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THE ROLE OF POLOXAMER 188 IN THE PREVENTION OF NECROTIC CELL DEATH FOLLOWING A SEVERE BLUNT IMPACT

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Daniel Michael Phillips

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Roger C. Haut
Major Professor's Signature

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THE ROLE OF POLOXAMER 188 IN THE PREVENTION OF NECROTIC CELL DEATH FOLLOWING A SEVERE BLUNT IMPACT

By

Daniel Michael Phillips

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ABSTRACT

THE ROLE OF POLOXAMER 188 IN THE PREVENTION OF NECROTIC CELL DEATH FOLLOWING A SEVERE BLUNT IMPACT

By

Daniel Michael Phillips

Clinical studies of traumatic joint injury often document early pain and development of chronic diseases, such as osteoarthritis (OA). Various studies from the literature suggest that OA can be initiated by cell death that occurs in articular cartilage during mechanical trauma to the joint. The current study investigates chondrocyte cell death following blunt trauma and the roles of apoptosis and necrosis. TUNEL analysis was used to determine that a small degree of apoptotic cell death was observed in bovine chondral explants 48 hours following blunt impact. In previous studies on chondral explants from our laboratory it was observed that 30 – 40 % of the cells die in the first 24 hours following this same mechanical insult, therefore it was concluded that necrosis, rather than apoptosis, must be the primary mechanism of cell death. A nonionic surfactant, Poloxamer 188 (P188), was explored as a potential tool for early intervention into the disease process, as this surfactant has been shown to repair damaged cell membranes in other cell types. Bovine cartilage explants were treated with P188 following trauma and showed a significant decrease in the amount of cell death versus explants not treated with P188. When explants pre-treated with P188 were compared with those pre-treated and post-treated following trauma, it was determined that the post-treatment of cartilage with P188 had a greater effect than pre-treatment. In these studies, P188 was found to have a greater effect at lower levels of impact stress than at higher levels of trauma. Finally, the effectiveness of P188 was investigated in the *in vivo*

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CHAPTER FIVE

Introduction

The annual cost of automobile accidents is estimated to be $47.8 billion (Rice et al., 1989). Lower extremity injuries (Figure 1) account for approximately $21.5 billion dollars annually for treatment, rehabilitation, and lost workdays (Miller et al., 1995).

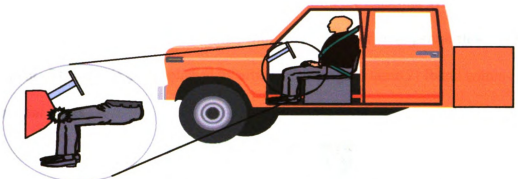

Figure 1. Lower extremity injuries account for more than $20 billion dollars annually, with a large percentage of these injuries occurring to the knee.

Studies show that the number of deaths from automobile accidents is declining, but a high rate of lower extremity injuries still occur (Dischinger et al., 1992). The most recent analysis of the National Accident Sampling System database shows that approximately 10% of total injuries per year are to the knee (Atkinson and Atkinson, 2000). The analysis also shows that greater that 50% of knee injuries per year result in contusions, while less than 5% result in bone fracture. Several studies have shown the presence of chronic joint disease with the absence of bone fracture (States et al., 1970; Upadhyay et al., 1983). The most common chronic disease affecting joints is osteoarthritis.

Osteoarthritis (OA) is a chronic disease of the articular cartilage in diarthrodial joints. Treatment of OA is both difficult and expensive, and rarely results in relief of conditions without completely replacing injured joints. Therefore, it is critical that new techniques for the treatment and prevention of OA are developed. Specifically, this

thesis investigates the possible prevention of OA by cellular rescue through the use of a surfactant, poloxamer 188.

The human body is a complex system composed of many tissues, each with their own composition and function. The skeletal system provides a framework for organs and structures that allows movement through interaction with the muscular system. Joints must transfer forces and absorb high loads with very little friction to permit this movement. This task is accomplished by articular cartilage (Figure 2) found within diarthrodial joints.

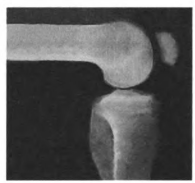

Figure 2. The bones of the knee joint are covered with cartilage, which is capable of distributing the loads produced across the joint. The cartilage provides the joint with a near frictionless surface for movement and minimal wear.

Articular cartilage, along with the synovial fluid, provides the joint with a near frictionless surface for movement and minimal wear. Cartilage is made up of two phases, a solid phase and a fluid phase. The solid phase is made up of collagen, proteoglycans and chondrocytes, while the fluid phase is made up of water and electrolytes. The macromolecules of the solid phase assemble in the extracellular space to form an electromechanically and biochemically functional extracellular matrix which is essential to the function of cartilage as a load-bearing tissue (Quinn et al., 1998). Collagen is an elastic protein that provides the cartilage with its tensile properties. Proteoglycans are proteins with one or more glycosaminoglycan (GAG) chains attached. The GAG chains

are negatively charged molecules that are closely associated with positive ions that attract water and impart many of the physical properties to cartilage. This provides an osmotic pressure to the cartilage matrix, causing tension in the collagen, and allowing the cartilage to support compressive loads. The integrity of articular cartilage is highly dependent on the matrix being intact. Damage to this structure can produce irreversible changes that accumulate over time because of the low metabolic rate of cells in this structure.

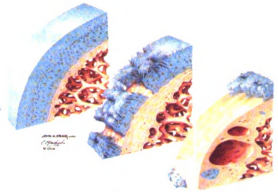

Figure 3. Osteoarthritis is a chronic disease of the articular cartilage found in load bearing joints. During the progression of the disease the cartilage is degraded, leaving bone on bone contact. This condition causes extreme pain and loss of mobility.

In OA patients, articular cartilage degrades (Figure 3), resulting in the bones of the joint coming into contact. This condition can cause extreme pain and loss of mobility. Acute trauma to the joint is known to increase the risk of OA. Destabilization of the knee joint due to anterior cruciate ligament rupture or meniscal damage is also known to cause radiographic signs of OA in patients (Kerin et al., 2002). Other mechanical influences that cause abnormal forces, such as obesity and muscle weakness, are also linked to the progression of OA. Degenerated tissue also has decreased stiffness and a thickening of the subchondral plate, which have been hypothesized to contribute to the eroding of the cartilage and the onset of OA (Ewers et al., 2000).

Chondrocytes, the resident cells of cartilage, are sensitive to mechanical stimuli, such as shear strains that develop as a result of blunt trauma. Cell death correlates with the degree of mechanical disruption on the surface of the cartilage (Oyen-Tiesma et al., 1999). These researchers also put forth the theory that the majority of cell death occurs near surface fissures, and with the absence of surface disruptions, minimal cell death would be observed. Morel et al made a similar observation that cell death appeared to be associated with superficial cracks for relatively high strain rates, but that cell death also occurred in the absence of cracks, suggesting different sets of contributing micromechanical factors (Morel et al., 2002). Early OA changes are often observed as fissures on the articular cartilage surface (Krueger et al., 2003).

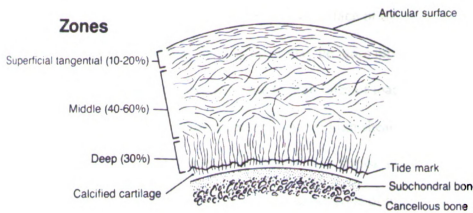

Figure 4. Cartilage is made up of three zones: the superficial, the middle and the deep. The zones are delineated by the orientation of the collagen fibers. In the deep zone the collagen fibers are arranged in a vertical orientation, in the superficial zone the fibers are arranged parallel to the cartilage surface. Chondrocytes are arranged along the collagen fibers in the matrix.

Chondrocytes are responsible for maintaining homeostasis within the matrix of cartilage. These cells mediate the synthesis, secretion and degradation of matrix proteoglycans, glycoproteins and collagen (Figure 4). Shear strains in the matrix of the

cartilage damage chondrocyte cell membranes and interrupt ion gradients leading to swelling and eventually death. The strain rate, peak stress and peak strain developed in cartilage during mechanical loads are important parameters for the determination of the extent of mechanical injury. Cell viability and matrix damage may be affected by the specific applications of strain, strain rate or peak stress (Kurz et al., 2000). Following injury a reduction in cell density may occur affecting the progression of lesions and their subsequent repair (D'Lima et al., 2001). This study also hypothesized that further matrix degeneration and the development of full thickness cartilage lesions may result from cell death and the absence of tissue homeostasis provided by cells.

It is therefore important to understand how mechanical forces affect cartilage. A theoretical model of cartilage as a transversely isotropic biphasic material under high rates of loading suggests high shear stresses on the surface of the explant (Ewers et al., 2001). For a low rate of loading shear stresses may be more uniformly distributed throughout the thickness of the explant. Due to high rates of loading, brittle material behavior of the cartilage may cause a smaller zone of high shear stresses coinciding with cell death adjacent to lesions (Ewers et al., 2000). In contrast, low rates of loading will cause a more ductile response in cartilage and a larger zone of high shear stress at the tip of the propagating cracks.

Chondrocytes die by one of two modes, necrosis or apoptosis (Blanco et al., 1998; Loening et al., 2000; Lucchinetti et al., 2002). Necrosis is the mode of cell death that occurs when a cell's membrane is damaged (Figure 5).

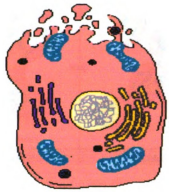

Figure 5. Necrosis is characterized by a disruption of the cell membrane. An osmotic imbalance is created by the loss of membrane integrity, the cell swells with water and eventually bursts releasing its cellular content into its surrounding environment.

The cell swells with excess water and eventually bursts. Necrosis has been referred to as 'cell murder' because otherwise healthy cells die due to an external influence. One study notes, following blunt impact, most of the observed cell death is necrotic, but after day 1, a small number of cells show characteristic ultrastructural features of apoptosis (Tew et al., 2000).

Necrotic cell death can be detected in tissues by using membrane exclusion dyes, which cannot penetrate intact cell membranes. Because necrotic cells have compromised cell membranes, ethidium homodimer can enter cells and become incorporated into the DNA of the cell and cause it to fluoresce. Viable cells are counterstained using a compound called Calcien AM that is metabolized in the cytoplasm resulting in a fluorescent byproduct. Tissue sections can then be viewed under a fluorescence microscope or assayed using flow cytometry.

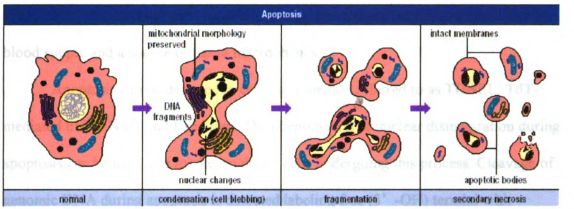

Figure 6. During apoptosis, the nucleus and cytoplasm of the cell condenses, the nuclear material is cleaved into small fragments and the cellular membrane blebs to package the cellular material into apoptotic bodies for easy removal by phagocytes.

Apoptosis or 'programmed cell death' is a normal physiological process that maintains tissue homeostasis by eliminating cells that may be harmful to the organism. During apoptosis, there is internucleosomal cleavage of DNA, the cytoplasm and genetic material condense, and the cell membrane 'blebs' to package fragments of cellular material into apoptotic bodies to facilitate removal by phagocytosis (Figure 6). Internal organelles retain their structure, but the nucleus, which is unaltered during necrosis, invariably changes dramatically during apoptosis (Duke et al., 1996). Chen et al found that apoptosis was a factor in the loss of cellularity in cartilage and that the percentage of apoptotic cells increased with the duration of loading following repeated 5 MPa impacts (Chen et al., 2001). Chen et al also make the suggestion that necrosis, rather than apoptosis, is the earlier event in chondrocyte cell death (Chen et al., 2001). The acutely traumatized joint is an ideal microenvironment for the occurrence of chondrocyte apoptosis due to the convergence of several known mediators of chondrocyte programmed cell death, including matrix injury, the influx of inflammatory cells, the release of proinflammatory cytokines, the generation of reactive oxygen species and nitric oxide, and the potential for continued joint injury from mechanical overload (Kim

et al., 2002). In the long term, apoptotic bodies are retained because cartilage lacks a blood supply and a source of tissue macrophages.

Apoptosis can be detected in cells by a method referred to as TUNEL: TdT-mediated dUTP Nick End Labeling. The phenomenon of nuclear disintegration during apoptosis can be used as a marker to detect cells undergoing this process. Cleavage of genomic DNA during apoptosis is detected labeling free (3'_-OH) termini with fluorescein labeled nucleotides. The reaction is catalyzed by terminal deoxynucleotidyl transferase in a template independent fashion. The fluorescent DNA is then detected on a single cell level by fluorescence microscopy or flow cytometry. Although the most efficient way of identifying apoptosis is by the detection of DNA with strand breaks by labeling them with biotin or fluorescein-conjugated nucleotides, there may be situations with extensive DNA degeneration, even selective to internucleosomal DNA, that may accompany necrosis (Blanco et al., 1998; Chen et al., 2000).

Several non-ionic surfactants have been shown to repair damaged cell membranes after injury from physical stress, electrical trauma, and chemical and thermal stresses (Hannig et al., 2000, Lee et al., 1992, Maskarinec et al., 2002, 2003). Poloxamer 188 (P188) repairs cells by incorporating itself into the damaged portion of the cell membrane and therefore sealing the defect preventing necrotic cell death (Maskarinec et al., 2002, Figure 7).

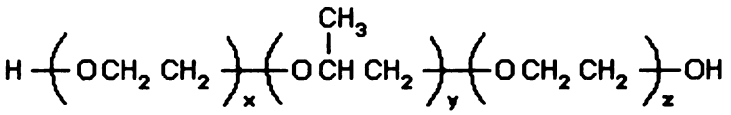

Figure 7. The chemical structure of Poloxamer 188. P188 is made up of two hydrophilic subgroups surrounding a central hydrophobic subgroup. Due to its structure, P188 integrates itself into biological membranes.

P188 is a triblock copolymer of the form poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) with a molecular weight of 8400 Daltons. P188 is a highly surface active copolymer, which probably aids in its absorption and facilitates its insertion into lipid monolayers (Maskarinec et al., 2002, 2003). As recent injection experiments have shown when surface pressure of an artificial lipid monolayer is decreased below a threshold level, therefore representing damage, P188 is easily inserted into the monolayer. On the other hand, at high surface pressures P188 does not insert itself into the artificial monolayer. Therefore, P188 is incorporated only into the damaged portions of membranes (Figure 8).

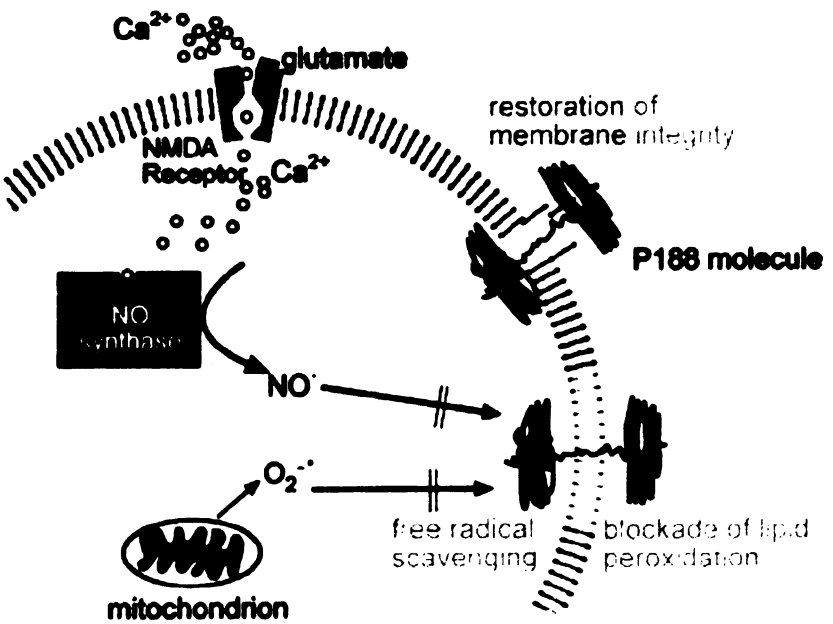

Figure 8. Diagram showing a possible mechanism for the insertion of P188 into the damaged portion of a cell membrane (Maskarinec, 2002, 2003). The hydrophobic portion of P188 remains on the inside of the membrane protected by the

P188 has been used for a range of purposes including as a mild detergent to cleanse wounds, as an emulsifier for non-polar lipids, and as a laxative. It is also used to coat the surface of membrane oxygenators in order to reduce erythrocyte lysis during

cardiopulmonary bypass (Lee et al., 1992). Because cells dying by apoptosis maintain intact membranes, P188 has no affect on these cells.

Chapter 1 of this thesis will investigate the role of apoptosis in cartilage explants following a single blunt trauma. Chapter 2 will investigate the ability of P188 to restore the integrity of damaged membranes, and 'save' these cells from necrosis. Chapter 3 will examine the role of P188 pre-treatment only, versus both pre- and post-treatment in preventing the loss of cell membrane integrity. Chapter 4 will document the effectiveness of P188 at varying levels of stress. Chapter 5 will deal with the potential action of P188 in a live animal joint following acute blunt trauma.

The potential for early intervention of a traumatized joint with P188 may represent an important advancement in the treatment of this trauma patient. The research presented in this thesis represents a first step in this process.

References

1. Atkinson T, Atkinson P. (2000) Knee injuries in motor vehicle collisions: a study of the National Accident Sampling System database for the years 1979-1995. *Accid Anal Prev*. 32(6):779-86

2. Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, Galdo F. (1998) Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. *Arthritis Rheum*. 41(2):284-9.

3. Borrelli Jr. J, Torzilli PA, Grigiene R, Helfet DL. (1997) Effect of impact load on articular cartilage: development of an intra-articular fracture model. *J Orth Trauma*. 11(5):319-326.

4. Chen CY, Burton-Wurster N, Lust G, Borden C, Bloom S. (2000) Cell death in articular cartilage after repeated impacts. *46th Annual Meeting Orthopaedic Research Society.*

5. Chen C, Burton-Wurster N, Borden C, Hueffer K, Bloom SE, Lust G. (2001) Chondrocyte necrosis and apoptosis in impact damaged articular cartilage. *J Orth Res*. 19:703-711.

6. Dischinger PC, Cushing BM, Kerns TJ. (1992) Lower extremity fractures in motor vehicle collisions: Influence of direction of impact and seatbelt use. *38th Annual Proceedings, Association for the Advancement of Automotive Medicine*. pp. 319-325.

7. D'Lima DD, Hashimoto S, Chen PC, Colwell CW, Lotz MK. (2001) Impact of mechanical trauma on matrix and cells. *Clinical Orth Related Res*. 391S:S90-S99.

8. Duke RC, Ojcius DM, Young JD. (1996) Cell suicide in health and disease. *Sci Am*. 275(6):80-7.

9. Ewers BJ, Haut, RC. (2000) Polysulphated glycosaminoglycan Treatments can mitigate decreases in stiffness of articular cartilage in a traumatized animal joint. *J Orth Res*. 18:756-761.

10. Ewers B, Dvoracek-Driksna D, Orth M, Altiero N, Haut R. (2000) Matrix damage and chondrocyte death in articular cartilage depends upon loading rate. *46th Annual Meeting Orthopaedic Research Society.*

11. Ewers B, Krueger J, Dvoracek-Driksna D, Orth M, Haut R. (2001) Chondrocyte viability decreases over 24 hours post-impact in a mechanically traumatized cartilage explant. *47th Annual Meeting Orthopaedics Research Society.*

12. Hannig J, Zhang D, Canaday DJ, Beckett MA, Astumian RD, Weichselbaum RR, Lee RC. (2000) Surfactant sealing of membranes permeabilized by ionizing radiation. *Rad Res.* 154(2):171-177.

13. Kim HT, Lo MY, Pillarisetty R. (2002) Chondrocyte apoptosis following intraarticular fracture in humans. *Osteo Cart.* 10(9):747-9.

14. Kerin A, Patwari P, Kuettner K, Cole A, Grodzinsky A. (2002) Molecular basis of osteoarthritis: biomechanical aspects. *Cell Mol Life Sci.* 59:27-35.

15. Krueger JA, Thisse P, Ewers BJ, Dvoracek-Driksna D, Orth MW, Haut RC. (2003) The extent and distribution of cell death and matrix damage in impacted chondral explants varies with the presence of underlying bone. *J Biomech Eng.* 125:1-6.

16. Kurz B, Jin M, Patwari P, Lark MW, Grodzinsky AJ. (2000) Biosynthetic response and mechanical propeties of articular cartilage after injurious compression: the importance of strain rate. *46th Annual Meeting Orthopaedic Research Society.*

17. Lee RC, River LP, Pan F, Ji L, Wollmann RL. (1992) Surfactant-induced sealing of electropermeabilized skeletal muscle membranes *in vivo*. *Proc Natl Acad Sci.* 89:4524-4528.

18. Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurtz B, Nuttall ME, Hung H, Blake SM, Grodzinsky AJ, Lark MW. (2000) Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. *Arch Biochem and Biophys.* 381(2):205-212.

19. Lucchinetti E, Adams CS, Horton Jr WE, Torzilli, PA. (2002) Cartilage viability after repetitive loading: a preliminary report. *Osteo and Cart.* 10:71-81.

20. Maskarinec SA, Hannig J, Lee RC, Lee KC. (2002) Direct Observation of Poloxamer 188 insertion into lipid monolayers. *Biophysical J.* 82:1453-1459.

21. Maskarinec SA, Lee, KC. (2003) Comparative study of poloxamer insertion into lipid monolayers. *Langmuir.* 19:1809-1815.

22. Miller TR, Levy DT. (1995) The effect of regional trauma care systems on costs. *Arch Surg.* 130(2):188-93.

23. Morel V, Quinn TM. (2002) Micromechanical factors in cartilage injury under ramp compression of explants for deformations applied near the "gel diffusion" rate. *48th Annual Meeting Orthopaedic Research Society.*

24. Oyen-Tiesma M, Deloria LB, Meglitsch T, Oegema TR, Lewis JL. (1999) Cell death after cartilage impact depends on matrix damage. *45th Annual Meeting Orthopaedic Research Society.*

25. Quinn TM, Grodzinsky AJ, Hunzinker EB, Sandy JD. (1998) Effects of injurious compression on matrix turnover anround individual cells in calf articular cartilage explants. *J Orth Res.* 16:490-499.

26. Rice DP, MacKenzie EJ. Cost of Injury in the United States –*A Report to Congress, National Highway Traffic SafetyAdministration, U.S. Department of Transportation and Centers for Disease Control, U.S. Department of Health and Human Services.* 1989

27. States JD. (1970) Traumatic arthritis: a medical dilemma. In: *Proceedings of the 14th Annual Conference of the American Association for Automotive Medicine.* 14:21-28

28. Tew SR, Kwan AP, Hann A, Thomson BM, Archer CW. (2000) The reactions of articular cartilage to experimental wounding: role of apoptosis. *Arthritis Rheum.* 43(1):215-25.

29. Upadhyay S, Moulton A, Srikrishnamurthy K. (1983) An analysis of the late effects of traumatic posterior dislocation of the hip without fractures. *J Bone Joint Surgery* 65-B(2):150-152.

Chapter One

The Role of Apoptotic Cell Death Following Blunt Trauma In Cartilage Explants

Introduction

Osteoarthritis (OA) affects more than 21 million Americans, and is the leading cause of disability in the U.S. each year (US census bureau, 2000). Clinically the disease is characterized by joint pain and narrowing of the joint, as diagnosed by radiological examination (Flores et al., 1998). Pathologically, the disease exhibits a loss of cartilage and sclerosis of underlying subchondral bone. Histologically there is fragmentation of the cartilage surface in the diseased joint, cloning of chondrocytes and eventually violation of the tidemark by blood vessels. Studies have associated the physiopathology of articular cartilage and the time course of OA with the existence of dead chondrocytes (Figure 1) (Blanco et al., 1998, Hashimoto et al., 1998, Hashimoto et al., 1998, Heraud et al., 2000, Kim et al., 2000).

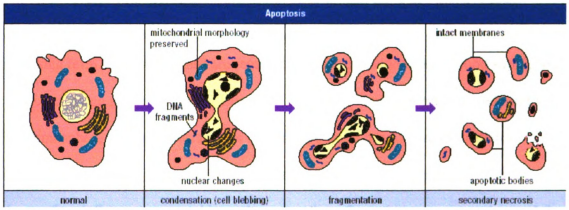

Figure 1. Diagram showing several steps an apoptotic cell undergoes. The nucleus and cytoplasm condense, there is blebbing of the cell membrane and packaging of the cellular contents into apoptotic bodies.

In recent times several new theories for the onset and development of OA have suggested that chondrocyte apoptosis might be involved as the mode of cell death

(Blanco et al., 1998, Loening et al., 2000). In these studies chondrocytes in the superficial tangential and middle zones of human OA cartilage show the characteristics of apoptotic cell death, such as nuclear chromatin condensation (Lucchinetti et al., 2002). Apoptosis, also known as programmed cell death or 'cell suicide', is a normal physiological process that maintains tissue homeostasis and is characterized by a condensation of cytoplasm and by internucleosomal cleavage of DNA. In its final stages of cellular death there is blebbing of the cell membrane in order to package fragments of cellular contents into structures known as apoptotic bodies (Figure 2) (Chen et al., 2001).

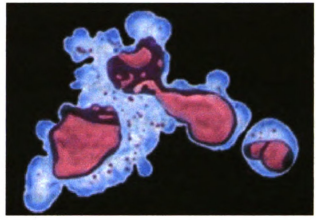

Figure 2. Picture showing cellular membrane blebbing that occurs during apoptosis. The blebbing of the membrane occurs to package the cellular contents into apoptotic bodies for easy removal by macrophages.

In the long term apoptotic bodies are retained because cartilage lacks a blood supply and a source of tissue macrophages (D'Lima et al., 1994). The acutely traumatized joint is an ideal atmosphere for the appearance of cellular apoptosis due to several mediators of programmed cell death including matrix injury, the influx of inflammatory cells, the release of proinflammatory cytokines, the generation of reactive oxygen species, and the potential for continued joint injury from mechanical overload (Kim et al., 2002). Cartilage wounding with a trephine shows cell death near the lesion due to a gradual increase in apoptosis, and eventually resulting in a secondary necrosis (Costouros et al.,

2003). In contrast, canine cartilage explants subjected to low levels of cyclic compression exhibit the development of cellular necrosis progressively increasing in time, whereas apoptosis (TUNEL-positive cells) is not seen until 48 hours or more post loading (Chen et al., 2001). Tew et al also documented that initially following trauma most of the observed cell death is necrotic, but after 24 hours post impact a small number of cells show the characteristic ultrastructural features of apoptosis (Tew et al., 2000). Chen et al also noted that necrosis rather than apoptosis appeared to be the earlier mode of cell death following impact loads, but that apoptosis was also involved, suggesting that cell loss in injured cartilage may involve both apoptosis and necrosis (Chen et al., 2001).

The hypothesis of the current study was that a single, severe blunt insult on cartilage would induce a small amount of apoptosis, but this type of cellular death would not be significant until 48 – 96 hours post impact, based on the above literature. In the current study bovine cartilage explants were subjected to a 30 MPa impact load. A TUNEL assay was used to assess apoptotic cell death at several time points within the first 96 hours. The impact load was applied at both a high (50 ms to peak) and a low (1 s to peak) rate of loading. These rates and levels of loading were chosen based on previous studies in the laboratory (Ewers et al., 2000, Krueger et al., 2003), and based on other studies in the literature (Atkinson and Haut, 1995, Atkinson et al, 1998, Borrelli et al., 1997).

Methods:

Four bovine forelegs from 2 mature animals were obtained from a local abattoir within six hours of slaughter. The legs were skinned and rinsed with water prior to exposing the metacarpal joint in a laminar flow hood. A biopsy punch (Miltex Instrument Company, Bethpage, NY) was used to make forty-four 6mm diameter chondral explants from the lower metacarpal surface of the limbs. The explants were separated from the underlying bone using a scalpel. The specimens were washed three times in Dulbecco's Modified Eagle Medium: F12 (DMEM, Invitrogen Co., Grand Island, NY, #12500-062). The explants were then equilibrated for 48 hours in DMEM:F12 (2 explants per well, approximately 1 mg of cartilage per 1 ml of media) supplemented with 10% fetal bovine serum (Invitrogen Co), 21.9 mg/ml glutamine, additional amino acids and antibiotics in a humidity controlled incubator (37° C, 5% CO_2, 95% humidity).

The explants were randomly assigned to three treatment groups, unimpacted controls, a high rate of loading group (50ms to peak load), and a low rate of loading group (1s to peak) (Figure 3).

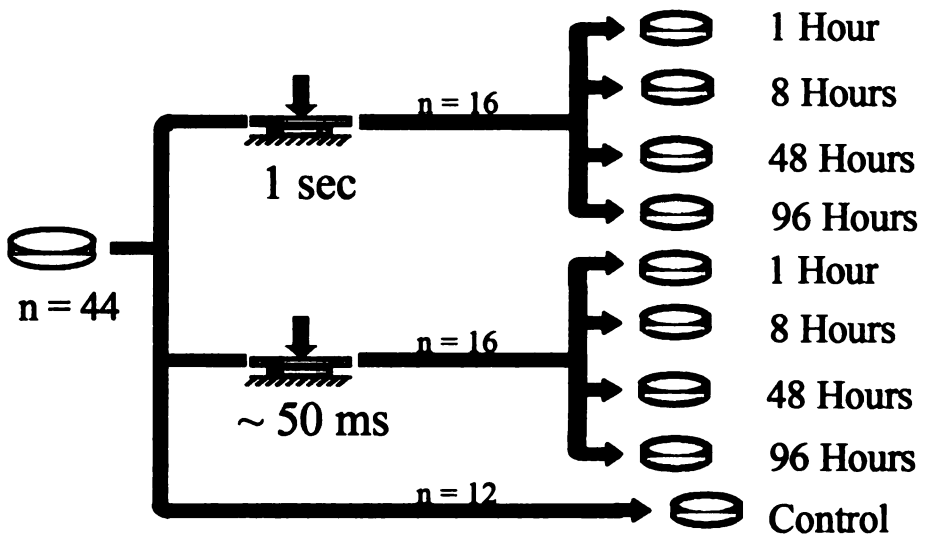

Figure 3. Diagram showing the experimental design. Explants were frozen at 1, 8, 48 or 96 hours post impact and stained for TUNEL.

All loaded explants were taken to 848 N (~30 MPa), following a 5 N preload, in unconfined compression between two highly polished stainless steel plates using a haversine loading protocol with a servo-controlled hydraulic testing machine (Instron, model 1331, Canton, MA). Three biomechanical parameters, peak load, time to peak, and maximum explant compression were documented in each experiment. The explants were then re-incubated for 1, 8, 48 or 96 hours in fresh DMEM:F12 supplemented medium. Following incubation, the explants were imbedded in Optimal Cutting Temperature media (OCT, Tissue-Tek O.C.T. compound, Sakura Finetek USA Inc., Torrance, CA), frozen, and stored at –80°C. 8μm thick slices were cut through the entire thickness of the explant using a cryotome (HistoSTAT microtome, AO Scientific Instruments, Buffalo, NY). The slices from the central area of the explant were immobilized on glass slides, air dried and stored at –80°C. A control explant was treated with DNase 1 (Promega, Madison WI) for 10 minutes at room temperature for use as a positive control. The explant slices were stained for apoptotic nuclei by the terminal

deoxynucleotide transferase mediated UTP nick end labeling method (TUNEL, Promega DeadEnd Fluorometric TUNEL System, Promega, Madison WI). Briefly, the explant slices were fixed in 4% formaldehyde and then permeabilized in 0.2% Triton X-100. The slices were then incubated in a 20 μg/ml proteinase K solution (Promega, Madison WI) for 8 minutes at room temperature. Next, the slices were incubated in an equilibrium buffer containing a nucleotide mix and TdT enzyme at 37°C for 1 hour to perform the reaction. Finally the slices were counterstained with a 1 μg/ml propidium iodide solution for 15 minutes at room temperature to highlight non-TUNEL positive cells. The specimens were then viewed under a fluorescence microscope (Leica DM LB (frequency: 50-60 Hz), Leica Mikroskopie und Systeme GmgH, Wetlzar, Germany) and photographed using a digital camera (Spot Digital Camera, Diagnostic Instruments Inc). Each explant slice was divided into three zones: superficial (top 20%), middle (middle 50%), and deep (bottom 30%). Two parallel lines were drawn approximately 2 mm apart and perpendicular to the explant to outline an area for determination of viable and non-viable cells. Each explant was divided into its upper 20%, middle 50%, and lower 30% using each line as a guide (Figure 4).

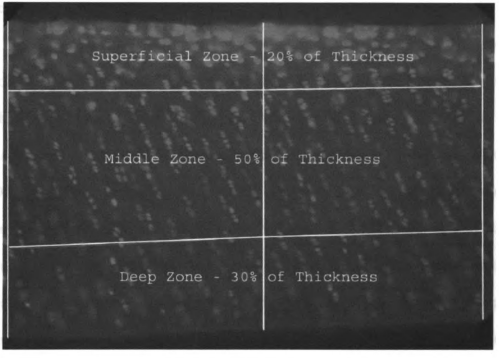

Figure 4. Using image software, the explants were divided into three zones, the superficial (top 20%), the middle (50%), and the deep (bottom 30%). Lines were drawn on the photo to represent the zones.

Another line was drawn centrally on the photograph to help construct the horizontal lines that demarcated each zone of the explant. This photographic processing was performed using a commercial program (Corel Draw, Corel Inc., Dallas, TX). A blinded observer, using image analysis software (Sigma Scan, SPSS INC., Chicago, IL), quantified cell viability in each zone of the explant.

The percentage of TUNEL-positive cells was quantified in each zone. The maximum applied load, time to peak load and maximum explant compression were determined from each experiment. A two-factor ANOVA was performed on these biomechanical parameters and the percentage of TUNEL-positive cells in each group of the explants. Time (1, 8, 48, 96 hours) and treatment (high rate, low rate or control) were the factors. A Student-Newman-Keuls (SNK) post-hoc test was used to determine differences between groups.

Results:

The experiment was performed to determine the percentage of TUNEL-positive cells following unconfined compression of chondral explants following a low (1s to peak) and a high (5ms to peak) rate of loading. Analysis of the high rate of loading data for the various groups (1, 8, 48, and 96 hours post trauma) indicated no significant differences ($p>0.05$) in any biomechanical parameter. The maximum applied load, maximum explant compression and time to peak load were (Mean± SD) 832.5± 7.5 N, 0.296± 0.068 mm and 0.056± 0.003 ms, respectively. In the low rate of loading experiments the biomechanical parameters also did not vary significantly ($p>0.05$) between groups, but the maximum load and time to peak were different than those in the high rate of loading experiment. The corresponding values of maximum load, maximum compression and time to peak load were 851.7± 1.1 N, 0.334± 0.041 mm and 1.00± 0.02 sec, respectively.

TUNEL staining of the impacted explants showed only a minimal (<0.5% of all cells) percentage of TUNEL-positive cells prior to 48 hours post trauma (Figure 5).

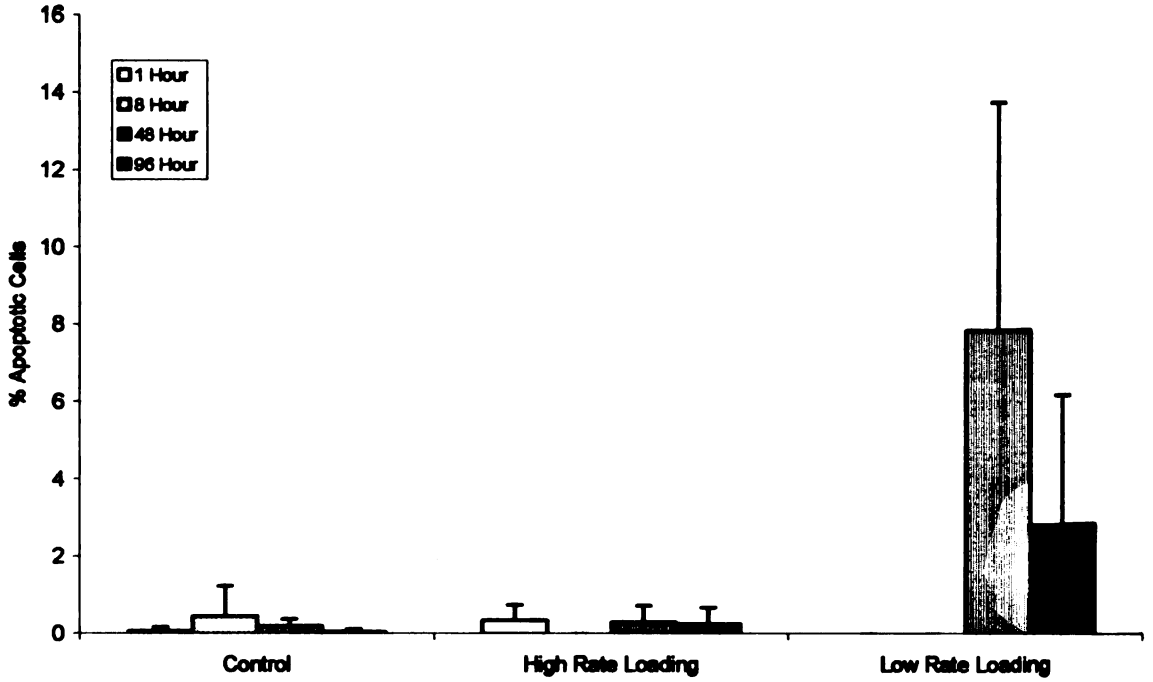

Figure 5. Histogram showing the total percentage of TUNEL positive cells following blunt impact loading at 1 (white bars), 8 (diagonal striped bars), 48 (gray bars), and 96 hours (black bars). Explants were randomly assigned to one of three groups: control, high rate of loading (50 ms to peak), and low rate of loading (1 s to peak).

Following a high rate of loading the chondral explants showed only a minimal (<0.5% of all cells) number of TUNEL-positive cells for each time point to 96 hours. Conversely, in the low rate of loading experiments the percentage of positively stained cells was 7.9± 5.9 % and 2.8± 3.4 % at the 48 and 96 hours, respectively. The percentage of TUNEL-positive cells in controls at each time point was minimal (<0.1%), while nearly all of the cells within the explant were stained in the DNase positive controls. In those explants showing TUNEL positive cells after loading, these cells were limited to the superficial zone in all groups (Figure 6). In the low rate of loading group at 48 hours and 96 hours the percentage of TUNEL-positive cells in the superficial zone was 38.1± 26.9 % (n=4) and 33.4± 35.7 % (n=4), respectively (Figure 6).

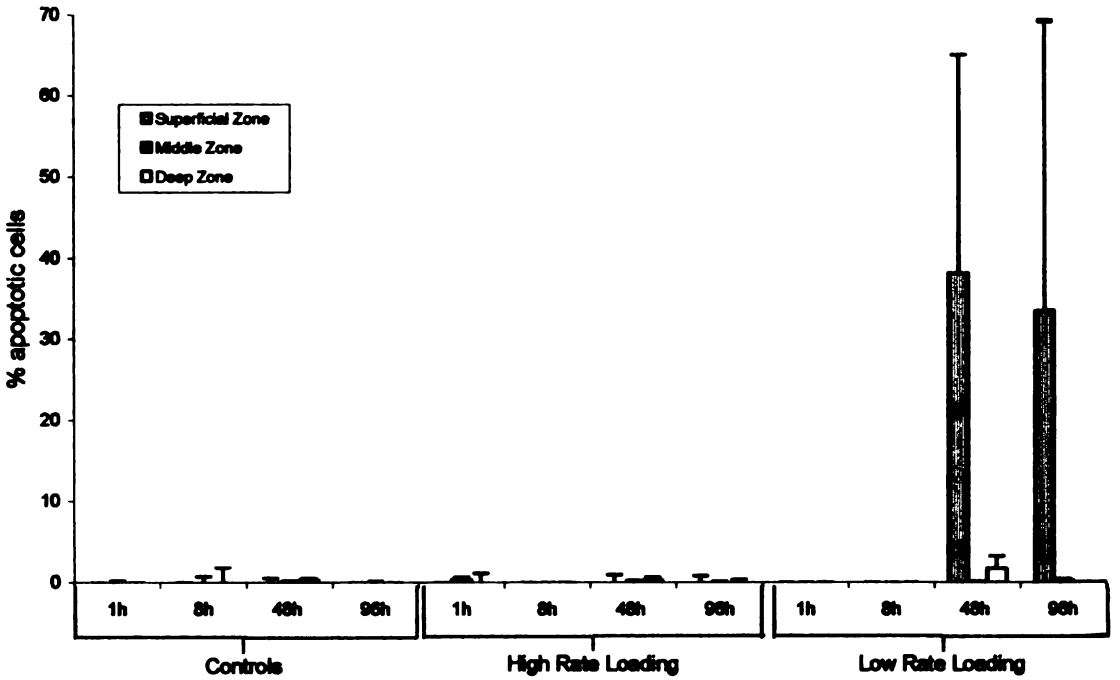

Figure 6. The percentage of TUNEL positive cells in the three zones of the cartilage: superficial (top 20%), middle (50%) and deep (bottom 30%). There was a significant difference in the percentage of TUNEL positive cells versus the control explants in the superficial zones at 48 and 96 hours post-impact for the low rate of loading groups.

Discussion:

This study documented the role of apoptosis in chondrocytes following blunt trauma to cartilage. Chondral explants were loaded to 30MPa in unconfined compression at low (1s to peak) and high (50ms to peak) rates of loading. The explants were examined at several time points following loading to determine the percentage of TUNEL-positive cells. The hypothesis of this study was that the majority of cells injured during compressive loading would not be TUNEL-positive.

In the study TUNEL-positive cells were observed after a low rate of loading at 48 and 96 hours, but only minimal (< 0.5%) staining was observed in the high rate of loading. Since the applied load was only slightly (2 %) greater in the low versus the high rate of loading experiments, we believe one possible factor responsible for the appearance of apoptotic cells was the rate of loading. Yet, the mechanism of this response is unknown. The percentage of TUNEL-positive cells following a low rate of loading was approximately 8 % at 48 hours and less than 3 % at 96 hours. In contrast, an earlier study by this laboratory documented approximately 66 % of cells dead at 24 and 96 hours in chondral explants exposed to 30MPa of loading (Ewers, 2001). Thus, the results of the current study suggest that the primary mechanism of cell death resulting from blunt mechanical loading is via the necrotic pathway. This result compared well with experimental data from adult canine chondral explants following low intensity (5MPa) cyclic impacts (Chen, 2001). In that study, cartilage cyclically loaded for 2 hours showed 32% of the chondrocytes had leaky cell membranes characteristic of necrosis. While only 1% of these chondrocytes were TUNEL-positive 4 hours later (at 6 hours), at 48 hours the percentage of TUNEL-positive cells rose to 73%. In this study and in the

current investigation, TUNEL-positive cells were limited to the upper zones of the explant. While TUNEL-positive cells have been thought to largely reflect cellular death via apoptosis, Chen et al (2000, 2001) and others have also documented TUNEL-positive responses in necrotic cells (Grasl-Kraupp, 1995, Tew, 2000, Blanco, 1998). Without verifying the existence of apoptotic bodies using transmission electron microscopy the mechanism of cell death in the TUNEL-positive cells of the current study is yet unknown. A possible scenario may be that these cells were less severely traumatized by the mechanical insult. On the other hand, death in these cells may have been due to a cell-mediated response produced by necrosis in adjacent cells (Levin, 2001).

A limitation of the current study was that osteochondral explants were not utilized. The attachment of the explant to underlying bone helps limit the extent of cell death in all zones for a given level of loading (Krueger, 2003). A reduction in the percentage of cell death in osteochondral versus chondral explants at 30 MPa of compressive loading is currently thought to be primarily due to a reduction in tissue strains. Especially in the deep zones of the tissue a reduction of tissue compressive stress in osteochondral versus chondral explants may produce more apoptosis at a given level of applied loading. But if cell death is primarily due to excessive cell strains, the distribution of apoptotic cells nearer the surface may be influenced by the fact that the deeper zones of cartilage are stiffer (Schinagl, 1997). Studies by others using intact joints also show that the location of cell apoptosis following blunt mechanical loading is primarily in the upper zones of the tissue, similar to the current study using chondral explants (D'Lima, 2001).

A study by another laboratory showed that a pan-caspase inhibitor, z-VAD.fmk [benzyloxycarbonyl-val-ala-asp (Ome) flouromethylketone] could help prevent chondrocyte apoptosis in human articular cartilage explants compressed to 30% (D'Lima, 2001). The Caspase chain reaction is the enzymatic process the cell uses to facilitate apoptosis. By interrupting this enzymatic chain reaction, it is believed that apoptosis can be prevented in the cell. Caspase inhibition reduced chondrocyte apoptosis in human chondral explants from 34% in non-treated specimens to 25% with z-VAD.fmk intervention at 48 hours.

Further studies are needed to conclude that indeed necrosis, not apoptosis is the primary cell death mode in cartilage trauma. An osteochondral model should be studied to investigate the importance of subchondral bone in limiting the extent of necrosis and apoptopsis. Further, an intervention in the necrotic pathway would be useful if OA is to be prevented. These ideas will be further developed in the subsequent chapters.

References

1. Atkinson PJ, Haut RC. (1995) Subfracture insult to the human cadaver patellofemoral joint produces occult injury. *J Orthop Res.* 13(6):936-44.

2. Atkinson TS, Haut RC, Altiero, NJ. (1998) Impact-induced fissuring of articular cartilage: An investigation of failure criteria. *J Biomech Eng.* 120:181-187.

3. Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, Galdo F. (1998) Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. *Arthritis Rheum.* 41(2):284-9.

4. Borrelli Jr. J, Torzilli PA, Grigiene R, Helfet DL. (1997) Effect of impact load on articular cartilage: development of an intra-articular fracture model. *J Orth Trauma.* 11(5):319-326.

5. Chen C, Burton-Wurster N, Borden C, Hueffer K, Bloom SE, Lust G. (2001) Chondrocyte necrosis and apoptosis in impact damaged articular cartilage. *J Orth Res.* 19:703-711.

6. Chen CY, Burton-Wurster N, Lust G, Borden C, Bloom S. (2000) Cell death in articular cartilage after repeated impacts. *46th Annual Meeting Orthopaedic Research Society.*

7. Costouros JG, Dang AC, Kim HT. (2003) Inhibition of chondrocyte apoptosis in vivo following acute osteochondral injury. *OA Cart.* 11(10) 756-9.

8. D'Lima DD, Hashimoto S, Chen PC, Colwell CW, Lotz MK. (2001) Impact of mechanical trauma on matrix and cells. *Clinical Orth Related Res.* 391S:S90-S99.

9. Ewers BJ, Haut, RC. (2000) Polysulphated glycosaminoglycan Treatments can mitigate decreases in stiffness of articular cartilage in a traumatized animal joint. *J Orth Res.* 18:756-761.

10. Ewers B, Krueger J, Dvoracek-Driksna D, Orth M, Haut R. (2001) Chondrocyte viability decreases over 24 hours post-impact in a mechanically traumatized cartilage explant. *47th Annual Meeting Orthopaedics Research Society.*

11. Flores RH, Hochberg MC. (1998) Definition and classification of osteoarthritis. In: Brandt KD, Doherty M, Lohmander LS (Eds). Osteoarthritis, Oxford University Press, New York. Pp 1-12.

12. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. (1995) In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology.* 5:1465-8.

13. Hashimoto S, Ochs RL, Komiya S, Lotz M. (1998) Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. *Arthritis Rheum.* 41(9):1632-8.

14. Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M. (1998) Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. *Arthritis Rheum.* 41(7):1266-74.

15. Heraud F, Heraud A, Harmand MF. (2000) Apoptosis in normal and osteoarthritic human articular cartilage. *Ann Rheum Dis.* 59(12):959-65.

16. Kim HA, Lee YJ, Seong SC, Choe KW, Song YW. (2000) Apoptotic chondrocyte death in human osteoarthritis. *J Rheumatol.* 27(2):455-62.

17. Kim HT, Lo MY, Pillarisetty R. (2002) Chondrocyte apoptosis following intraarticular fracture in humans. *Osteo Cart.* 10(9):747-9.

18. Krueger JA, Thisse P, Ewers BJ, Dvoracek-Driksna D, Orth MW, Haut RC. (2003) The extent and distribution of cell death and matrix damage in impacted chondral explants varies with the presence of underlying bone. *J Biomech Eng.* 125:1-6.

19. Levin A, Burton-Wurster N, Chen C, Lust G. (2001) Cellular signaling as a cause of cell death in cyclically imapcted cartilage explants. *47th Annual Meeting Orthopaedics Research Society.*

20. Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurtz B, Nuttall ME, Hung H, Blake SM, Grodzinsky AJ, Lark MW. (2000) Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. *Arch Biochem and Biophys.* 381(2):205-212.

21. Lucchinetti E, Adams CS, Horton Jr WE, Torzilli, PA. (2002) Cartilage viability after repetitive loading: a preliminary report. *Osteo and Cart.* 10:71-81.

22. Schinagl R, Gurskis D, Chen A, Sah R. (1997) Depth-dependent confined compression modulus of full-thickness bovine articular cartilage. *J Orth Res.* 15(4):499-506

23. Tew SR, Kwan AP, Hann A, Thomson BM, Archer CW. (2000) The reactions of articular cartilage to experimental wounding: role of apoptosis. *Arthritis Rheum.* 43(1):215-25.

24. US census bureau, 2000

Chapter Two

The Use of a Non-Ionic Surfactant (P188) to Save Chondrocytes from Necrosis Following Impact Loading of Chondral Explants

Introduction

Injury of the lower extremities is a common occurrence in automobile accidents. The consequence of these injuries is often the development of a chronic joint disease, osteoarthritis (OA). OA is a condition that develops as the cartilage of the joint wears away, resulting in bone on bone contact accompanied by severe pain and loss of mobility. It is believed that a loss of cellularity and damage of the cartilage matrix are the causes of the degradation of the cartilage. Ewers et al. noted that both the amount of matrix damage and cell death following trauma depends on the rate of loading applied to the cartilage (Ewers et al., 2001). This study also observed that in a low rate of loading test, cell death was more uniformly distributed throughout the matrix of the cartilage. In the previous chapter the role of apoptotic cell death in bovine cartilage explants following a low rate of blunt trauma was investigated. TUNEL positive (apoptotic) cells were increased 48 hours post-impact, but only slightly above background levels. This finding suggests that primary necrosis contributes to the degradation of cartilage following trauma.

A defining feature of cell necrosis is swelling, due to the inability of the injured cell to maintain ionic gradients across the damaged plasma membrane. Ultimately, the necrotic cell ruptures (Figure 1) (Duke et al., 1996).

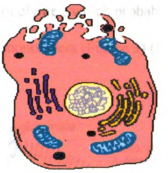

Figure 1. A cell with a damaged membrane will swell and burst. This mode of cell death is called necrosis.

Within the first 24 hours following blunt trauma, cell death increases steadily in explants injured by blunt trauma (Ewers et al., 2000). These data suggest that a "window of opportunity" may exist to intervene in the progressive disease process in the joint and limit the degree of cell death following a single, blunt insult.

Because of their amphiphilic properties, some mild surfactants are able to interact with the lipid bilayer of cell membranes to restore their integrity after injury from physical stress (Clarke et al., 1992, Papoutsakis et al., 1991), electroporation via electrical trauma (Lee et al., 1999), and chemical and thermal stresses (Hellung-Larsen et al., 2000). Recent studies of brain trauma also suggest that these surfactants, particularly poloxamer 188 (P188) can help "save" severely traumatized neurons from developing necrosis (Barbee et al., 1992, Marks et al., 2001). P188 is a triblock copolymer of the form poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) with a molecular weight of 8400 Daltons (Figure 2).

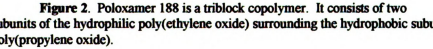

Figure 2. Poloxamer 188 is a triblock copolymer. It consists of two subunits of the hydrophilic poly(ethylene oxide) surrounding the hydrophobic subunit poly(propylene oxide).

P188 is a highly surface active copolymer, which probably aids in its absorption and facilitates its insertion into lipid monolayers (Figure 3) (Maskarinec et al., 2002, 2003).

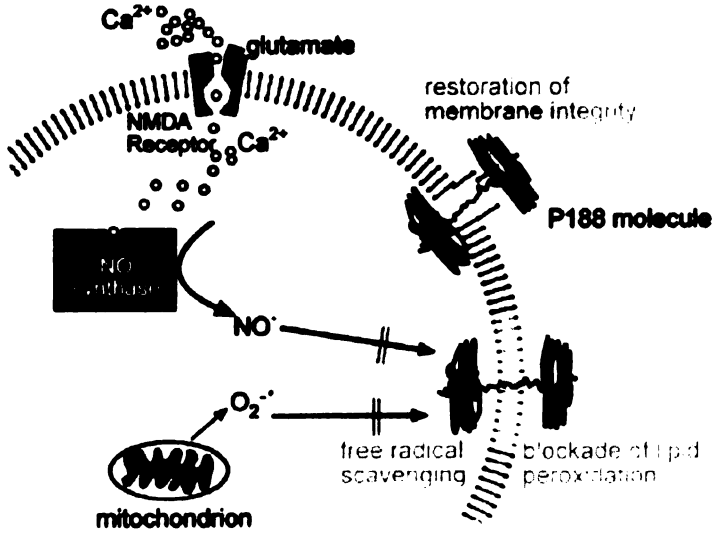

Figure 3. It is hypothesized that P188 inserts into the damaged portion of cell membranes to arrest leakage and prevent cellular necrosis. The cell may then repair itself naturally and the P188 is squeezed out.

The hypothesis of this study was that administration of the nonionic synthetic surfactant P188 would be effective in limiting the development of cellular necrosis within the first 24 hours post trauma.

Methods:

Six bovine forelegs from 3 mature animals were obtained from a local abattoir within six hours of slaughter. The legs were skinned and rinsed with water prior to exposing the metacarpal joint in a laminar flow hood. A biopsy punch (Miltex Instrument Company, Bethpage, NY) was used to make 72 6mm diameter chondral explants from the lower metacarpal surface of the limbs. The explants were separated from the underlying bone using a scalpel. The specimens were washed three times in Dulbecco's Modified Eagle Medium: F12 (DMEM, Invitrogen Co., Grand Island, NY, #12500-062). The explants were then equilibrated for 48 hours in DMEM:F12 (2 explants per well, approximately 1 mg of cartilage per 1 ml of media) supplemented with 10% fetal bovine serum (Invitrogen Co), 21.9 mg/ml glutamine, additional amino acids and antibiotics in a humidity controlled incubator (37° C, 5% CO_2, 95% humidity).

Eight explants were randomly assigned to a non-impact, control group (Figure 4).

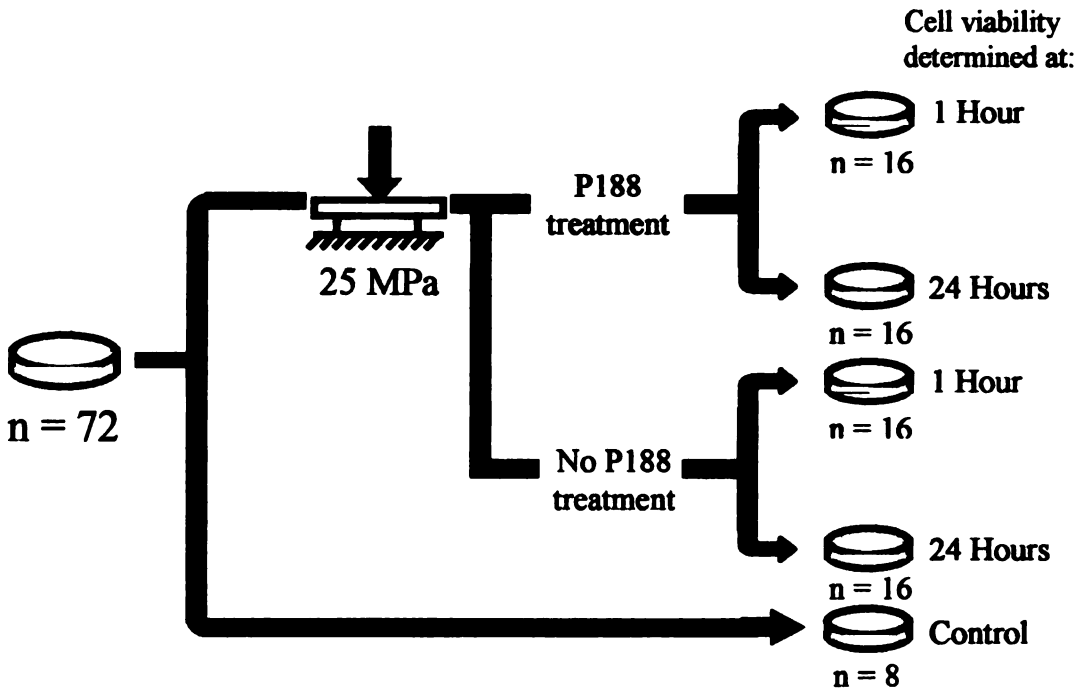

Figure 4. Diagram showing experimental setup. 64 chondral explants were impacted with 25 MPa. 32 explants were treated with P188 and 32 were untreated. 16 explants from each group were stained for cell viability at 1 and 24 hours.

The remaining explants were randomly assigned to two groups, poloxamer-treated and no poloxamer treatment (n=32 each group). All explants, except the control group, were loaded to 707 N (~25 MPa), following a 5 N preload, using a low rate of loading (1s to peak). The loading was performed in unconfined compression between two highly polished stainless steel plates using a haversine loading protocol with a servo-controlled hydraulic testing machine (Instron, model 1331, Canton, MA). Eight mg/ml poloxamer 188 (P188, Pluronic F-68, Sigma Aldrich Co., St. Louis, Mo) was added to the media within 2 minutes of impact for all 16 explants in the P188-treated group. This concentration was chosen based on the *in vivo* concentration used in human patients (Orringer, 2001). The explants were manually submitted to 10 cycles of pressure at (~1 MPa) to encourage P188 uptake into the cartilage matrix. Sixteen explants from each group were used to determine cell viability at either 1 or 24 hours post impact. The 24

hour explants were washed three times in DMEM:F12 after loading and placed into supplemented DMEM:F12 media with P188 and incubated overnight. Following incubation the explants were cut through the thickness into thin sections using a specialized cutting tool with parallel blades spaced 0.5 mm. The slices were stained with Calcein AM and Ethidium Homodimer, according to the manufacturer's specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR). Briefly, 10µl of Ethidium Homodimer and 2.5µl of Calcien AM were added to 10ml of PBS, vortexed and approximately 500µl of the staining solution was added to each well containing explant slices.

Approximately 3 slices from each explant were viewed across approximately 1 mm near the center in a fluorescence microscope (Leitz Dialux 20 (frequency: 50-60 Hz), Leitz Mikroskopie und Systeme GmgH, Wetlzar, Germany). Photographs were taken using a digital camera (Polaroid DMC2, Polaroid Corporation, Waltham, MA, USA). Each digital photo group from the explant slices was divided into three zones: superficial, middle and deep. Using a drawing program (Corel Draw,) parallel lines were drawn on either side of the explant through the thickness to outline the area of the explant to be counted. These end lines were drawn perpendicular to the surface. Starting from the superficial surface of the explant, a line was started on one of the existing end lines at 20% distance from the superficial surface, and extending to the other end line at the 20% mark. The line was drawn to parallel the contours of the superficial surface, as well as the program would allow. Another line was drawn at 70% distance from the superficial surface, or 30% from the deep zone surface. These lines defined the zones of the cartilage: superficial, middle and deep.

Cell viability was quantified in each zone of the explant using the software described above. A live cell, with an intact membrane, will metabolize the calcien AM resulting in a green fluorescent byproduct. A necrotic cell, with a compromised cell membrane, will not exclude the ethidium homodimer from its cytoplasm and its nuclear material will be stained a red fluorescent color.

The percentage of dead cells was quantified in each zone. The maximum applied load, time to peak load and maximum explant compression were determined from the instron data. A two-factor ANOVA was performed on these biomechanical parameters and the percentage of total cell death in each group of explants. The two factors were time (1 and 24 hours) and treatment (P188 vs. no P188). A Student-Newman-Keuls (SNK) post-hoc test was used to determine differences between groups.

Results:

In the experiment, a random sampling of explants was administered 8 mg/ml P188 within 2 minutes of loading. An analysis of the biomechanical properties in the 4 impacted groups (1 and 24 hours, with and without P188 treatment, low rate of loading) showed no significant differences. The average maximum applied load, maximum explant compression and time to peak load were 644.0± 70.6 N, 0.298± 0.054 mm and 0.928± 0.017 sec, respectively (n = 64).

While the percentage of dead cells in control explants was 0.19± 0.17 % at 1 hour (n = 4) and 1.24± 0.76 % at 24 hours (n = 4), the levels of cell death were dramatically increased with compressive loading (Figure 5). The percentage of dead cells significantly (p=0.005) increased between 1 hour and 24 hours post trauma (Figure 5).

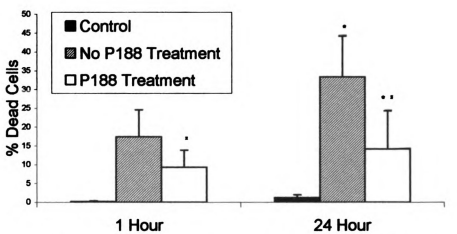

Figure 5. Histogram showing percentage of cell death at 1 and 24 hours for each treatment group: control (black bar), no poloxamer 188 treatment (stripped bar) and P188 treatment (white bar). There was a significant increase in cell death following impact from 1 to 24 hours in both the treated and non-treated groups (*). Conversely, there was a decrease in cell death with the addition of P188 treatment at both 1 and 24 hours post impact (x).

Extensive cell death was specifically noted around impact-induced surface fissures, which were limited to the superficial zone of the explant at 1 hour (Figure 6). A

significant increase in cell death between 1 and 24 hours also occurred in each zone of the explant ($p=0.007$ superficial, $p<0.001$ middle, $p=0.005$ deep) (Figure 6). While the overall percentage increase in cell death between 1 and 24 hours was approximately 16 %, this increase was largely due to that occurring in the middle zone (approximately 80 %).

The percentage of dead cells in the P188 treated explants was significantly less than that in the no treatment group at 1 hour ($p=0.001$) and 24 hours ($p<0.001$) (Figure 5). In the 1 hour group P188 treatment of the loaded chondral explants significantly reduced the percentage of cell death in the superficial ($p<0.001$) and the middle zones ($p=0.022$) versus the no treatment group, but did not reduce the extent of cell death in the deep zone of the explant (Figure 6).

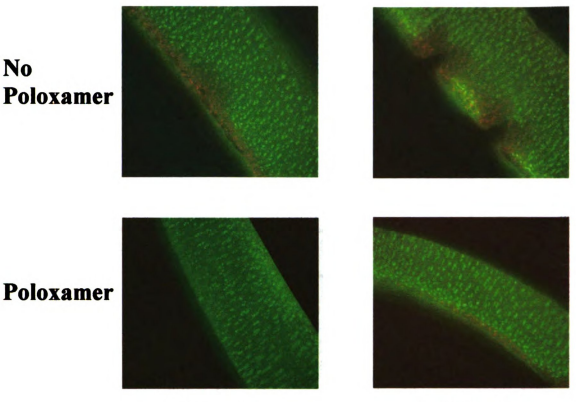

Figure 6. Cartilage explants were impacted with 25 MPa and explant slices were stained for cell viability. Selected explants were treated with 8 μg/ml Poloxamer 188 immediately following impact. Cells stained green were metabolically active and therefore alive, while cells stained red have compromised cell membranes and therefore were necrotic.

Twenty-four hours after loading, however, there were significant reductions in cell death in all zones of the explant following P188 treatment (in the superficial, middle and deep zones $p<0.001$).

While early treatment of the loaded chondral explants with the non-ionic surfactant (P188) significantly decreased the percentage of dead cells, cellular death still increased significantly (p=0.047) in these explants between 1 and 24 hours (Figure 7).

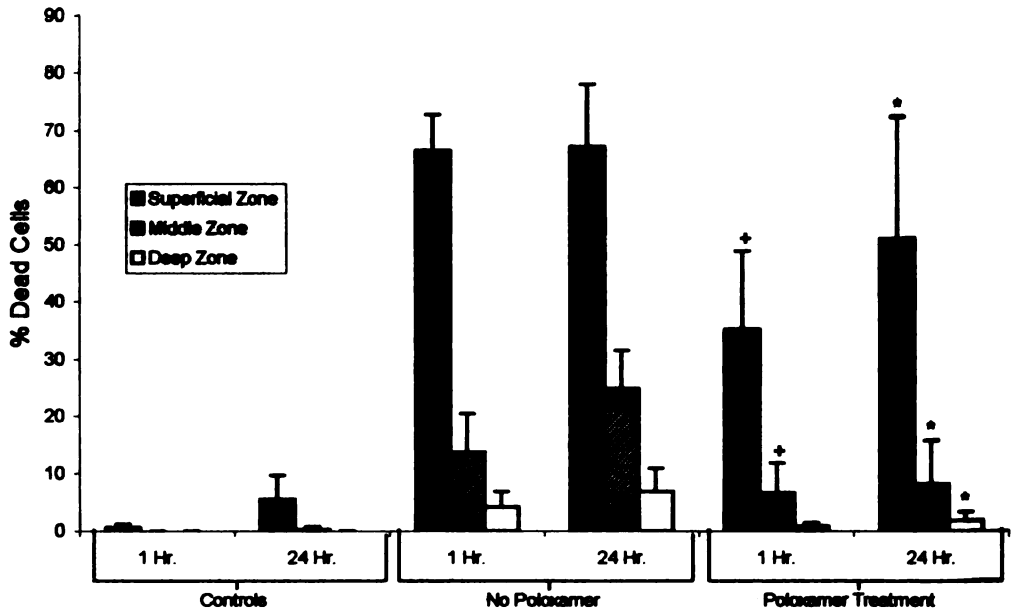

Figure 7. Histogram showing percentage cell death in each zone of cartilage explants following blunt loading. The explants were divided into three zones: superficial (top 20%, black bars), middle (50%, striped bars), deep (bottom 30%, white bars). At 1 hour post-impact, P188 treatment significantly (+) reduced cell death in the superficial and middle zones of the cartilage versus the untreated groups. At 24 hours post-impact, P188 treatment significantly (*) reduced cell death in all zones of the explant versus the untreated groups.

The apparent rate of increase in the percentage of cell death with time, however, tended to be less in the treatment group than in the no treatment group (Figure 5). Between 1 and 24 hours in the P188-treated group there was a significant (p=0.008) increase in percentage of cell death in the superficial layer, but in contrast to explants without treatment no significant increases in the percentage of cell death were noted between 1 and 24 hours in the middle (p=0.259) and the deep (p=0.725) zones of the treated explants.

Discussion

The potential for "saving" traumatized chondrocytes was explored in this study using the mild, nonionic surfactant P188. Bovine cartilage explants were loaded with 25 MPa of stress in unconfined compression, and assayed for cell viability at 1 or 24 hours. The hypothesis was that chondrocytes damaged during blunt mechanical loading would be "saved" from necrosis by early administration of P188.

Studies, by others, have documented the membrane restoring qualities of P188, as this surfactant has been shown to be selectively absorbed into damaged regions of a cell membrane. The "patch" then arrests the leakage of intracellular materials and helps maintain ionic concentrations across this semi-permeable layer (Maskarinec et al, 2002). In studies of chemical damage to cells with N-methyl-D-aspartate, P188 treatment normalizes membrane receptor and intracellular functions with fast (within 20s) action (Marks et al, 2001). In the current study the action of early P188 treatment of mechanically traumatized chondrocytes by 25 MPa of unconfined compressive pressure was explored for the first time. Studies by others have shown that P188 treatment has no effect on the viability of apoptotic cells (Marks et al, 2001). While the applied pressure in the viability study (25 MPa) was less than that used in the apoptosis study (30 MPa, Chapter 1), cell necrosis was evident, even with the addition of P188 treatment, which had a significant effect on the percentage of cell death at 1 hour post trauma.

The current study also showed there was a "window of opportunity" to save cells from death, as the percentage of cell death rose from approximately 17 % to 33 % between 1 and 24 hrs without P188 treatment. The increase in the percentage of cell death rose from approximately 12 % to 25 % in the middle zone of the tissue from 1 to 24

hours. While P188 treatment yielded a reduction in cell death of approximately 20% in the superficial zone at 1 and 24 hours, the reductions in cell death in the middle zone were approximately 5% at 1 hour and 21% at 24 hours. The dramatic difference in the ability of P188 to restore membrane integrity in the middle zone at 24 hours versus 1 hour may relate to the time required for the surfactant to penetrate the cartilage matrix. In pilot studies we found the need to cyclically load the cartilage to allow P188 to have a significant effect on the percentage of cell death. The cyclic loading, it was believed, squeezed water out of the cartilage; when the cartilage relaxed, fresh water along with P188 was sucked into the matrix and in contact with the cells. A limitation of the current study was that this protocol was not further investigated.

Additionally, only one level of applied loading was investigated in the current study. While approximately 25 MPa of pressure has been previously recorded on retro-patellar cartilage during blunt impact loading of the human knee (Haut et al, 1989), this level of pressure applied to a chondral explant likely generated significantly larger cell strains than for an intact joint. With the degree of cellular strain reduced for an intact joint, P188 treatment may be more effective.

A study by another laboratory showed that a pan-caspase inhibitor, z-VAD.fmk [benzyloxycarbonyl-val-ala-asp (Ome) flouromethylketone] could help prevent chondrocyte apoptosis in human articular cartilage explants compressed to 30% (D'Lima et al, 2001). While a reduction of apoptotic cell death within 48 hours may have a significant effect on the long-term survival of articular cartilage, a higher percentage of cell death by necrosis, as evidenced by the current study, increases the potential role of P188 intervention clinically.

References

1. Barbee K, Ford C, Blackman B, Thibault L. Neural cell injury: Characterization and treatment strategy. Yang, K. 2. (1992). 2nd Injury Prevention through Biomechanics Symposium Proceedings: CDC.

2. Clarke MS, McNeil PL. (1992) Syringe loading introduces macromolecules into living mammalian cell cytosol. *J Cell Sci.* 102:533-41.

3. D'Lima DD, Hashimoto S, Chen PC, Colwell CW, Lotz MK. (2001) Impact of mechanical trauma on matrix and cells. *Clinical Orth Related Res.* 391S:S90-S99.

4. Duke RC, Ojcius DM, Young JD. (1996) Cell suicide in health and disease. *Sci Am.* 275(6):80-7.

5. Ewers B, Krueger J, Dvoracek-Driksna D, Orth M, Haut R. (2001) Chondrocyte viability decreases over 24 hours post-impact in a mechanically traumatized cartilage explant. *47th Annual Meeting Orthopaedics Research Society.*

6. Ewers, BJ, Weaver, BT, Sevensma, ET, Haut, RC. (2002) Chronic changes in rabbit retro-patellar cartilage and subchondral bone after blunt impact loading of the patellofemoral joint. *J. Orth Res.* 20(3):545-550.

7. Haut RC. (1989) Contact pressures in the patellofemoral joint during impact loading on the human flexed knee. *J Orthop Res.* 7(2):272-80.

8. Hellung-Larsen P, Assaad F, Pankratova S, Saietz BL, Skovgaard LT. (2000) Effects of Pluronic F-68 on Tetrahymena cells: protection against chemical and physical stress and prolongation of survival under toxic conditions. *J Biotechnol.* 76(2-3):185-95.

9. Lee RC, River LP, Pan F, Ji L, Wollmann RL. (1992) Surfactant-induced sealing of electropermeabilized skeletal muscle membranes *in vivo*. *Proc Natl Acad Sci.* 89:4524-4528.

10. Lee RC, Hannig J, Matthews KL, Myerov A, Chen C. (1999) Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. *Annals New York Acad Sciences.* 888:266-273.

11. Luchtman-Jones L, Files B, Ballas SK. (1999) Phase I study of FLOCOR in patients with acute chest syndrome of sickle cell disease. *1999 Meeting American Society Hematology.*

12. Marks JD, Pan C, Bushell T, Cromie W, Lee RC. (2001) Amphiphilic, tri-block copolymers provide potent membrane-targeted neuroprotection. *FASEB J.* 15:1107-1109.

13. Maskarinec SA, Hannig J, Lee RC, Lee KC. (2002) Direct Observation of Poloxamer 188 insertion into lipid monolayers. *Biophysical J.* 82:1453-1459.

14. Maskarinec SA, Lee, KC. (2003) Comparative study of poloxamer insertion into lipid monolayers. *Langmuir*. 19:1809-1815.

15. Papoutsakis ET. (1991) Media additives for protecting freely suspended animal cells against agitation and aeration damage. *TIBTECH.* 9:316-324.

16. Simon WH, Richardson S, Herman W, Parsons JR, Lane J. (1976) Long-term effects of chondrocyte death on rabbit articular cartilage in vivo. *J Bone Joint Surgery* 58(4):517-526.

Chapter Three

The role of Poloxamer 188 in Saving Chondrocytes from Necrotic Cell Death Following Various Mechanical Loads

Introduction

Osteoarthritis (OA), a disease of the articular cartilage, has been shown to be induced by impact loading diarthroidal joints. It is thought that one factor that may cause OA is a loss of cellularity due to chondrocyte death (D'Lima et al., 2001). Chondrocytes mediate the synthesis, secretion and degradation of matrix proteoglycans, glycoproteins, and collagen, and thus are integral to tissue homeostasis (Quinn et al., 1998).

Tensile stresses developed in the membranes of cells during compressive loading of cartilage can disrupt this semi-permeable membrane to cause an imbalance in the ionic concentrations of the cell leading to cellular necrosis (Ewers et al., 2000). Compressive stresses as low as 14 MPa have been shown to kill cartilage cells, however the level of applied stress necessary to damage cell membranes is at present unknown (Morel et al., 2002). In a previous study a 35 MPa impact stress significantly decreased proteoglycan biosynthesis and increased water content in cartilage explants (Borrelli et al., 1997); the study suggests that the decreased biosynthesis could be due to either decreased chondrocyte metabolism or chondrocyte death, or both. A similar study showed a stress dependent inhibition in chondrocyte metabolic activity, an increase in water content, and a decrease in viable cell population when cartilage is impacted with a single load producing stresses of approximately 15 to 20 MPa (Torzilli et al., 1999). This same study observed that there was a correlation of the density of cell death with stress intensity.

Cell death appeared to be initiated in the explant at approximately 15 MPa with complete cell death throughout the explant's thickness at $\geq$ 20 MPa (Torzilli et al., 1999).

A non-ionic surfactant, Poloxamer 188 (P188), has been shown to repair cell membranes (Phillips et al., 2004). At a stress of 25 MPa P188 can save nearly 50 % of the chondrocytes from necrotic death. But at lower stress levels (less than 15 MPa), the degree to which P188 is effective is unknown. The mechanism by which P188 functions in saving cells is likely by patching holes in the cell membrane caused by tensile stresses. P188 may be less effective at repairing cell membranes with greater degrees of damage.

At 25 MPa cell death occurs along the superficial tangential zone and adjacent to fissures in the cartilage, which stretch into the middle zone. Shear stresses are generally higher at the tips of propagating fissures; therefore cell death is higher around these cracks (Ewers et al., 2000). At lower stress levels, chondrocytes in the deeper zones may not be affected by damaging shear stresses. Due to lower stresses developed in the matrix, fissures are generally smaller, and less common, than at higher stresses; therefore the shear stresses that occur near these fissures are smaller. Cell death is observed mostly in the superficial tangential zone of the cartilage at lower stresses and is only observed at the higher levels of the middle zone of the cartilage.

We hypothesize that at lower stress levels P188 will be more effective in lowering the amount of cell death than at higher stresses due to the lower tensile stresses developing in the matrix. At lower stress levels, total cell death will be lower in the cartilage, as well as the degree to which cell membranes are damaged will be lower, therefore P188 will be more effective.

Methods:

Two bovine forelegs from a mature animal were obtained from a local abattoir within six hours of slaughter. The legs were skinned and rinsed with water prior to exposing the metacarpal joint in a laminar flow hood. A biopsy punch (Miltex Instrument Company, Bethpage, NY) was used to make forty-four 6mm diameter chondral explants from the lower metacarpal surface of the limbs. The explants were separated from the underlying bone using a scalpel. The specimens were washed three times in Dulbecco's Modified Eagle Medium: F12 (DMEM, Invitrogen Co., Grand Island, NY, #12500-062). The explants were then split into two treatment groups, poloxamer 188 (P188) pre-treatment (n=18) and no P188 treatment (n=18), and the remaining explants were assigned to a non-impact control group (n=8). The explants were equilibrated for 48 hours in DMEM:F12 (2 explants per well, approximately 1 mg of cartilage per 1 ml of media) supplemented with 10% fetal bovine serum (Invitrogen Co), 21.9 mg/ml glutamine, additional amino acids and antibiotics in a humidity controlled incubator (37° C, 5% CO_2, 95% humidity). Eight mg/ml P188 was added to the media of the 18 explants in the P188-treated group. The explants were manually submitted to 10 cycles of pressure (~1 MPa) to encourage P188 uptake into the cartilage matrix.

The explants of the two treatment groups were then randomly assigned to one of nine impact groups (n=2). Each impact group was loaded to one of nine different loads at a low rate of loading (1s to peak). The nine load levels were 254.5N (9 MPa), 311.0N

(11 MPa), 367.6N (13 MPa), 424.1N (15 MPa), 480.7N (17 MPa), 537.2N (19 MPa), 593.8N (21 MPa), 650.3N (23 MPa), and 706.9N (25 MPa) (Figure 1).

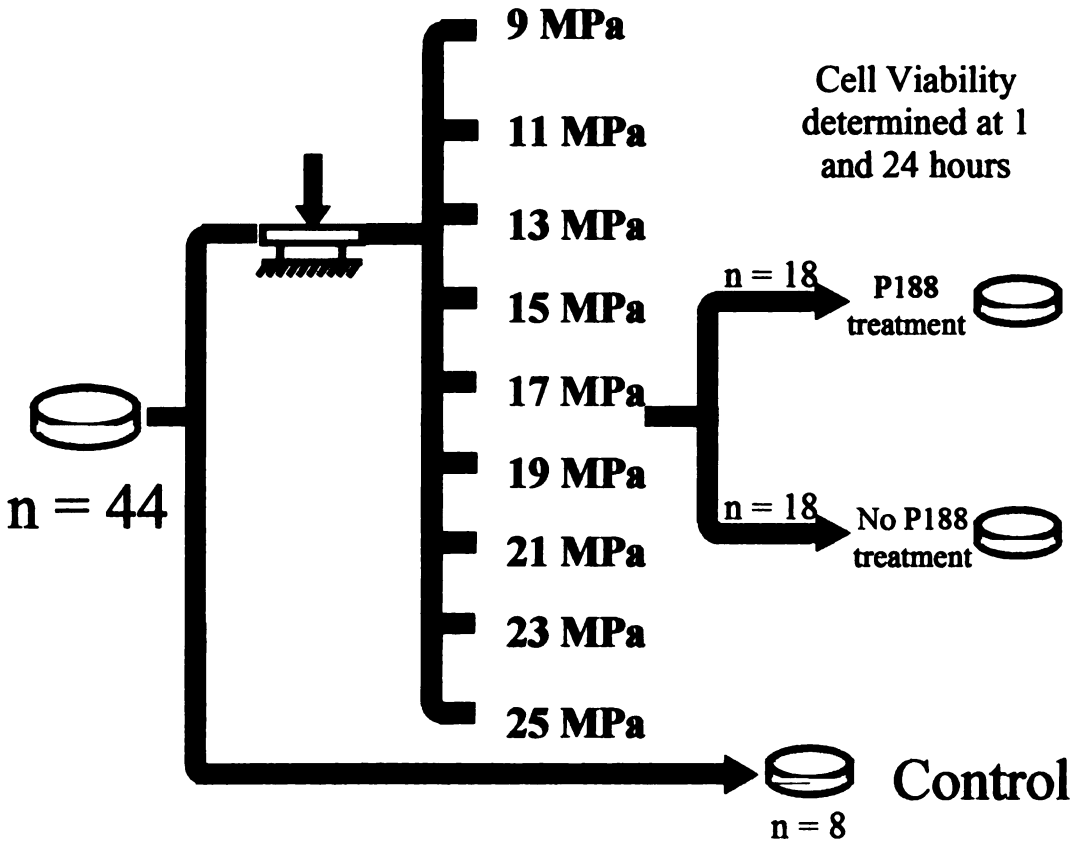

Figure 1. Diagram showing the experimental design. 36 explants were impacted at one of nine impact levels. Two explants from each impact group were incubated in P188, and two were untreated. Cell viability was determined at 1 and 24 hours.

The explants were loaded in unconfined compression between two highly polished stainless steel plates using a haversine loading protocol with a servo-controlled hydraulic testing machine (Instron, model 1331, Canton, MA). The following biomechanical parameters, peak load, time to peak, and maximum explant compression were documented in each experiment.

Nine explants from each treatment group were used to determine cell viability at either 1 or 24 hours post impact. The 24 hour explants were washed three times in DMEM:F12 after loading and placed into DMEM:F12 media supplemented with 8μg/ml P188 and incubated overnight. Following incubation the explants were cut through the thickness into thin sections using a specialized cutting tool with parallel blades spaced 0.5 mm apart. The slices were stained with Calcein AM and Ethidium Homodimer, according to the manufacturer's specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR). Approximately 2 slices from each explant were viewed across approximately 1 mm near the center in a fluorescence microscope (Leitz Dialux 20 (frequency: 50-60 Hz), Leitz Mikroskopie und Systeme GmgH, Wetlzar, Germany) and photographed using a digital camera (Polaroid DMC2, Polaroid Corporation, Waltham, MA, USA). A blinded observer, using image analysis software (Sigma Scan, SPSS INC., Chicago, IL), quantified cell viability of the explant.

A two-factor ANOVA was performed on the biomechanical parameters. A two-factor ANOVA for total cell death in each explant was also performed. The two factors were time (1 and 24 hours) and treatment (P188 and no P188). An SNK post-hoc test was used to determine differences between groups.

Results

The experiment evaluated the cell death observed in bovine cartilage explants following severe blunt impact at varying stress levels. Cartilage explants were subjected to stresses ranging from 9 MPa to 25 MPa in increments of 2 MPa for a total of nine stress levels and incubated for either 1 or 24 hours post impact. Half of the explants were treated with the surfactant poloxamer 188 (P188) immediately following impact. Explants were then stained for cell viability using Calcein AM and Ethidium homodimer.

There was a trend towards increasing cell death found in the explants at 1 hour with an increase in the stress applied. The slope of the linear regression line was 0.971 with an R^2 value of 0.343 (P=0.163).

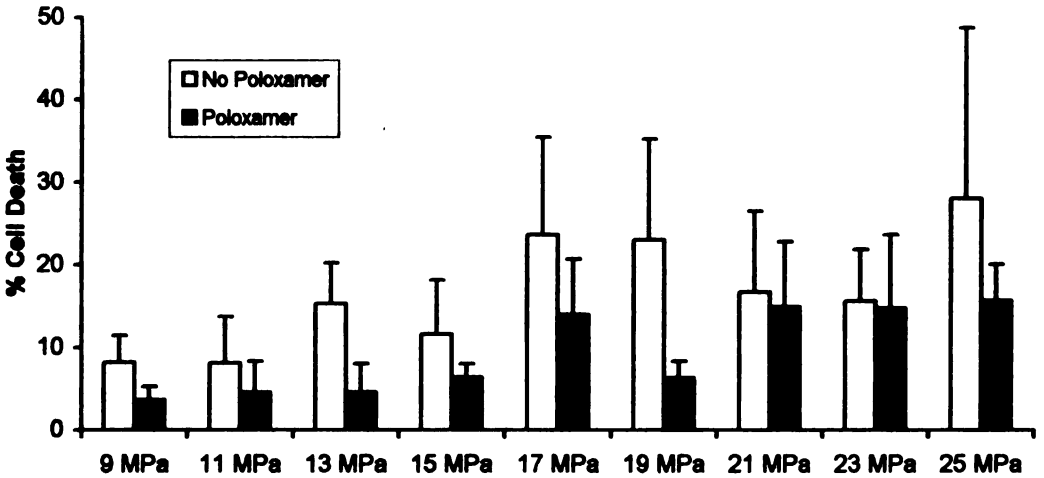

Figure 2. Histogram showing the percentage of cell death observed in explant impacts with various levels of stress at 1 hour post impact. The white bars represent data from explants that were untreated, while the black bars represent data from explants that were treated with 8 mg/ml poloxamer 188.

A stress of 9 MPa resulted in approximately 8% cell death at 1 hour post-impact, whereas a stress of 25 MPa resulted in approximately 28% cell death at 1 hour post-impact (Figure 2). At 24 hours post-impact this trend increased with the slope of the regression

line of 1.148 and an R^2 value of 0.827 ($P<0.05$). A stress of 9 MPa resulted in approximately 7.3% cell death at 24 hours post-impact, and a stress of 25 MPa resulted in approximately 25% cell death (Figure 3).

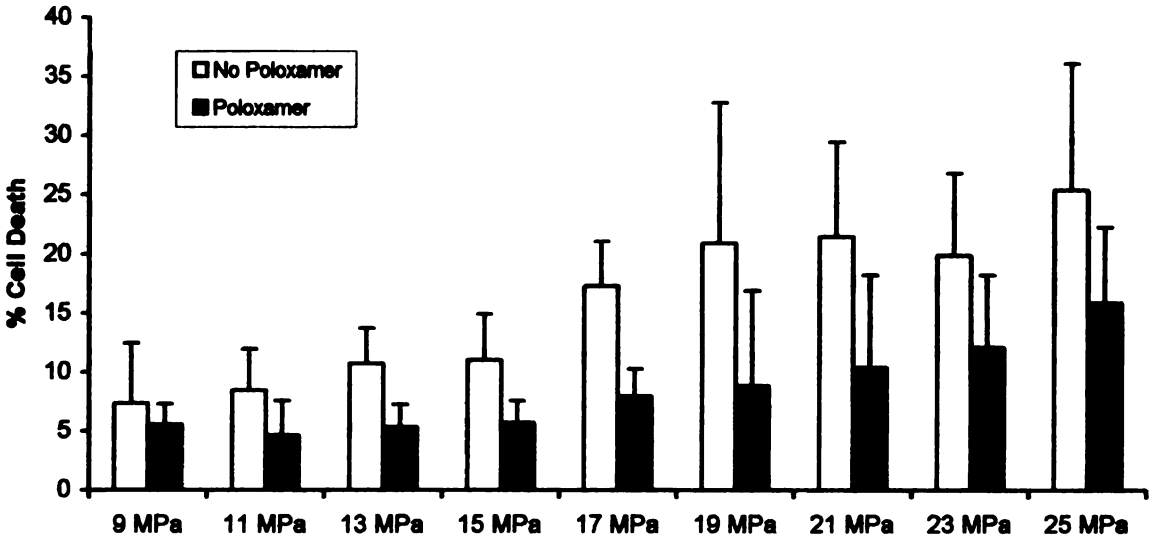

Figure 3. Histogram showing the percentage of cell death observed in explants impact with various levels of stress at 24 hour post impact. The white bars represent data from explants that were untreated, while the black bars represent data from explants that were treated with 8 mg/ml poloxamer 188.

With the addition of P188 the slope of the regression line was reduced to 0.826 (R^2=.750, $P<0.05$) at 1 hour post-impact, though it was not significantly different then from the no P188 group. At a stress of 9 MPa, cell death was reduced to 3.6%, a reduction of greater than 50% versus the no P188 group, while at 25 MPa cell death was reduced to 15.6%, nearly a 50% reduction (Figure 2). At 24 hours, the slope of the regression line was greatly reduced to 0.638, a significant difference from the no P188 group (R^2=.609, $P<0.05$). At a stress of 9 MPa, cell death was reduced to 5.6% versus the no P188 group, and at 25 MPa cell death was reduced to 15.8% versus the no P188 group (Figure 3).

Discussion

The objective of the study was to determine the effectiveness of P188 following various levels of mechanical stresses. Cartilage explants were loaded to one of 9 stress levels ranging from 9 MPa to 25 MPa and cell viability was assessed at either 1 or 24 hours. Half of the explants from each stress level were administered P188 immediately following impact.

The hypothesis of the study was that an increase in the stress applied to chondral explants would correspond to an increase in cell death. The amount of cell death correlates with the degree of mechanical disruption of the cartilage surface (Lewis et al,), and a higher applied load would yield more surface disruption. Also, P188 administration is hypothesized to have an effect in decreasing the percentage of chondrocytes killed following various levels of applied stress. Previously, we demonstrated that the surfactant had the ability to save chondrocytes from necrotic cell death following a mechanical load of 25 MPa (Phillips et al, 2004). It is also theorized that at the lower stresses, P188 would have a greater result in preserving cell viability due to the lower shear forces that occur under these mechanical stimuli.

The percentage of cell death increased as the applied stress increased at both 1 and 24 hours in the untreated groups (Figure 2). This finding agrees with the original hypothesis. With the addition of P188, the trend of increasing cell death with increasing stress continued, but the relationship between cell death and stress lessened. At 1 hour, P188 treatment was reduced at every stress level versus the untreated groups with the larger effect observed at the lower stresses (9 MPa – 17 MPa). The effectiveness of P188 at 1 hour may possibly be due to chondrocytes that are less severely injured being

rescued by the surfactant. At higher stress levels, these cells may be fewer in number compared to more severely injured cells.

At 24 hour, P188 treatment reduced cell death at every stress level as well, but it appeared to have a greater effect at the higher stress levels (17 MPa – 25 MPa) as opposed to the groups at 1 hour. This may be due to the time it takes for P188 to repair the more severely damaged cells, of which there is a higher number than at the lower stress levels.

There appeared to be a threshold stress level above 17 MPa in which cell death was greatly increased above the lower stress levels at both 1 and 24 hours. This threshold level of stress also appeared in the poloxamer treated groups as well. This threshold could represent an increased level of shear stresses that develop within the matrix, above the levels of shear stresses present at lower stresses. This increase in shear stress may be responsible for the death of a certain population of cells not injured at lower stresses, but susceptible to a higher level of shear stresses. It is this population of cells in which P188 is most effective as demonstrated by the larger reduction in cell death with treatment observed above 17 MPa.

References

1. Borrelli J, Torzilli PA, Grigiene R, Helfet DL. (1997) Effect of impact load on articular cartilage: development of an intra-articular fracture model. *J Orth Trauma.* 11(5):319-326.

2. Clements KM, Bee ZC, Crossingham GV, Adams MA, Sharif M. (2001) How severe must repetitive loading be to kill chondrocytes in articular cartilage? *Osteo and Cart.* 9:499-507.

3. D'Lima DD, Hashimoto S, Chen PC, Colwell CW, Lotz MK. (2001) Impact of mechanical trauma on matrix and cells. *Clinical Orth Related Res.* 391S:S90-S99.

4. Ewers B, Dvoracek-Driksna D, Orth M, Altiero N, Haut R. (2000) Matrix damage and chondrocyte death in articular cartilage depends upon loading rate. *46th Annual Meeting Orthopaedic Research Society.*

5. Lee RC, River LP, Pan F, Ji L, Wollmann RL. (1992) Surfactant-induced sealing of electropermeabilized skeletal muscle membranes *in vivo*. *Proc Natl Acad Sci.* 89:4524-4528.

6. Lee RC, Hannig J, Matthews KL, Myerov A, Chen C. (1999) Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. *Annals New York Acad Sciences.* 888:266-273.

7. Maskarinec SA, Hannig J, Lee RC, Lee KC. (2002) Direct Observation of Poloxamer 188 insertion into lipid monolayers. *Biophysical J.* 82:1453-1459.

8. Maskarinec SA, Lee, KC. (2003) Comparative study of poloxamer insertion into lipid monolayers. *Langmuir.* 19:1809-1815.

9. Morel V, Quinn TM. (2002) Micromechanical factors in cartilage injury under ramp compression of explants for deformations applied near the "gel diffusion" rate. *48th Annual Meeting Orthopaedic Research Society.*

10. Phillips DM, Haut RC. (2004) The use of a nonionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants *J Orth Res.* (In Print).

11. Quinn TM, Grodzinsky AJ, Hunzinker EB, Sandy JD. (1998) Effects of injurious compression on matrix turnover anround individual cells in calf articular cartilage explants. *J Orth Res.* 16:490-499.

12. Togo T, Alderton JM, Steinhardt RA. (2000) The mechanism of cell membrane repair. *Zygote.* 8(Supplement):S31-S32.

13. Torzilli PA, Grigiene R, Borrelli J, Helfet DL. (1999) Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content. *J Biom Eng.* 121:433-441.

Chapter Four

The Effect of Pre-treatment Versus Post-treatment of Poloxamer 188 in Treating Chondrocytes Damaged by Mechanical Loads

Introduction

Traumatic injury is one of the most common causes of secondary osteoarthritis. Osteoarthritis (OA) is classified by the degeneration of articular cartilage in diarthroidal joints, resulting in bone on bone contact that causes pain and loss of mobility. Blunt trauma has been shown to cause chondrocyte cell death, which may lead to OA. Tensile stresses caused by impact loading damage cell membranes, which disrupt ion gradients across the cell membrane and cause the cell to absorb water (Torzilli et al., 1999). Necrosis, a mode of cell death, occurs as the cell swells with water and then bursts. The prevention of a post-traumatic OA may start with the inhibition of cell death by repairing damaged cell membranes.

Administration of poloxamer 188 (P188) can save chondrocytes damaged by severe blunt trauma from death by necrosis (Phillips et al., 2004). P188 is a triblock copolymer of the form poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) with a molecular weight of 8400 Daltons; it is a highly surface active copolymer, which probably aids in its absorption and facilitates its insertion into lipid monolayers (Maskarinec et al., 2002, 2003). The surfactant is similar in structure to the lipid bi-layer of the cell membrane, and is incorporated into the damaged portion when trauma occurs, thereby sealing the tear in the membrane. As recent injection experiments have shown, when surface pressure of an artificial lipid monolayer was decreased below a threshold level, therefore representing damage, P188 was easily inserted into the monolayer. On

the other hand, at high surface pressures, P188 did not insert itself into the artificial monolayer, therefore it was concluded that P188 could only insert itself into the damaged portions of membranes (Maskarinec et al., 2002, 2003). At membrane surface pressures of 25 mN/m or higher, the P188 is squeezed out of the membrane (Maskarinec et al., 2002). Some studies suggest that P188 also has the ability to strengthen the matrix of the cartilage whereby lowering the shear stresses resulting from blunt trauma.

Chapter 2 investigated the ability of P188 to save cells when added post-impact. The percentage of cell death was greatly reduced in the explants treated with P188. The current study investigates the role of P188 applied to the cartilage pre-impact versus post-impact. The application of P188 as a supplement used prior to trauma could be an important step in preventing the initial stages of OA. It is unknown how long P188 may last in the cartilage matrix when used as a supplement and whether it is degraded over time. However, recent investigations on the use of poloxamer in protecting model drug carriers from phogocytosis indicate that surfactant micelles themselves possibly have a longer *in vivo* half-life time and thereby can act as a slow release vehicle (Lee et al., 1999). The effects of pre-treating cartilage with P188 are important in order to use P188 as a supplement instead of an immediate treatment following blunt trauma. The hypothesis of the current study was that pre-treatment of chondral explants with P188 surfactant would reduce the degree of necrosis that develops in the initial 24 hours following a severe impact trauma to joint cartilage.

Methods:

Two bovine forelegs from a mature animal were obtained from a local abattoir within six hours of slaughter. The legs were skinned and rinsed with water prior to exposing the metacarpal joint in a laminar flow hood. A biopsy punch (Miltex Instrument Company, Bethpage, NY) was used to make seventy-six 6mm diameter chondral explants from the lower metacarpal surface of the limbs. The explants were separated from the underlying bone using a scalpel. The specimens were washed three times in Dulbecco's Modified Eagle Medium: F12 (DMEM, Invitrogen Co., Grand Island, NY, #12500-062). The explants were then split into two treatment groups (3 groups total). All treatment groups and controls were equilibrated for 48 hours in DMEM:F12 (2 explants per well, approximately 1 mg of cartilage per 1 ml of media) supplemented with 10% fetal bovine serum (Invitrogen Co), 21.9 mg/ml glutamine, additional amino acids and antibiotics in a humidity controlled incubator (37° C, 5% CO_2, 95% humidity). The equilibration media of the first group was supplemented with 8 mg/ml poloxamer 188 (P188). The explants were submitted to 10 manual cycles of loading (~ 1MPa) prior to impacting to encourage P188 uptake into the matrix (n=20). The media of the second group was also supplemented with P188 during equilibration and following impact. The explants of the second treatment group were submitted to 10 manual cycles of loading prior to impacting and 10 cycles of manual loading following impacting (n=20). The final treatment group received no P188 treatment (n=20). The remaining sixteen explants were assigned to a non-impact control group that also underwent 10 cycles of manual loading.

All explants from the treatment groups were loaded to 706 N (~25 MPa), at a low rate of loading (1s to peak) (Figure 1).

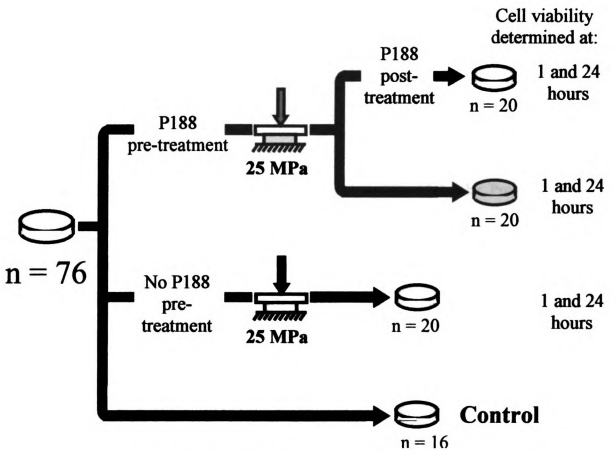

Figure 1. Diagram showing the experimental design. 40 chondral explants were pre-treated with P188, and were then loaded to 25 MPa. 20 explants were untreated and loaded to 25 MPa. 20 explants from the pretreated group were post-treated with P188. Cell viability was determined at 1 and 24 hours.

The explants were loaded in unconfined compression between two highly polished stainless steel plates using a haversine loading protocol with a servo-controlled hydraulic testing machine (Instron, model 1331, Canton, MA). Peak load, time to peak and maximum explant compression were documented in each experiment. The explants were then incubated for 1 or 24 hours following impact, in fresh DMEM:F12 supplemented medium according to treatment group.

Six explants from each treatment group were used to determine cell viability at either 1 or 24 hours post impact. The 24 hour explants were washed three times in DMEM:F12 after loading and placed into DMEM:F12 supplemented media and incubated for 24 hours. The media of the second treatment group was supplemented with 8 mg/ml P188. Following incubation the explants were cut through the thickness into thin sections using a specialized cutting tool with parallel blades spaced 0.5 mm. The slices were stained with Calcein AM and Ethidium Homodimer, according to the manufacturer's specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR). Approximately 2 slices from each explant were viewed across approximately 1 mm near the center in a fluorescence microscope (Leitz Dialux 20 (frequency: 50-60 Hz), Leitz Mikroskopie und Systeme GmgH, Wetlzar, Germany) and photographed using a digital camera (Spot Digital Camera, Diagnostic Instruments Inc). A blinded observer, using image analysis software (Polaroid DMC2, Polaroid Corporation, Waltham, MA, USA), quantified cell viability of the explant. The data from the two explant slices from a single explant were averaged prior to statistical analysis.

The data in this chapter represents three experiments, the experiment described was repeated to increase the power of the study, and a pilot study was performed prior to investigate P188 pre-treatment. The second experiment was performed similar to the first with a total of 28 explants and group sizes of n=4. The third experiment was a pilot experiment and only consisted of two treatment groups, P188 pre-treatment (n=14) and no P188 treatment (n=14). Both groups were treated according to the treatment groups described above.

The maximum applied load, time to peak load and maximum explant compression were determined from each experiment. A two-factor ANOVA was performed on these biomechanical parameters. The percentage of dead cells in each explant was quantified using the image software. A two-factor ANOVA for total cell death in each explant was performed. The two factors were time (1 and 24 hours) and treatment (P188 pretreatment and P188 pre and post treatment and no P188). An SNK post-hoc test was used to determine differences between groups.

Results

The study was designed to compare untreated explants with explants pre-treated with 8 mg/ml P188 prior to loading and explants pre-treated with P188 as well as incubated in P188 post impact. An analysis of the biomechanical properties in the 6 impacted groups (1 and 24 hours, no poloxamer, P188 pre-treatment, and P188 pre- and post-treatment) showed no significant differences. The average maximum applied load and maximum explant compression were 713.84± 7.03 N and 0.304± 0.04 mm, respectively (n = 60).

The percentage of dead cells in the control explants was 5.87± 2.19% at 1 hour (n=8), and 9.71± 4.29% at 24 hours (n=8) (Figure 2).

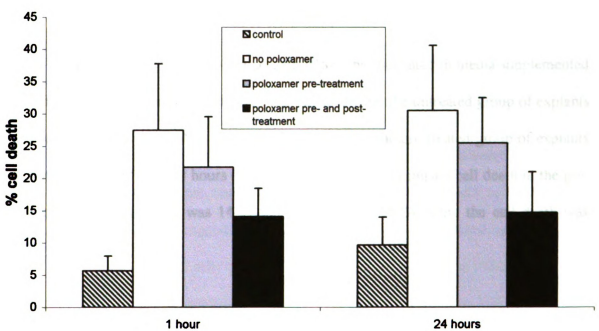

Figure 2. Histogram showing the percentage of cell death documented at 1 and 24 hours post-trauma in each of the treatment groups: control (striped bar), no poloxamer (white bar), poloxamer pre-treatment (gray bar), and poloxamer pre-and post-treatment (black bar).

As previously noted, cell death was increased around fissures which occurred on the superficial surface of the explants (Phillips, 2004). On several explants a characteristic 'butterfly' pattern of cell death occurred around the fissures. There was a trend of increasing cell death in the explants from 1 to 24 hours following impact, though it was not statistically significant ($p=0.216$). Following a 25 MPa impact, cell death in untreated explants was 27.5$\pm$ 10.3% at 1 hour post impact ($n=10$), and 30.5$\pm$ 10.1% at 24 hours ($n=10$).

With the addition of 8 mg/ml P188 to the media of the explants during the 48 equilibrium period, cell death was significantly reduced from the untreated groups at both 1 ($p=0.020$) and 24 ($p=0.035$) hours post impact. At 1 hour cell death in the pre-treated group of explants was 21.75$\pm$ 7.84% ($n=10$), and at 24 hours cell death was 25.5$\pm$ 7.06% ($n=10$).

When explants were pre-treated with P188, and incubated in media supplemented with P188 following impact, cell death was reduced from the untreated group of explants at both 1 ($p<0.001$) and 24 hours ($p<0.001$), as well as the pre-treated group of explants at both 1 ($p=0.002$) and 24 hours ($p<0.001$). At 1 hour post impact cell death in the pre- and post-treatment group was 14.1$\pm$ 4.4% ($n=10$) and at 24 hours the cell death was 14.7$\pm$ 6.4% ($n=10$).

Discussion

The study documented the use of a surfactant, poloxamer 188, in the intervention of cell death following compressive blunt trauma. Bovine cartilage explants were impacted with 25 MPa of impact stress, and cell viability was assessed at either 1 or 24 hours. In one group of explants, the surfactant was added prior to impact to investigate the role of pretreatment. In a second group the surfactant was added both prior to impact as well as following the impact.

Cell death increased in untreated explants from 1 to 24 hours, though not statistically ($p=0.22$), which supports the data reported in Chapter 2. In Chapter 1, it was reported that following blunt trauma, apoptosis was not observed until 48 hours; this point along with the current data suggests that the increase in cell death in this study is through the mechanism of necrosis. The observed increase in cell death in the first 24 hours following trauma is a vital event if intervention is desired. It is within the first 24 hours that this study focused its attempt at saving cells.

The spread of necrotic cell death can be by several methods. First, cells damaged during impact, may remain viable at 1 hour, but may lose the ability to maintain membrane integrity over time, resulting in the onset of necrosis. Second, cells in the area of necrotic cells may be damaged by the contents of the cytoplasm spilled into the cellular milieu by damaged cells. Lastly, cells damaged initially by the trauma may release a cell signaling molecule alerting other cells of its damaged, and initializing a necrotic pathway in neighboring cells.

With the addition of P188 treatment prior to impact, cell death was reduced significantly at both 1 and 24 hours post impact. The mechanism by which P188 works

is not yet fully understood, but according to studies by others, P188 repairs defects in the membranes of cells damaged by mechanical stresses (Lee et al, 1992, Maskarinec et al, 2002). In the plasma membrane of living cells, the rigid cytoskeleton greatly increases the surface tension of the membrane (Togo et al, 2000). When membrane integrity is lost, a drop in surface tension occurs. As shown by recent injection experiments, when surface pressure of an artificial lipid monolayer was lowered below a threshold level, as occurs during membrane damage, P188 was easily inserted into the monolayer. Conversely, at high surface pressures, P188 did not insert itself into the artificial monolayer, therefore it was concluded that P188 could only insert itself into the damaged portions of membranes (Maskarinec et al, 2002, 2003). Apoptosis, a cellular event in which the cell 'self-destructs' from within, does not exhibit compromised cell membranes, and therefore would not be affected by P188 treatment. On the other hand, the repair of cell membranes may prevent necrotic cell death if the process occurs early enough in the necrotic event. Recent investigations on the use of poloxamer in protecting model drug carriers from phagocytosis indicate that surfactant micelles themselves possibly have a longer *in vivo* half-life time and thereby can act as a slow release vehicle (Lee et al, 1999).

The objective of the current study was to document the timeframe for p188 treatment most effective in preventing cell death following mechanical trauma. P188 pre-treated explants and P188 pre- and post-treated explants were compared with untreated explants to determine the scenario in which P188 was most effective in preserving cell viability. The present experiment shows that while pre-treatment of P188 is effective in preserving cell viability, pre- and post-treatment of cartilage was more

effective. Pre- and post-treatment with P188 reduced cell death significantly versus the untreated group, and the pre-treatment only group at both 1 and 24 hours.

Comparing these data with data presented in chapter two shows a similar reduction in cell death for explants that were only treated post-impact. In chapter two, data was presented showing the ability of P188 to preserve cell viability when added post-impact only; a reduction in cell death of approximately 50% at both 1 and 24 hours versus an un-treated group showed that following injury, addition of P188 would help to preserve cell viability threatened by mechanical trauma.

A possible conclusion is that at the time of impact, a portion of P188 may be squeezed out of the explants, and would not be available to the cells to repair damage. By post-treating the explants, P188 is reintroduced to the matrix, and therefore is present to preserve cell viability. A second possible conclusion that can be made from these data is that it is more important to have a dynamic influx of p188 available to the cells, than to have the P188 present at the time of injury. There may be a physical mechanism associated with the insertion of P188 into membranes, and this could have been facilitated by the ten cycles of 1 MPa loading that the explants were subjected to following P188 addition.

References

1. Lee RC, Hannig J, Matthews KL, Myerov A, Chen C. (1999) Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. *Annals New York Acad Sciences.* 888:266-273.

2. Maskarinec SA, Hannig J, Lee RC, Lee KC. (2002) Direct Observation of Poloxamer 188 insertion into lipid monolayers. *Biophysical J.* 82:1453-1459.

3. Maskarinec SA, Lee, KC. (2003) Comparative study of poloxamer insertion into lipid monolayers. *Langmuir.* 19:1809-1815.

4. Oyen-Tiesma M, Deloria LB, Meglitsch T, Oegema TR, Lewis JL. (1999) Cell death after cartilage impact depends on matrix damage. *45th Annual Meeting Orthopaedic Research Society.*

5. Phillips DM, Haut RC. (2004) The use of a nonionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants *J Orth Res.* (In Print).

6. Torzilli PA, Grigiene R, Borrelli J, Helfet DL. (1999) Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content. *J Biom Eng.* 121:433-441.

Chapter Five

The Use of a Rabbit Animal Model to Determine the *in vivo* Effectiveness of Poloxamer 188 in Saving Chondrocytes Damaged by Mechanical Trauma

Introduction

Osteoarthritis (OA), a disease of diarthrodial joints, has been shown to be induced by blunt trauma (Gelber et al., 2000, Nagel et al., 1977). OA is characterized by cartilage loss on the ends of weight bearing bones, resulting in pain and loss of function (Flores et al., 1998). It is believed that a loss of cellularity within cartilage is a major factor in the onset of OA (Simon, et al., 1976). Chondrocytes, the resident population of cells in cartilage, are damaged by tensile stresses in membranes that arise from blunt trauma (Ewers et al., 2001). The cell membrane of chondrocytes is compromised and delicate ion balances are interrupted within the cell leading to a necrotic cell death.

Poloxamer 188 (P188), a nonionic synthetic surfactant, has been shown to repair the membranes of cells damaged by physical stresses, electrical trauma and chemical and thermal stresses (Hannig et al., 2000, Lee et al., 1992, Maskarinec et al., 2002, 2003, Chapters 2, 3, 4). The surfactant inserts itself into damaged portions of cell membranes (Chapter 4) and repairs the defect, thereby saving the cell from necrotic cell death (Maskarinec et al., 2002). The effectiveness of P188 in preventing cell death in cartilage explants has been documented (Phillips et al., 2004), but the role that P188 may play in the *in vivo* environment remains unknown. This chapter will try to elucidate the role of P188 *in vivo*.

In the previous chapters, the role of P188 in preventing cell death in bovine cartilage explants was documented. P188 reduced the amount of cell death in the

explants at 1 and 24 hours post impact, though a long-term study would be needed to elucidate the chronic effects of this treatment. In the current study, a live animal model was used to investigate the effectiveness of P188 *in vivo* (Figure 1).

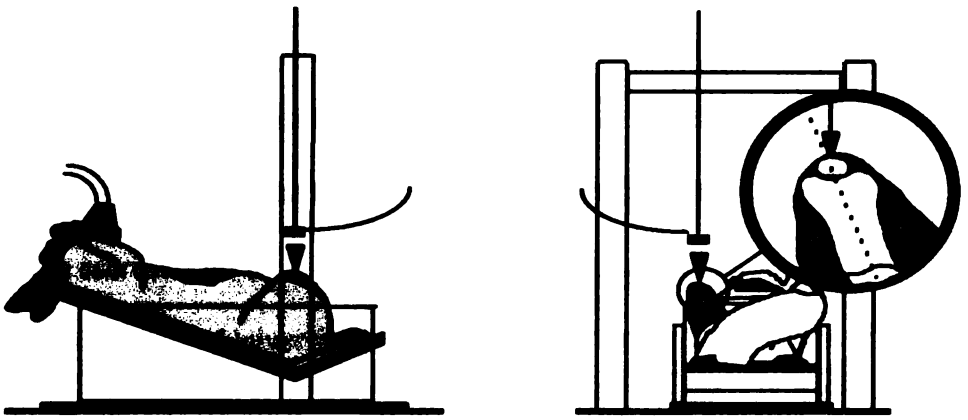

Figure 1. Flemish giant rabbits received a blunt impact to the left patellofemoral joint. Several rabbits received an injection of P188 immediately following impact.

Flemish Giant rabbits were used as part of an animal model that was developed in our lab to study the long-term effects of blunt trauma to the patellofemoral joint (Haut et al., 1995). The effectiveness of P188 in preventing cell death at 96 hours post impact will be investigated.

Methods

Fourteen skeletally mature Flemish Giant rabbits (age range 8-11 months, average weight 5.7± 0.34 kg) were used for this study. Seven rabbits were impacted with 6 Joules (J) of energy to the patello-femoral joint and seven received an impact of 10J of energy. Three rabbits from each impact group received a 1.5ml injection of poloxamer 188 (8mg/ml) to the knee joint immediately following impact. Four animals were sacrificed at time zero and the remaining animals at 96 hours post impact. Michigan State Universities All-University Committee on Animal Use and Care approved the study.

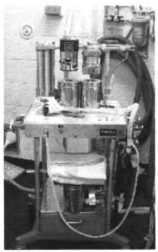

Figure 2. The apparatus used to maintain the animals under general anesthesia

The rabbits were maintained under general anesthesia using a 2% isoflurane and oxygen mixture (Figure 2). The right hind limb was arbitrarily chosen to be impacted over the left limb for every rabbit. The animal's right hind limb was shaved and the animal was placed in a specially designed chair that held the right hind limb rigid and flexed at a 120° angle with the animal laying on its back. Special care was

taken to align the femur vertical, with the patella resting in the femoral groove and the impact head away from the tibia. A canvas strap was fastened across the animals left hind limb to keep the pelvis from rotating during impact.

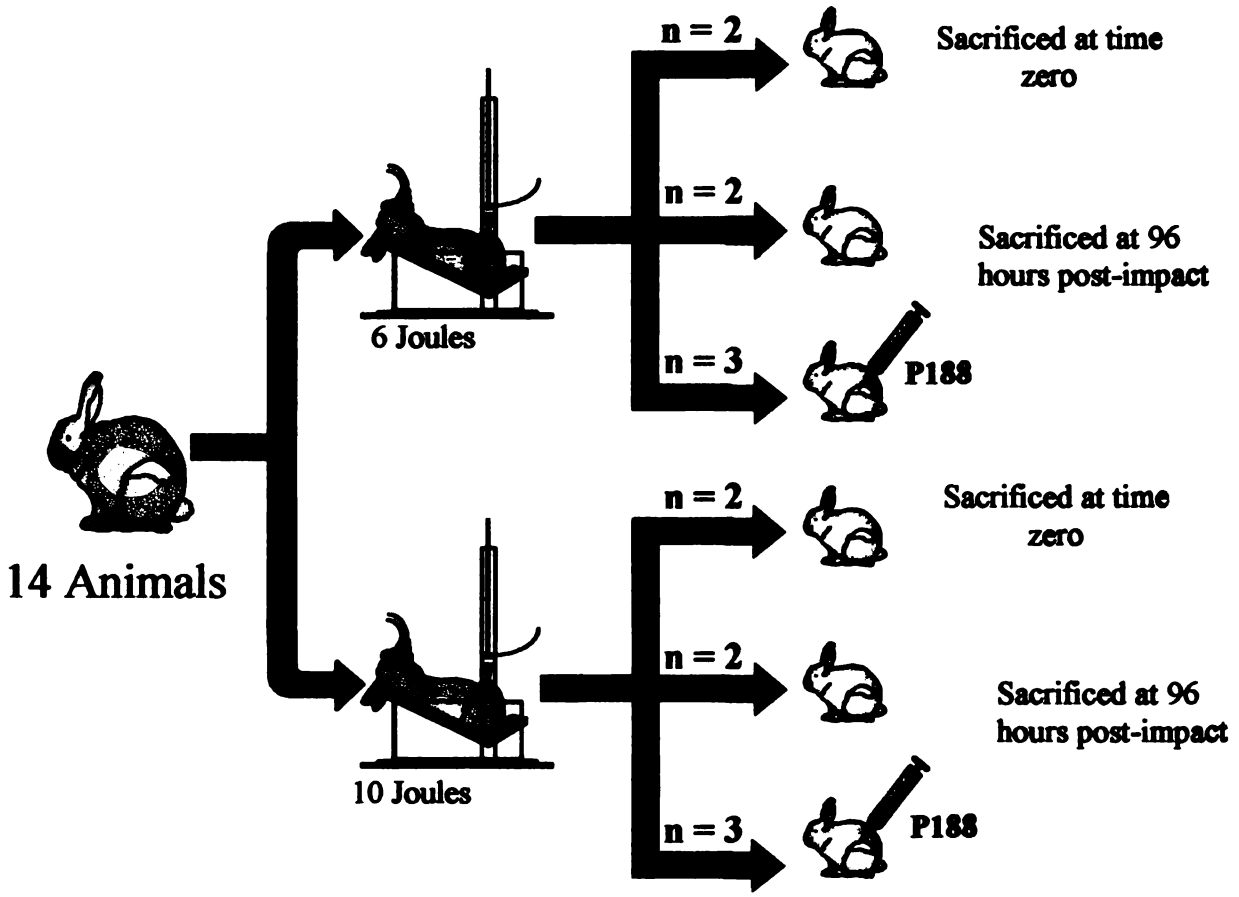

Figure 3. Diagram showing the experimental design. Fourteen animals were used for the study; eight animals each were impacted with either 6 or 10 Joules of impact energy. Three animals from each group were given a 1.5 cc injection of 8mg/ml Poloxamer 188.

The impacts were delivered to the patello-femoral impact of the rabbit via a gravity-driven drop fixture (Figure 5). The experiment was performed by dropping a weight of either 1kg or 1.33kg from a height of 1m or 0.46m to generate a 10J or 6J of impact energy load. The dropped sled was prevented from making multiple impacts electronically, by arresting the sled following the first impact. A load

transducer (model 31/1432: Sensotec, Columbus, OH) with a 2,224 N capacity was in series with the impact head attached to the drop sled to acquire the impact loads generated. Using a PC

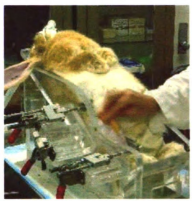

Figure 4. The impact chair (holding an animal) that oriented the femur vertical and allowed a solid impact to the patellofemoral joint of the animal.

equipped with an analog-to-digital board, the loads generated were collected at 10 kHz for 1 sec prior to impact (100 samples), and following a 30 N analog trigger, 400 samples

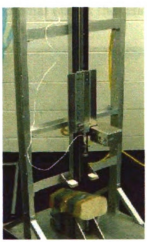

Figure 5. The gravity-driven impact fixture. The impact chair was situated under the fixture with the animal's femur vertical and the patella directly under the impact sled. Note the impact sled stopped at one of the electronic braking fixtures that prevented multiple

of data were saved. The peak load and time to peak were recorded from the load versus time curve.

Following impact, three animals from each impact group were administered a 1.5ml injection of P188 (in phosphate buffered saline, PBS at a concentration of 8μg/ml) to the patello-femoral joint-capsule. The P188 was filter sterilized using a 0.2μm vacuum filter (Nalgene, Nalge Nunc Int., Rochester, New York, USA). The animal's limb was exercised manually to incorporate the P188 into the patellar cartilage. All animals were exercised on a treadmill for 10 minutes a day at 0.3 mph starting the day of impact. Animals were housed in individual cages (122 cm x 61 cm x 49 cm) when not exercising. At 96 hours post-impact (four days), the animals were euthanized with a lethal injection of Pentobarbital.

Following sacrifice, the patellas were excised from the animal using a scalpel. The skin was cut away from the knee with the knee flexed. The patellar tendon was then cut using a scalpel and the proximal end of the tendon was pulled distally using

Figure 6. Picture showing animals right hind limb situated beneath impactor head prior to impact. The animal's femur was positioned vertically, and the impactor head was aligned to impact the patella.

forceps. The soft tissue on both sides of the patella was removed and the patella was removed by cutting the quadriceps tendon. The patellas were wrapped in PBS soaked gauze to maintain hydration. The patellas were then stained with India ink to highlight surface fissures and were photographed using a digital camera (Polaroid DMC2, Polaroid Corporation, Waltham, MA, USA) under a dissecting microscope (Wild TYP 374590,

Heerbrugg, Switzerland). The soft tissue was removed from the back of the patella, and the patella was glued to a custom cylindrical base using cyanoacrylate glue. Approximately half of the bone was removed from the back of the patellas using an Isomet low speed saw (model# 11-1180-170, Buehler, Inc., Lake Bluff, IL) by attaching the cylindrical base to the lever arm of the saw. The patella was placed on the edge of the blade, while the saw was turned off. After aligning the patella on the blade to ensure that the cartilage face of the patella was to the right of the blade, the saw was started at a very low speed, and the speed was increased slowly, as the blade cut through the bone (Figure 7).

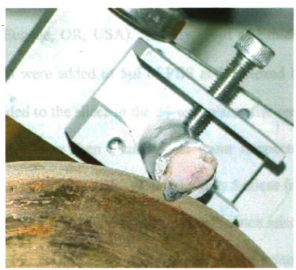

Figure 7. The excised patellae were cut through their thickness using an Isomet saw as shown in the photo. Patellae were glued to a metal base, and kept moist with PBS during cutting.

When the blade was completely through the bone, a scalpel was used to cut the soft tissue holding the patella together. The remaining portion of the patella was sliced into sections using a custom cutting tool with steel blades spaced 0.5mm with fixed vertical movement using linear ball bearings (Figure 8). The slices were placed into a 24-well plate containing approximately 1 ml of PBS.

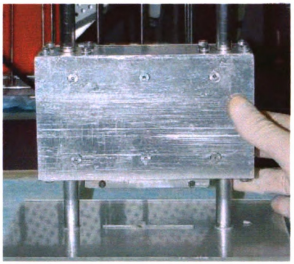

Figure 8. Patellae were sliced into 0.5mm sections prior to staining using the device shown.

The patellar slices were stained with Calcein AM and Ethidium Homodimer, according to the manufacturer's specifications (Live/Dead Cytotoxicity Kit, Molecular Probes, Eugene, OR, USA). Briefly, 10μl of Ethidium Homodimer and 2.5μl of Calcein AM were added to 5μl of PBS and vortexed briefly. The staining mixture was then added to the slices in the 24-well plate after removing the PBS from the wells. The slices were then incubated at room temperature for 15 minutes, followed by several washes with PBS. Approximately 5 slices from each patella were viewed across the center 1 mm of the slice in a fluorescence microscope (Leitz Dialux 20 (frequency: 50-60 Hz), Leitz Mikroskopie und Systeme GmgH, Wetlzar,

Germany) and photographed using a digital camera (Polaroid DMC2, Polaroid Corporation, Waltham, MA, USA). A blinded observer, using image analysis software (Sigma Scan, SPSS INC., Chicago, IL, USA), quantified cell viability of the retropatellar cartilage.

Results

This study was performed to investigate the efficacy of poloxamer 188 (P188) in mitigating cell death in Flemish giant rabbits *in vivo*. Fourteen animals received a blunt impact to the right patellofemoral joint using a gravity driven drop fixture at an impact energy of either 6 J or 10 J. Six (3 @ 6J, 3 @ 10J) rabbits received 1.5 ml of 8 mg/ml P188 into the patellofemoral joint immediately post impact. Cell viability was assessed at 96 hours post impact for 10 animals, and at approximately 1 hour post impact for four animals. All rabbits appeared healthy at time of sacrifice, and did not favor one leg over the other.

The average peak load of the 6 J impact animals was 686.6± 120.0 N, and the average time to peak was 0.003± 0.0008 seconds. The average peak load for the 10 J impacts was 996.3± 123.4 N, and the average time to peak was 0.0024± 0.0005 seconds. The documentation of the extent of surface fissuring was not completed for the writing of this thesis. These data will be utilized in subsequent peer-reviewed publications. Briefly, no difference has been shown, to date, between subsequent groups. The average percentage of cell death in the retropatellar cartilage for the 10 J time zero rabbits was 8.3± 5.8% for control patellae and 9.3± 5.2% for the test patellae (Figure 9).

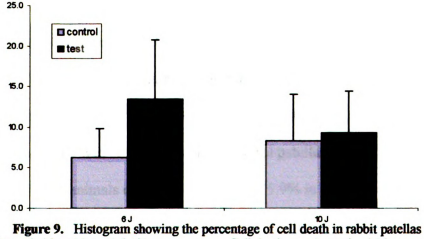

Figure 9. Histogram showing the percentage of cell death in rabbit patellas administered either 6 J or 10 J of impact energy. Cell death was determined 1 hour post impact for both control and test patellas.

The percentage of cell death for the 6 J time zero rabbits was 6.2± 3.6% for the control patellae and 13.4± 7.3% for the test patellae. At four days post impact, there was a significant difference between control and test patellae in the 10 J rabbits. The percentage of cell death for the 10 J control patellae was 8.5± 4.5% and 22.9± 17.3% for the test patellae. There was not a significant difference in cell death between control and test patella in the 6 J rabbits at 96 hours post impact, however there was a strong trend (p=0.051). The cell death in the 6 J patellae was 14.8± 8.1% for the control side and 24.8± 17.3% for the test side (Figure 10).

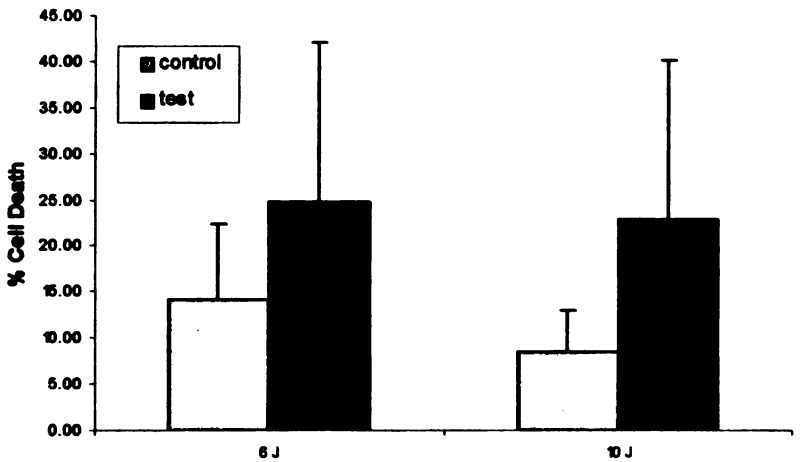

Figure 10. Histogram showing percentage cell death in untreated rabbit patellas administered either 6 J or 10 J of impact energy. Cell death was assessed at 96 hours post impact for both control and test patellas. There is a significant difference in percentage of cell death in the control versus test patella of the 10 J animals.

There was a small amount of background cell death observed in the patellae of the animals used in the study. This could be due to the genetics of the animals, or the conditions in which the animals were raised. After P188 treatment, the percentage of cell death in the 10 J animals was 10.9± 6.1% in the control patellae and 12.7± 8.1% in the test patellae. In the 6 J animals cell death was 20.4± 15.9% in the control patellae and

17.5± 8.1% in the test patellae. There was not a significant difference between the percentage of cell death in the control limb versus the test limb in either the 6 J or 10 J animals. Likewise, there was no significant difference between the test limbs of the 6 J and 10 J animals (Figure 11).

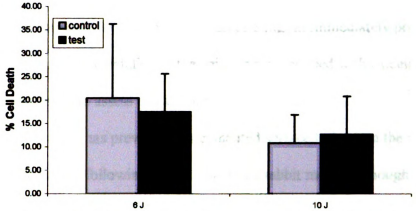

Figure 11. Histogram showing the percentage of cell death in rabbit patellas treated with poloxamer 188 *in vivo* and administered either 6 J or 10 J of impact energy. Cell death was determined 96 hours post impact for both control and test patellas.

Discussion

The objective of the experiment was to investigate the efficacy of P188 in preserving chondrocyte cell viability in an *in vivo* rabbit model. Fourteen Flemish giant rabbits were administered a blunt trauma to their right patellofemoral joint of either 6 Joule or 10 Joule (n=7 each). Six of the impacted rabbits (3 @ 6J, 3 @ 10J) received 1.5 ml injection of sterile P188 at a concentration of 8 mg/ml immediately post-impact. At 96 hours post-impact, the patellae of the animals were stained with calcein AM and ethidium homodimer to assess cell viability.

Our laboratory has previously documented surface lesions on the retropatellar cartilage of the patella following blunt impact in a rabbit model, although not reported in this study, surface lesions were observed on the impacted patellae of the animals (Newberry et al, 1997). The study by Newberry also documented thickening of the subchondral bone and degeneration of the overlying cartilage at 12 months post-trauma, both early signs of OA. Our laboratory and others believe that cell death may play a significant role in the onset of OA. In Chapter 2 of this thesis, cell death was reduced in cartilage explants treated with P188 versus untreated explants following a blunt impact. It was therefore the hypothesis of this study that cell death would be reduced in the patellae of animals treated with P188 versus those that were untreated. The degradation of cartilage that occurs during OA may arise from the death of chondrocytes within the cartilage and fissuring of the surface of the cartilage matrix caused by a blunt impact to the joint (Simon et al, 1976, D'Lima et al, 2001, Borrelli et al, 1997). The prevention of chondrocyte death may prevent the OA like changes that occur following a blunt impact.

In the 4 animals sacrificed at time zero, cell death was increased in the 6 Joule impacted rabbits versus their control limbs, but no increase was seen in the 10 Joule rabbits over their control limbs. The amount of cell death in the test limb of the 6 Joule animals was also higher than in the test limb of the 10 J animals. In a previous study by our laboratory, a 6 Joule impact delivered to the patello-femoral joint of Flemish Giant rabbits produced a peak average contact pressure of 25 MPa, while a 4.2 Joule impact produced 16 MPa (Haut et al, 1995). It can reasonably be stated that a 10 Joule impact would therefore produce contact pressures of greater than 25 MPa. However, the higher impact did not produce greater cell death in the retropatellar cartilage. The increase in cell death for the 6 Joule impact may be due to an uneven distribution of the forces on the retropatellar cartilage, while the 10 Joule impact was more evenly spread across the patella, thereby more evenly distributing the impact energies (unpublished data). The development of high hydrostatic pressures in the 10 Joule versus the 6 Joule animals may help protect cartilage cells.

After 96 hours post-impact, there was an increase in cell death in the test limbs of both the 6 Joule and 10 Joule animals versus their controls. This increase further expands a conclusion of Chapter 2 that cell death increases in the first 24 hours following a blunt impact (Phillips et al, 2004, Ewers et al, 2001). Damaged chondrocytes may stain viable within the first hour following impact, but succumb to the injuries and lose membrane integrity after the first hour. It may also be that degradative enzymes are released from cells heavily damaged by the impact, and spread causing further damage to surrounding undamaged cells.

With the addition of the P188 injection, cell death was reduced in both the 6 J and 10 J animals versus those animals not treated with P188. P188 therefore had a positive effect in preserving cell viability. This result supports a previous study by our laboratory that showed a 41 % reduction in cell death in bovine cartilage explants treated with Poloxamer following blunt trauma (34 % in untreated explants and 14 % in P188 treated explants; Phillips et al, 2004). An important difference between this previous study and the current study was the active delivery of the surfactant to the deeper layers of the cartilage matrix. Although zonal data was not collected in the current study, in the previous study 'pumping' of the cartilage allowed the surfactant to reach the deeper zones of the cartilage. The cartilage explants received 10 cycles of 1 MPa of pressure immediately following impact as well as approximately 22 hours later. Following injection of P188 in the current study, the animal joint was flexed several times, and regular exercise was initiated on the date of impact. It was believed that this exercise would accomplish the same goal as pumping the explants. It is possible that further mechanical pumping of the patellofemoral joint in vivo may show P188 to be even more effective at saving chondrocytes from mechanical trauma.

A limitation of the current study was that the impacted, control animals were not given a placebo injection of phosphate buffered saline without the surfactant. This control procedure would show the effect of the injection on the joint capsule and whether an inflammatory reaction occurs. The increase in cell death between the test and control limbs of the untreated animals in this study may have been affected.

The chondrocyte cells that stain viable at 96 hours are not necessarily viable after this time point. The effect of P188 on chondrocytes in the long term is not known and

needs further study. It may be found that chondrocytes saved by P188 in the first 96 hours following impact die by apoptosis at a later time due to further damage within the cell not repaired by the surfactant. A possible treatment would therefore be an apoptosis inhibitor such as the z-VAD.fmk pan-caspase inhibitor that interrupts the apoptosis cascade and prevents cell death (D'Lima et al., 2001). Further study is needed to study this theory.

The next step in studying the effects of the surfactant would be an animal study to determine the long-term success of the surfactant in preserving cell viability. In previous work published by our laboratory, positive effects were observed for the mechanical stiffness of retropatellar cartilage following early polysulfated glycosaminoglycan treatments in our rabbit model (Ewers et al, 2000). However, the gross appearance of surface lesions on the retropatellar surface was unaffected at the time of sacrifice. It was thought that the loss in the number of chondrocytes at the time of impact caused the degradation of the cartilage. It is possible that a combination of P188 and polysulfated glysosaminoglycan treatment could further limit OA changes to cartilage *in vivo*, by limiting cell death and stiffening the cartilage to lessen surface disruptions such as fissures. Our laboratory has developed a 7 month and 1 year chronic animal model to study the OA changes found following severe blunt impacts. This length of time would give a definitive answer as to the long-term progress that chondrocytes make following repair with P188.

The conclusions to this study are that an increase in cell death shown following both a 6 and 10 Joule impact to the patello-femoral joint of rabbits. Following P188 treatment, cell death was reduced in both the 6 and 10 Joule impacts. The long-term

success of this treatment is unknown and must be investigated. This treatment along with others may severely limit the death of chondrocytes in retropatellar cartilage following blunt trauma and may postpone or eliminate the onset of OA.

References

1. Borrelli Jr. J, Torzilli PA, Grigiene R, Helfet DL. (1997) Effect of impact load on articular cartilage: development of an intra-articular fracture model. *J Orth Trauma.* 11(5):319-326.

2. D'Lima DD, Hashimoto S, Chen PC, Colwell CW, Lotz MK. (2001) Impact of mechanical trauma on matrix and cells. *Clinical Orth Related Res.* 391S:S90-S99.

3. Ewers B, Dvoracek-Driksna D, Orth M, Haut R. (2001) The extent of matrix damage and chondrocyte death in mechanically traumatized articular cartilage depends on rate of loading. *J. Ortho. Res.* 19(5)779.

4. Ewers B, Haut RC. (2000) Polysulfated glycosaminoglycan treatment can mitigate decreases in stiffness of articular cartilage in a traumatized animal joint. *J. Ortho. Res.* 18(5)756-761

5. Flores RH, Hochberg MC. (1998) Definition and classification of osteoarthritis. In: Brandt KD, Doherty M, Lohmander LS (Eds). Osteoarthritis, Oxford University Press, New York. Pp 1-12.

6. Gelber AC, Hochberg MC, Mead LA, Wang NY, Wigley FM, Klag MJ. (2000) Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. *Ann Intern Med.* 133(5):321-8.

7. Hannig J, Zhang D, Canaday DJ, Beckett MA, Astumian RD, Weichselbaum RR, Lee RC. (2000) Surfactant sealing of membranes permeabilized by ionizing radiation. *Rad Res.* 154(2):171-177.

8. Haut RC, Ide TM, DeCamp CE. (1995) Mechanical responses of the rabbit patello-femoral joint to blunt impact. *J Biomech Eng.* 117:402-8.

9. Lee RC, River LP, Pan F, Ji L, Wollmann RL. (1992) Surfactant-induced sealing of electropermeabilized skeletal muscle membranes *in vivo*. *Proc Natl Acad Sci.* 89:4524-4528.

10. Maskarinec SA, Hannig J, Lee RC, Lee KC. (2002) Direct Observation of Poloxamer 188 insertion into lipid monolayers. *Biophysical J.* 82:1453-1459.

11. Maskarinec SA, Lee, KC. (2003) Comparative study of poloxamer insertion into lipid monolayers. *Langmuir.* 19:1809-1815.

12. Nagel DA, Burton DS, Manning J. (1977) The dashboard knee injury. *Clin Orthop.* 126:203-8.

13. Newberry WN, Zukosky DK, Haut RC. (1997) Subfracture insult to a knee joint causes alterations in the bones and in the functional stiffness of overlying cartilage. *J Orth Res.* 15:450-455.

14. Phillips DM, Haut RC. (2004) The use of a nonionic surfactant (P188) to save chondrocytes from necrosis following impact loading of chondral explants. *J Orth Res.* 22:1135-1142.

15. Simon WH, Richardson S, Herman W, Parsons JR, Lane J. (1976) Long-term effects of chondrocyte death on rabbit articular cartilage in vivo. *J Bone Joint Surgery* 58(4):517-526.

APPENDIX A

STANDARD OPERATING PROCEDURE

Staining of cartilage explants for Apoptosis using the Promega kit

Kit: Promega Apoptosis Detection System, Fluorescein

Materials needed: PBS, Propidium iodide, APES coated microscope slides, 4% formaldehyde in PBS, dH_2O, incubator, fluorescence microscope, covered tray

Prior to day of experiment

1. Remove each explant from the 24 well plate and embed the explant in OCT freeze media on a cork disk. This should be done on dry ice. Wrap each OCT embedded explant in Aluminum foil along with a label, and place at –80C.
2. Using the cryotome make slices of the explant and attach the slice to an APES coated microscope slide. Warm back of slide with finger to attach slice to slide. Place slide into microscope box and place box at –80C. Explants can remain at –80C for several weeks.

Day of Experiment

1. Prepare all solutions including the 4% formaldehyde and PBS. Remove kit from freezer and thaw nucleotide mix, equilibration buffer, and TdT enzyme on ice. Place all microscope slides on a tray covered with paper towels.
2. Fix the explants by applying an amount of 4% formaldehyde sufficient to cover the slices. Incubate for 15 minutes at room temperature. Gently remove liquid.
3. Wash the slices 2x with PBS for 5 minutes each at room temp. Gently remove liquid.
4. Prepare a 20μg/ml Proteinase K solution from the reconstituted Proteinase K.

$$C \quad V \quad = \quad C \quad V$$

$$10x10^{-3}g/ml * X = 20x10^{-6}g/ml * 100\mu l$$

$$X \quad = \quad 0.2ul \text{ of stock Proteinase K per } 100ul \text{ needed}$$

Add approximately 100µl of Proteinase K per slide or enough to cover tissue sections. Incubate slides at room temperature for 8 minutes. Longer incubations may release sections from slide.

5. Wash the samples with PBS for 5 minutes at room temperature.
6. Fix the sections with 4% formaldehyde for 5 minutes at room temperature.
7. Wash samples with PBS for 5 minutes at room temperature.
8. Be sure nucleotide mix is thawed on ice.
9. Remove as much liquid from the slides as possible. Cover the tissues with approximately 100µl of Equilibration buffer. Equilibrate at room temperature for 10 minutes.
10. Prepare TdT incubation buffer according to following chart while tissues are equilibrating.

Buffer component	Component volume	# of reactions	Component volume
Equilibration buffer	45µl	_____ =	_____ µl
Nucleotide Mix	5µl	_____ =	_____ µl
TdT enzyme	1µl	_____ =	_____ µl
		Total TdT Incubation buffer volume =	_____ µl

11. Remove all liquid and blot with tissue paper around tissues. Add 50µl of TdT incubation buffer to the tissue sections and be sure sections are completely

covered. Cover the slides with the plastic coverslips provided to ensure even distribution of reagent. BE SURE TO WORK IN THE DARK FROM THIS STEP ON!!!!!

12. Wet the paper towels that line the tray and cover the tray. Cover the container with aluminum foil to protect from direct light. Place into 37^{0}C incubator and incubate for 60 minutes to allow reaction to occur. Be sure that a tray of water is in the bottom of the incubator to provide moisture.
13. Dilute the 20X SSC 1/10 with dH_2O and add enough of the resulting 2X SSC to cover the tissue sections and terminate the reaction. Incubate the slides for 15 minutes at room temperature in the dark.
14. Wash tissues with PBS for 5 minutes at room temperature. Repeat 2 times for a total of three washes.
15. Prepare a solution of 1μg/ml propidium iodide. Add 1mg of PI to a ml of PBS. Vortex to mix. Add 1μl of PI solution to 1ml of PBS. Vortex to mix. Add enough PI to completely cover tissue sections. Incubate for 15 minutes at room temperature in the dark.
16. Wash the samples with dH_2O for 5 minutes at room temperature. Repeat 2 times for a total of 3 washes.
17. Add approximately 50μl of dH_2O to the tissue sections to keep tissues from drying out. Cover the slide tray with foil to protect from light. Analyze slides on fluorescence microscope using a fluorescein filter set to view green fluorescence at 520 $\pm$ 20nm. View red fluorescence of PI at >620nm. On Orth's scope, set dial to filter 3.

Procedure for obtaining Cartilage Explants from Bovine Metacarpal Joints

1. Call Bellinger's meat packaging/slaughter house Monday's and ask for (?) pairs of Bovine Knees (1-800-778-4577). Pick up the knees Tuesday.
2. Prepare all media and make sure 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. Place skin in biohazard bag. Wrap plastic bag around hoof and leg up to joint.
4. Set up the Laminar flow hood. Lay down laboratory paper in hood, place scalpel, biopsy punches, tweezers, a beaker with ethanol soaked kim wipe, 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 explants 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. Take petri dish of explants into Laminar flow hood. Perform three washes by using tweezers to place the explants in a new petri dish with media. Allow 5 min incubation for each wash. Place two explants into each well of a 24 well plate with 1 ml of media (w/ 10% FBS) in each well. Explants may (or not) be cultured for two days before impacting. If explants are cultured for more than 2 days, change media every two days.
8. Take cultures to Instron room. Impact each explant.

9. Place 24 well plate of explants into the hood. Label three petri plates with the impact categories of the explants and add media to the plates. Transfer the explants from the 24 well plate to the first petri plate using tweezers and incubate for 5 minutes. Repeat for total of three washes.
10. Explants can now be examined for fissures, Live/Dead cell assay, or frozen for Apoptosis study.

RABBIT PATELLOFEMORAL IMPACT SOP

What to bring with you:

1. LPS lubricant, ethanol, meter stick, rabbit data sheets, program disk, white silicon grease, duct tape, and crescent wrench.

Pre-test set up:

1a Plug in Valadyne strain gage amp, attach load cell plug to plug on back of resistor box, and plug resistor box into back of computer.

1b. Turn on and start up computer, then insert program disk.

1. Turn on the Valadyne resistor box and insure that the trigger release switch is turned off to protect from accidental triggering.
2. Connect the channel #2 output A of the Valadyne strain gage amp, to the A/D box channel #1 input (Load cell output). This should be done already.
3. Connect the channel #3 output B of the Valadyne strain gage amp, to the A/D trigger input signal.
4. Wait for 15 minutes before calibrating the load cell to allow the electronics to stabilize.
5. Spray some LPS greaseless lubrication on a rag and wipe down the rail of the impact cart.
6. Clean, with alcohol, the side of the impact cart that is gripped by the brakes.
7. Hook the volt meter up to the strain gage amp. Put the volt meter probes into the holes to the right and above and below the black dial. Switch the volt meter on and set to voltage.
8. Using the small screwdriver, zero the load cell on the Valadyne strain gage amp. Use the opening to the top left of the black dial. Use the readout of the volt meter.
9. Calibrate the load cell by depressing the shunt cal on the Valadyne strain gage amp. If needed readjust the set point to <u>-7.53</u> by using the Gain knob (black knob).
10. Double check to make sure the load cell is still zeroed, adjust if necessary.

Rabbit preparation:

1. On the Data Sheet, record the Rabbit name, weight (kg), and sex.
2. Once the rabbit is fully sedated, pull the left hind foot through the very bottom hole of the leather strap of the holding chair and tuck the rest of the strap under the rabbit so it can be fixated to the underside of the chair.
3. Position the right leg so that the femur is pointing vertical and the impactor is directed to hit the middle of the patella.
4. Place the black strap around the right hind foot and pull tight to insure the foot is fully constrained. Fix the end of the strap, as well as the leather strap, to the Velcro pad on the underside of the chair.
5. Move the clamping bar into position and attach the free end to the distal clamp.
6. Slowly apply even pressure to both clamps until the clamps lock in place.
7. Slide the chair into position so the patella is directly under the head of the impacting cart.
8. Lower the cart so the head of the impacting cart is just above the patella, checking to insure the patella is centered under the head.
9. Raise the cart to the desired height.
10. The position of the beam holding the upper brake may need repositioning to accommodate the desired height. Do this by removing the screws of the beam and moving the beam to the desired height above the patella.
11. The height and weight of the sled should be:

Energy	Height	Mass
6 J	0.46meters	1.33kg
10 J	1 meter	1 kg

Computer set-up:

1. Start the "CDC RABBIT DROP TEST" program by double clicking on the icon in the Windows atmosphere.
2. Answer the A/D set-up questions from the computer

Start channel = 1

End channel = 1

Time as ref. = "Y"

Inverting load cell = "N"

Rotary encoder = "N"

3. Change the filename.

   Type "F" [enter]

   File to change = 1

   Filename = rabbit's name (ID).csv

   Enter

4. Check the plot control file data

   # of channels to plot = 1

   X label = time (seconds)

   Y label = Newtons

   Data file name = rabbit's name being tested

   Channel number 0 cal factor = 1

   (time calibration must be set to 1)

   Channel number 1 cal factor = 444.8 N/V

5. Press E for exit when all values are set correctly
6. Sample rate 10,000 samples / sec (Hz), Type y for yes
7. Number "N" = 10,000, Type y for yes
8. Trigger level for data acquisition = 30(N)
9. Number of samples to save after the trigger = 400
10. Press space bar

Impacting the rabbit:

1. Turn on the trigger enable switch on the Valadyne strain gage amp.
2. Press the red release button on the Valadyne resistor box to drop the cart.
3. Impact cart will drop and the A/D board will trigger and save the data.
4. Check status of D.M.A. operation = "N"
5. If you select "Y" then it should read:

Op type = 1

Status = 0

Word count = 10000

6. Plot using line mode = "L"
7. After the data scrolls to screen, it will be plotted. On the data sheet note the peak load and the time to peak.
8. Sketch the graph on the data sheet and write any comments about the test that were unusual, i.e. if the impact cart hit the rubber stoppers during the test, etc.
9. ***FOLLOWING IMPACTION OF ALL ANIMALS, REMOVE DISK FROM DRIVE (a:\) AND COPY ALL FILES TO THE (g:\user\bimgrad) DIRECTORY FOR PERMANENT DOCUMENTATION!***