shows how to get the PG content (μg PG / mg Wet Weight) from x. For convenience, here we use "a" to represent the "x" in the spreadsheet.

a μg/ml PG

1. $0.25 \times a$ = PG amount (μg) in well, where 0.25 (ml) comes from 50 μl diluted sample plus 200 μl DMB.

2. $\frac{0.25 \times a}{0.05}$ = concentration (μg/ml) of diluted sample, where 0.05 comes from 50 μl diluted sample.

3. $\frac{0.25 \times a}{0.05} \times 0.4$ = PG amount (μg) in diluted sample, where 0.4 (ml) comes from 4μl of sample plus 396μl of dilution buffer.

4. $\frac{0.25 \times a}{0.05} \times 0.4 \Big/ 0.004$ = concentration (μg/ml) of sample, where 0.004 comes from 4μl of sample.

5. $\left[\frac{0.25 \times a}{0.05} \times 0.4 \Big/ 0.004 \right] \times 0.5$ = PG amount (b μg) in sample, where 0.5 comes from 500 μl papain solution and "b" represents the final PG amount in sample.

6. b / mg Wet Weight = PG content (μg PG / mg Wet Weight).

APPENDIX I: HYDROXYPROLINE DETERMINATION

Proteoglycan extraction:

1) Day 1 Record wet weight of samples.
2) Add 2ml 4M Guanidine HCl and 2mL incubation buffer to each sample well (12 well plate).
3) Place the plate on a roller bank for 24hrs at 4°C.
4) Day 2 Wash the explants with 1ml incubation buffer two times (on roller bank for 3hrs at 4°C).

Digestion/hydrolyzation of collagen:

5) Add each sample to a centrifuge vial containing 500μL of incubation buffer with 1mg/mL αCT. Allow the denatured collagen to digest overnight in a 37°C water bath.
6) Day 3 Pipette 500μL of the supernatant (liquid) from each vial into new vials and dilute 1:1 with 12M HCl.
7) Place the remaining tissue samples into new vials and immerse in 1mL of 6M HCl.
8) Allow both the tissue and the supernatant to hydrolyze in a 70°C water bath for 24hrs.
9) Day 4 Dry the tissue and supernatant hydrolyzates overnight (possibly two nights) in an oven.
10) Day 5 Add 300μL of dH_20 to the samples, vortex, and dry overnight again to ensure the removal of any residual HCl.
11) Day 6 Add 300μL of dH_20 to the samples again, vortex.

Preparation of microplate wells:

*** Vortex between each step!!!

1) Mix 4µL of the 1mg/ml hydroxyproline standard solution + 286µL of 10mM HCl in vial 1.
2) Add 150µL 10mM HCl to vials 2-8.
3) Take 150µL from 1st vial and add to 2nd.
4) Take 150µL from 2nd vial and add to 3rd. Continue for all remaining vials.
5) Mix 5µL of sample + 495µL of 10mM HCl in vial 1.
6) Add 250µL 10mM HCl to vials 2-5.
7) Take 250µL from 1st vial and add to 2nd.
8) Take 250µL from 2nd vial and add to 3rd. Continue for all remaining vials.
9) For each sample, repeat step 5–8.
10) Add 50µl of standards to wells A11-H11 & A12-H12 (columns 11 & 12 are duplicates).
11) Each sample will occupy a single row. Add 50µl of each concentration of diluted samples to two wells (duplicates). Ex: add the 1st dilution to A1 & A2, the 2nd dilution to A3 & A4, and so on.
12) Add 100µL of oxidizing solution to each of the sample wells.
13) Place plate on shaker or roller bank for 5 minutes.
14) Add 100µL of Erlich's Reagent to each well. Place an adhesive seal on the plate and incubate in a water bath at 60°C for 45 minutes.
15) Place microplate in reader and run test. Absorbance readings are taken at a wavelength of 570 nm.

<u>Stock Solutions:</u>

- 4M Guanidine HCl:

Mix 250mL dH_2O with 95.53g Guanidine HCl.

- Incubation buffer:

  In 800mL dH_2O, dissolve 15.76g Tris HCl, 184.96mg iodoacetamide, 372.24mg EDTA, and 10mg pepstatin A. add dH_2O to 1L.

- Citrate/acetate buffer:

  Mix 6.8g sodium hydroxide, 6.8g citric acid monohydrate, and 24g sodium acetate trihydrate in 180mL dH_2O. Adjust to pH 6.0 with glacial acetic acid (<5mL), make up to final volume of 200mL.

- Oxidizing solution:

  Mix 22.727mL isopropyl alcohol, 12.5mL dH_2O, and 14.773mL citrate/acetate buffer. Dissolve 300mg chloramine T in solution. Store in dark.

- Ehrlich's reagent:

  Mix 52mL isopropyl alcohol with 5.7mL 70% perchloric acid and 10.3mL dH_2O. Dissolve 6g p-DAB into the mixture.

- 10 mM HCl:

  Mix 50mL dH_2O with 40.5μL Hydrocloric acid (37.9% HCl)

- 1mg/ml hydroxyproline standard:

  Mix 1mg hydroxyproline in 1mL 10mM HCl in a centrifuge tube. Store frozen.

Sample Calculations:

From the spreadsheet in OBL's z-drive we can get x μg/ml HYP (unadjusted) by solving the straight line equation, this sample calculation shows how to get the HYP content (μg

HYP / mg Wet Weight) from x. For convenience, here we use "a" to represent the "x" in the spreadsheet.

a μg/ml HYP

1. $0.25 \times a$ = HYP amount (μg) in well, where 0.25 (ml) comes from 50μl diluted sample plus 100μl of oxidizing solution and 100μL of Erlich's Reagent.
2. $\frac{0.25 \times a}{0.05}$ = concentration (μg/ml) of diluted sample, where 0.05 comes from 50 μl diluted sample.
3. $\frac{0.25 \times a}{0.05} \times 0.5$ = HYP amount (μg) in diluted sample, where 0.5 (ml) comes from 5μl of sample plus 495μl of 10mM HCl.
4. $\left.\frac{0.25 \times a}{0.05} \times 0.5 \right/ 0.005$ = concentration (μg/ml) of sample, where 0.005 comes from 5μl of sample.
5. $\left[\left.\frac{0.25 \times a}{0.05} \times 0.5 \right/ 0.005\right] \times 0.3$ = HYP amount (b μg) in sample, where 0.3 comes from 300μL of dH_20 and "b" represents the final HYP amount in sample.
6. b / mg Wet Weight = HYP content (μg HYP / mg Wet Weight).