

ABSTRACT

SIRAVURI, KRISHI. Quantification of Chondrocyte Apoptosis in Mechanically Impacted Articular Cartilage. (Under the direction of Peter L. Mente).

Osteoarthritis is a severely debilitating joint disorder. It is slowly progressing chronic disease and has no precise known causes. Impact injury can lead to cellular and matrix changes in articular cartilage similar to those seen in osteoarthritis. Impact injury models are often used as models for osteoarthritis. The purpose of this study was to use an *in vitro* impact injury model to examine the distribution and timing of apoptosis cell death following an impact injury. In this study it was hypothesized that there will be an increase in apoptosis cells with increasing impact load; that over time in culture the number of apoptotic cells will increase due to lingering effects from the impaction; and that apoptosis will be most severe directly below the impaction. Paired porcine knees were obtained fresh and patellae were removed using sterile techniques. Controlled mechanical impactions were made on 12 patellae at 25 mm/s, using MTS load frame, to pre selected force levels of 1000N (characterized as medium) and 12 patellae were subjected to 2000N (characterized as high). The twelve non-impacted patellae were used as controls. Following impaction, the impact patellae were placed in organ culture for 0, 3, 7, or 14 days and subsequent degenerative changes over time were assessed. Apoptotic cells were quantified using terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique. The stained cells were quantified as a percentage of the total number of cells. Changes in percentage of apoptotic cells was analyzed with experimental factors impact level, days in culture (0, 3, 7, 14), distance from center of impaction, and depth in tissue. There was a significant depth and time dependent increase

in the percentage of apoptotic cells for high impactions. A significant increase in percentage of apoptotic cells was observed from high impactions after 14 days culture time and a significant increase in percentage of apoptotic cells, for 0 days culture time was observed from medium impactions. In conclusion magnitude of load has significant effect on chondrocyte apoptosis throughout the depth of the cartilage tissue, but depends on culture time.

**QUANTIFICATION OF CHONDROCYTE APOPTOSIS IN
MECHANICALLY IMPACTED ARTICULAR CARTILAGE**

By

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1. Introduction

Osteoarthritis is a severe degenerative joint disease especially in the aged population and has no precise known cause. Osteoarthritis is an increasing threat to the human quality of life. The pathology of osteoarthritis is far from being elucidated and treatment of the disease currently is related to the alleviation of the symptoms. The symptoms associated with osteoarthritis are well documented, but the primary changes in the cartilage that can be accounted to initial stages of osteoarthritis are yet to be fully understood. It involves all the tissues in the joint, but is primarily associated with cartilage degeneration, subchondral bone remodeling, projections on the bone surface, and loss in tissue cellularity (34, 233). Reduction in chondrocyte numbers or increase in empty lacunae in articular cartilage tissue are reported in studies looking at osteoarthritis (19, 153, 238). Apoptosis plays an important role in the degenerative diseases of the nervous system and other pathological processes (61). Chondrocyte apoptosis and apoptotic pathways in pathology of osteoarthritis is yet to be elucidated. However apoptosis is understood to have prominent role in the osteoarthritis disease process (23, 83, 85, 93, 109, 133, 204). Impact injury can lead to cellular and matrix changes similar to Osteoarthritis (47, 60). An *in vitro* impact injury model was designed in the study to investigate the distribution and timing of apoptosis cell death during the first two weeks following an impact injury. One way to begin to understand the role of apoptosis in the initiation and progression of this degenerative disease, is to study its time-dependent behavior in response to mechanical injury which is known to cause osteoarthritic like changes in the cartilage. The objective of this study was to investigate chondrocyte

apoptosis in relation to distance from the site of injury, its time dependent development, and to correlate apoptosis with mechanical injury forces.

2. Articular Cartilage

Articular cartilage has a smooth, glossy appearance and resists compression upon probing. Articular Cartilage is a hydrated tissue which serves two important functions; to distribute the loads over wide areas so that the joint surfaces sustain minimum forces (16, 91), and to provide frictionless movement between the opposing joint surfaces (157). Articular cartilage has good stiffness in compression and has an exceptional ability to distribute the loads, minimizing the stresses on subchondral bone. The tissue consists of an extracellular matrix and sparse population of cells. It lacks blood vessels, lymphatic vessels and nerves. Being avascular, articular cartilage has limited ability to repair itself and hence has higher risk of degeneration upon injury or with age (202)



Figure 1. Healthy Articular Cartilage Surface (Porcine Patella) (132)

Collagen, proteoglycan, and water interact to form a porous-permeable, fiber reinforced matrix that possesses the mechanical properties to sustain daily high stresses upon the articular cartilage (11). The macromolecules and chondrocytes of the tissue are in-homogeneously distributed in the tissue giving it a zonal character. Based on the

morphologic changes in chondrocytes and matrix from cartilage surface to subchondral bone, articular cartilage can be divided into four zones or layers (160): superficial zone; transitional or middle zone; deep zone and zone of calcified cartilage (Figure 1: Zonal arrangement). The zones differ in chondrocytes, collagen, proteoglycan, water, and other matrix component concentrations.

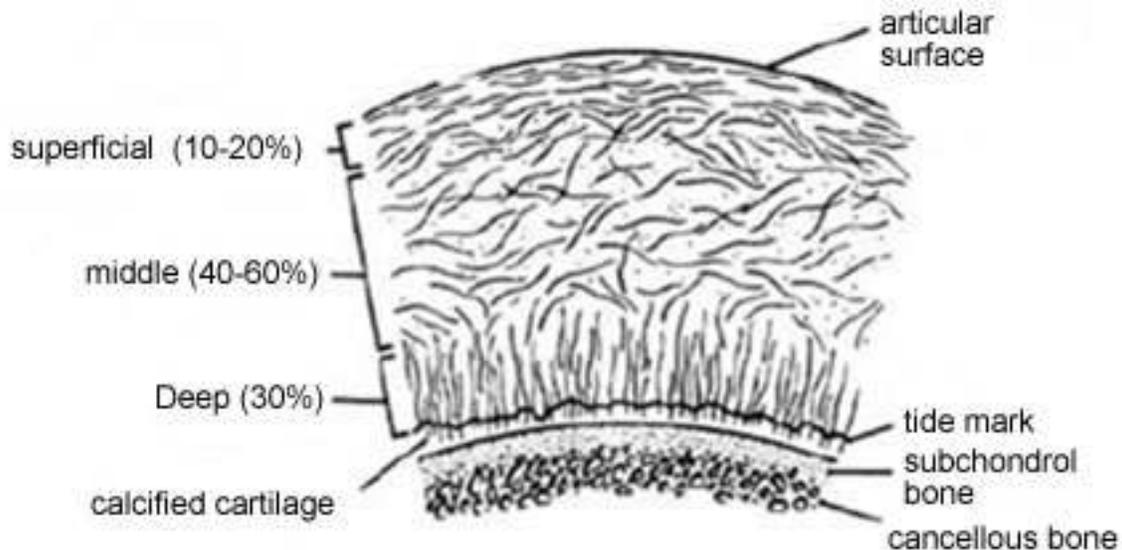


Figure 2. The Zones of Articular Cartilage (99)

Superficial zone: It is the thinnest and uppermost zone. It can be subdivided into two regions. The joint surface is covered with a thin sheet of fine fibrils and do not have cells. This surface overlay the region with flattened cells arranged such that their axis is parallel to the joint surface. Collagen fibrils are tightly woven into a sheet parallel to the articular surface. The superficial zone has a higher concentration of collagen, fibronectin, water and a lower concentration of proteoglycans relative to the other zones (202). The chondrocytes in this zone degrade proteoglycans more rapidly compared to other zones,

in maintenance processes or in response to external stimuli. They also synthesize less proteoglycan compared to the cells from deeper zones. The dense mat of collagen fibrils parallel to the joint surface gives it a relatively high tensile stiffness, strength and good resistance to shear during joint movements (202). Disruption or remodeling of the collagen on the joint surface is the first detectable change in artificially induced cartilage degeneration, suggesting any changes in this zone may lead to osteoarthritis (75). The superficial zone acts as a barrier between the outer and internal environments of the cartilage.

Middle zone: In the middle zone, collagen fibrils are less densely packed to accommodate the higher concentration of proteoglycans. The chondrocytes in this zone have spheroidal shape and have a higher concentration of organelles compared to surface zone cells. They produce a matrix with higher collagen fibril diameter and higher proteoglycan concentration, but lower concentrations of collagen and water than surface zone. The collagen fibrils are randomly oriented.

Deep zone: In the deep zone, the collagen fibrils are perpendicular to the articular surface and cross the tidemark (basophilic line that separates calcified zone from uncalcified cartilage) to enter the calcified zone and hence anchor the tissue to the calcified bone. The chondrocytes in this zone are more round and stacked in cylindrical columns above each other (159). The cells in this zone synthesize matrix with the highest concentration of proteoglycan and largest collagen fibril diameter, but with the lowest concentration of water (141).

Calcified zone: This zone separates the deep zone from the subchondral bone. The chondrocytes in this zone have the least volume of endoplasmic reticulum and golgi membranes. The metabolic activity of the chondrocytes in this zone is the lowest. The cartilage in this zone has calcified and acts to cement the cartilage to the underlying bone.

Articular cartilage extracellular matrix is also divided into three regions depending on its proximity to the chondrocytes (141): pericellular, territorial, and interterritorial. These regions too have inhomogeneous distribution of collagen, proteoglycan and water. The pericellular region engulfs the cell and attaches to the chondrocyte membrane. This matrix region is rich in proteoglycan and contains little or no fibrillar collagen. It contains non collagenous protein (Anchorin CII, cell membrane associated molecule) and type VI collagen (80, 158, 179, 180, 202). The pericellular matrix is very sensitive to stimulation, and mechanical stimuli can rapidly affect aggrecan synthesis (184). The chondrocytes together with its pericellular matrix is called a chondron. The territorial matrix surrounds the pericellular matrix and primarily contains collagenous fibrils that form a network distinct from the interterritorial region. The interterritorial matrix covers the entire region between the territorial matrices of the cells and contributes to the majority of the material properties of the cartilage (202). This variation in collagen fiber density as well as collagen-proteoglycan interactions throughout the tissue are thought to be responsible for the anisotropic nature of articular cartilage behavior (8, 160).

2.1 Biochemical Composition of Articular Cartilage

The extracellular matrix primarily consists of collagen fibrils (mostly type II), proteoglycans (protein-polysaccharides) and water. There are other components in the extracellular matrix which include inorganic salts, lipids, phospholipids, proteins and glycoprotein (Table 1).

The matrix macromolecules comprise 20-40% of the wet weight of the cartilage. Collagens contribute about 60% of the dry weight and proteoglycans about 25 to 35% of the dry weight and non collagenous proteins and glycoproteins about 15 to 20%. They differ in volume and composition in different zones (202).

Table 1. Biochemical Composition of Articular Cartilage

| Component | % Wet Weight |
|---|---------------------|
| Quantitatively Major Components | |
| Water | 65 to 80 |
| Collagen (type II) | 10 to 20 |
| Aggrecan | 4 to 7 |
| Quantitatively Minor Components (less than 5%) | |
| Proteoglycans | |
| - biglycan | |
| - decorin | |
| - fibromodulin | |
| Collagens | |
| - type V | |
| - type VI | |
| - type IX | |
| - type X | |
| - type XI | |
| Link Protein | |
| Hyaluronate | |
| Fibronectin | |
| Lipids | |

Source: Orthopaedic Basic Science; edited by Sheldon R. Simon, MD (207)

The Collagen fibrillar meshwork gives articular cartilage its tensile stiffness. The fibrillar network entraps proteoglycans, restricting its expansion and thereby creating swelling pressure, which gives compressive stiffness to the cartilage. Other non-collagenous proteins either bind to this meshwork or get entrapped. The non-collagenous proteins help in organizing the matrix or help in the binding of the chondrocytes to the extracellular matrix (202). Small non-aggregating proteoglycans - decorin, biglycan, and fibromodulin (90, 200, 247), are believed to participate in matrix stabilization and fibrillogenesis by cross-bonding. Cartilage matrix molecules are susceptible to degradation by several families of proteinases (164), including metalloproteinases, serine proteinases (108) and the aggrecanase family (227).

2.2 Proteoglycans

Proteoglycan is a protein-polysaccharide molecule with glycosaminoglycan side chains (65). Aggrecan (large proteoglycans found in articular cartilage) is an approximately 200-nanometer-long protein to which glycosaminoglycan chains and oligosaccharides are covalently attached (65, 162). Glycosaminoglycans are inhomogeneously distributed along the protein core giving it “bottle brush” appearance (162). Glycosaminoglycans found in the cartilage include hyaluronan, keratan sulfate (13 disaccharide units, (162)), Chondroitin sulfate (25 to 30 disaccharide units, (162)) and dermatin sulfate (202). Glycosaminoglycans have long polysaccharide chains consisting of repeating disaccharides, which have at least one negatively charged carboxylate (COO^-) or sulfate group (SO_3^-). Hence glycosaminoglycans have large strings of negative charges that repel each other and other negative charges, and attract cations in the fluid

thus holding the fluid and creating a tissue swelling pressure (Donan osmotic pressure) which resists compression.

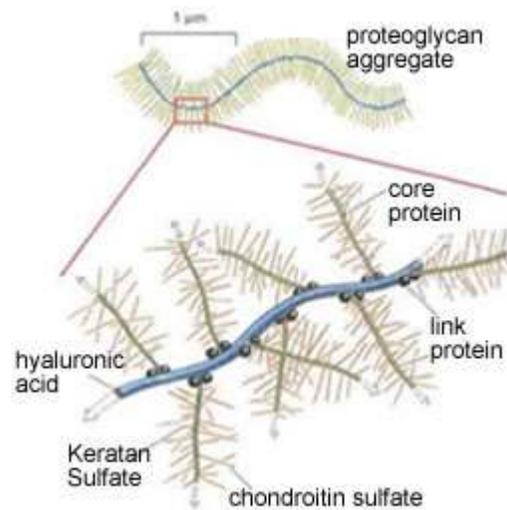


Figure 3. Proteoglycan Aggregate (100)

Based on size, there are two types of proteoglycans found in the articular cartilage: large aggregating aggrecans ($1-4 \times 10^6$ mw, they contribute about 90% of the total proteoglycan mass) and smaller or non-aggregating proteoglycans (1×10^4 mw) such as decorin, biglycan and fibromodulin. Small proteoglycans contribute about 10% of the total proteoglycans (77, 202). Smaller proteoglycans though contribute less to the total mass, can be present in equal or higher molar concentrations compared to the aggregating proteoglycans.

Hyaluronan, the back bone of the large proteoglycans, is a non sulfated disaccharide chain. Many aggrecan molecules link to the hyaluronan molecule via hyaluronan binding region (HABR) to form large aggregates. This binding is stabilized by a link protein. Aggregate formation helps organize and stabilize the proteoglycans in the collagen meshwork, where they get entrapped preventing proteoglycans from slipping through the meshwork and providing stability and rigidity to the extracellular matrix (161, 162). Aggrecan molecules have a large number of chondroitin and keratin sulfate chains. Decorin, biglycan, and fibromodulin have one, two, and more than two dermatan sulfate chains respectively (202). Aggrecan molecules differ in length, molecular weight and composition.

Non-aggregating proteoglycans play a role in the stabilization of the matrix by bridging the larger macromolecules. Decorin and fibromodulin bind with type II collagen and may have an important role in organizing and stabilizing the type II collagen meshwork (202). Biglycan interacts with type VI collagen in the pericellular matrix. The small proteoglycans may also bind to the growth factors and may influence the activity of these cytokines.

The composition and concentration of proteoglycans is not same throughout the cartilage. It changes with age, cartilage injury and disease (202). The water content and carbohydrate/protein ratio decreases with age (14, 69, 194). This is mirrored in decrease in chondroitin sulfate (CS) and increase in Keratan sulfate (KS). CS/KS ratio which is 10:1 at birth becomes 2:1 in adult articular cartilage (194, 225). These changes might be a result of age related functional demand on articular cartilage. Proteoglycans are synthesized and eliminated continuously as part of the homeostasis. Turnover of

aggrecans is highly regulated by molecules such as matrix metalloproteinases (MMPs) (128).

2.3 Water

Articular cartilage is a material of high porosity and low permeability with approximately 65% to 80% porosity (ratio of fluid volume to total volume). Water contains gases, small proteins, metabolites and a high concentration of cations to balance the negative charges on the proteoglycans. Water interacts with matrix macromolecules and this interaction influences the mechanical properties of the cartilage. Water's volume, concentration and behavior depend on its interaction with matrix macromolecules, specifically the proteoglycans which hold the water inside the matrix. The macromolecules have a large concentration of negative charges. These attract cations like sodium (Na^+) and repel anions like chloride (Cl^-), thereby increasing the concentration of cations in the fluid. This increases osmolarity of the fluid causing Donnan effect (irregular distribution of charged particles). The collagen network resists the Donnan effect by not allowing the proteoglycans to swell. Permeability is inversely proportional to the frictional drag force and hence articular cartilage with low permeability has high frictional drag forces on the order of 10^{14} to 10^{15} Ns/m^4 (141). The high frictional drag forces require large pressure to move the water through the interstitial space. Compressive forces on the tissue raise the interstitial pressure which causes the water to flow. The rate of efflux is controlled by drag forces which in turn are determined by the compressive forces. Compressive forces are shared between the liquid and solid phase. This apportion of the forces depends on the porosity, loading rates and type of loading (141). This allows the cartilage to resist the rapid exudation of water at high

compressive forces and allocate the higher compressive forces to liquid phase without causing damage to the solid matrix.

2.4 Collagen

Collagen is the most abundant protein in the body. It has high structural organization and provides fibrous ultra structure to the tissues. The basic unit of collagen is tropocollagen, that polymerizes to form collagen fibrils (18). Tropocollagen is made up of three procollagen polypeptide strands, each of which is a left handed helix. These three left handed helices are coiled about each other into a right-handed helix, forming a triple helix that is stabilized by numerous hydrogen bonds. The tropocollagen molecules cross links give the collagen fibril its high tensile stiffness. The collagen fibrils group together to form collagen fibers.

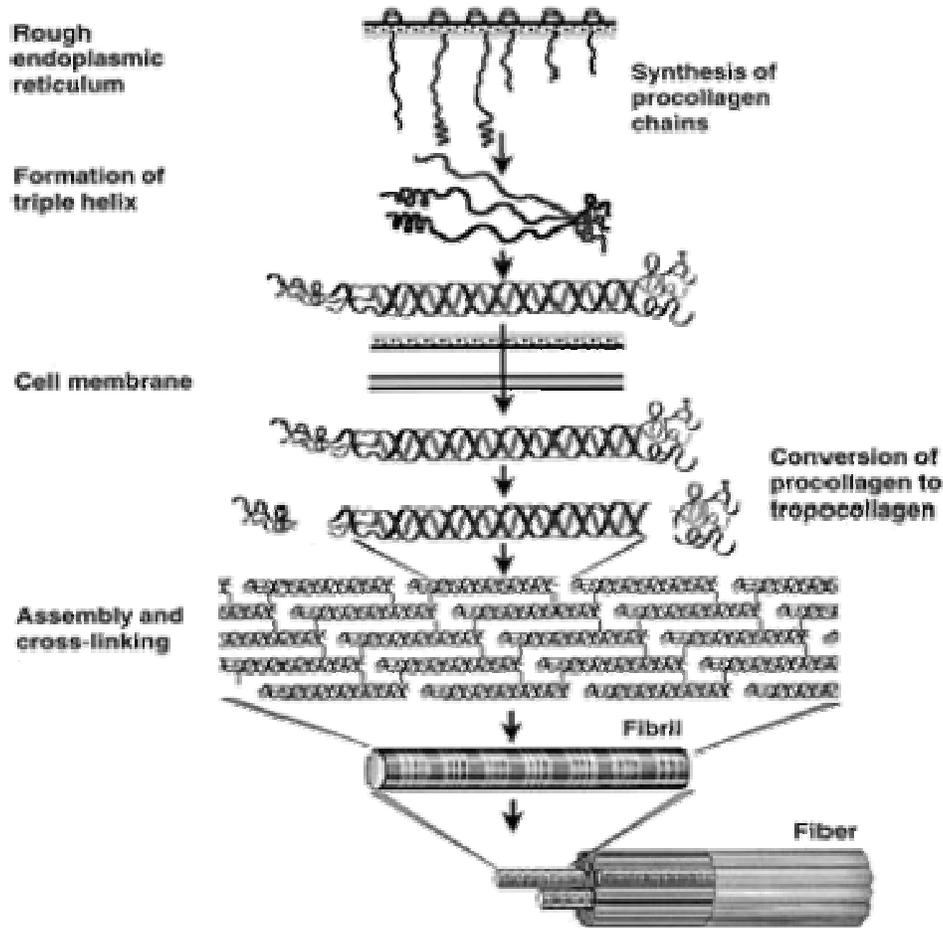


Figure 4. Synthesis of Collagen Fiber (132)

Cartilage contains several different types of collagen – II, VI, IX, X, XI. Types II, IX and X form the cross banded fibrils. The fibrils form the meshwork throughout the cartilage giving the cartilage its tensile properties and strength (202). Type II collagen accounts for 90-95% of collagen volume and forms the primary component of cross banded fibrils. Type IX collagen covalently bonds to the type II collagen fibril and project into the matrix, where they also can bind to other type IX collagen molecules. Type IX and XI are thought to aid in the building of the collagen fibril network and its stabilization. Type VI collagen, primarily found in the pericellular matrix (78, 124), has an important role in forming an interface between the chondrocyte and cartilage matrix

(16). Type X collagen is only found in the calcified cartilage zone and the hypertrophic zone of the growth plate suggesting that it plays an important role in cartilage mineralization (202).

Very few of the non-collagenous proteins have been studied thus far. They primarily consist of protein and attached monosaccharide and polysaccharides. These help to maintain the structure of the matrix. Anchorin CII, a chondrocyte surface protein attaches to the collagen fibril and hence forms a bridge between the chondrocytes and the fibrillar network (211). Similarly oligomeric protein, only found in the territorial matrix of cartilage, can attach to the chondrocytes (121). Other non collagenous proteins in the matrix that have been identified include fibronectin and tenascin (202). Their functions in the articular cartilage are still poorly understood, but they are thought to have a role in matrix stability, matrix-chondrocyte interaction, and in the responses of the tissue in osteoarthritis.

2.5 Chondrocytes

Only one type of specialized cells, chondrocytes, exists in articular cartilage. Chondrocytes only account for about 10% of the cartilage tissue but the value varies with species (215). In humans, chondrocytes occupy approximately 1% of the cartilage volume, while other small animals like mice, rabbits etc have much higher chondrocytes density than humans (202). Despite their small number, chondrocytes are responsible for synthesis and maintenance of the entire tissue extracellular matrix (65). Synthesis and degradation of macromolecules is influenced by mechanical stimuli. However cellular transduction mechanisms are poorly understood.



Figure 5. Articular Cartilage Chondrocytes (showing different zones Of articular cartilage with different orientation of chondrocytes) (244)

Chondrocytes from different zones not only differ in shape, size and concentration but also in metabolic activities (202). The metabolism and metabolic response of chondrocytes to external stimuli alter with aging. With age their ability to respond to anabolic stimuli and growth factors decrease, their capacity to produce some types of proteoglycan decrease, and their proliferative capacity decrease (144, 145),.

The chondrocytes sense and respond to various mechanical stimuli through multiple regulatory pathways. The response might be changes at the transcriptional level (187, 229) or at the translational and post translational level (111) or there might be change in the cell mediated assembly and matrix degradation mechanisms (185). Certain mechanical regimens can effect the chondrocyte behavior, either building up the matrix or compromising the biomechanical properties by cartilage degradation. Various *in vitro* models have been developed to study the chondrocyte-matrix interactions and signal transduction mechanisms of chondrocytes in response to mechanical stimuli (87, 88, 155,

188). The mechanical response of chondrocyte depends on compression frequency and amplitude. Biosynthesis is not affected by low strain amplitude (1%-4%), unconfined compression at low frequency ($<0.001\text{Hz}$), whereas protein synthesis is stimulated by low strain amplitudes at higher frequencies (0.01-1Hz) (197). Quinn TM *et al.* (184) using quantitative autoradiography techniques reported that spatial proteoglycan biosynthesis matched the profile of interstitial fluid flow and matrix deformation. These findings emphasize the importance of physical stimuli in regulating chondrocyte response. Physical stimuli might be sensed by fluid shear at the cell surface or by alterations in the pericellular concentration of cytokines, growth factors, degradative enzymes, newly synthesized macromolecules, or other nutrients.

3. Osteoarthritis

3.1 Definition

The World Health Organization Conference updated the definition of osteoarthritis in 1991 as a disease and it now reads as follows: “Osteoarthritic diseases (OA) are a result of both mechanical and biologic events that uncouple the normal balance between degradation and synthesis by articular cartilage chondrocytes and extracellular matrix, and subchondral bone. OA diseases involve all of the tissues of the diarthrodal joint. Ultimately, OA diseases are manifested by morphologic, biochemical, and biomechanical changes. When clinically evident, OA diseases are characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of inflammation.” (156)

3.2 Osteoarthritis and Articular Cartilage

The pathomorphology of the disease is poorly understood. Various factors like injury, age, genetics, trauma etc might contribute to the initiation and progression of disease (secondary osteoarthritis), although osteoarthritis (primary or idiopathic) occurs mostly in the absence of known factors. Investigation of these factors is an ongoing research in the field, an attempt to find medical intervention for the disease. OA involves all the tissues (e.g. ligaments, tendons etc. in knee) in the joint but the primary changes are loss of articular cartilage, remodeling of subchondral bone and projections on the subchondral bone surface (osteophyte formation) (34) .

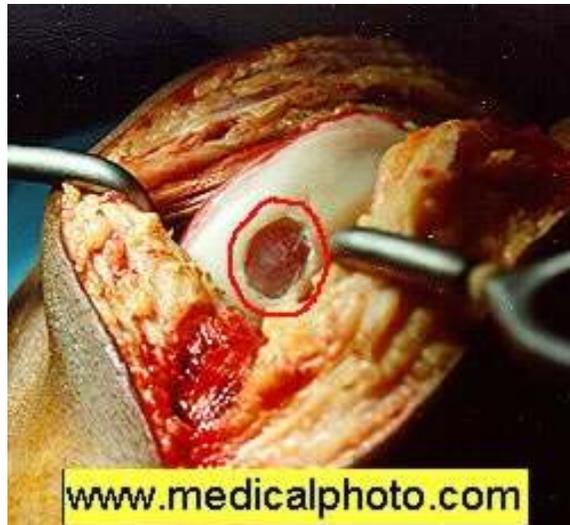


Figure 6. Articular Cartilage Surface Defect (circled in red) - after removing damaged articular cartilage (243)

Osteoarthritis is a joint degenerative disease that results from the imbalance between physiochemical resistance and mechanical stress, leading to destruction and ultimately failure of the matrix. The different changes that are observed in the matrix occur at microscopic, macroscopic, molecular and supramolecular levels. They include microscopic fissuring, degeneration of molecular components, destabilization of the collagen network at the macroscopic level, change in chemical composition of the molecular components, changes in chondrocyte phenotype, and cell death. All these changes together compromise the tissue's ability to withstand applied stresses and ultimately lead to the destruction of articular cartilage.

OA is a progressive disease that is generally divided into three stages: cartilage matrix degeneration, chondrocyte response to tissue damage, and progressive loss of tissue (34). These stages have significant overlap and a definite boundary between stages cannot be defined. Initial tissue damage may occur for unknown reasons (primary or idiopathic OA), or may occur due to mechanical injury, or changes in the metabolic

activities of the tissue (33). Fissuring at a microscopic level can be observed after initial surface tissue damage (5), with no observed change in the collagen concentration. However there can be macromolecular disruption including decreased aggrecan concentration and glycosaminoglycan chain length, and increased water content (34, 58, 60, 137-139). The main changes that are observed at the macromolecular level are degradation of proteoglycans and loosening of the collagen network (137), though which activity occurs first is not yet elucidated. Although the collagen type II concentration remains intact, the fibrillar framework loosens due to the disruption of the interaction between minor collagens, aggrecans and collagen fibrils. This disruption and loosening of the network allows the aggrecan to swell, redistribute the ionic charges and increases water intake. These changes lead to hyper-hydration of the cartilage. This can be seen macroscopically as a swelling and softening of the tissue. The loosening and the disruption also release anabolic cytokines which act on the chondrocyte receptors.

The second stage in the OA disease is the chondrocyte response to the tissue damage. The chondrocytes sense the changes in the osmolarity, mechanical stress and tissue matrix components. These changes in the cells environment and action of anabolic cytokines, leads to increased anabolic activity, cell proliferation, and cell death. Increases in biosynthesis and enhanced anabolic activity of the chondrocytes following tissue damage, due to biomechanical and biochemical changes in its environment, is now widely accepted. This anabolic activity of the damaged tissue cells is different from that of chondrocytes in healthy articular cartilage. The anabolism of the chondrocytes in healthy cartilage is mainly concerned with proteoglycans turnover and taking care of the homeostasis functions of the tissue (142). The chondrocytes in damaged tissue synthesize

extracellular matrix with excessive collagen type II, as evidenced by higher expression of collagen type II than aggrecans (149, 192). Alteration in the chondrocyte phenotype alters the macromolecular expression, which is observed in the *in vitro* studies (21, 40). Increased catabolism in OA cartilage is an effect of the enzymatic degradation and alteration in the genomic expression of the chondrocytes. The presence of many matrix metalloproteinases (MMPs) such as MMP-13 (17, 22), MMP-7 (172), MMP-8 (206), and aggrecanase (131), are reported in OA cartilage, thus signifying the role of enzymatic degradation of cartilage. An imbalance between the catabolic and anabolic activities alters the matrix composition and the biomechanical properties of the OA cartilage ultimately leading to the tissue failure.

Chondrocytes also respond to a variety of stimuli including catabolic cytokines and mechanical stress, by expressing degradative enzymes and mediators. The downstream degradative pathways mostly involve the aggrecanase and MMPs (64). MMPs are proenzymes that are activated by physiological agents in the matrix (163). Nitric Oxide (NO) is believed to play a mediator role in the activation of the MMPs (163, 199). NO is synthesized by nitric oxide synthase which is found in the chondrocytes (10, 24). It is highly diffusible free radical. The NO production signal cascade is initiated by interleukin1- β (IL1- β) and Tumor Necrosis factor- α (TNF- α) that also initiate the expression of MMPs (151). IL1- β and TNF- α are thought to be the key catabolic cytokines in articular cartilage. NO also plays an important role in mediating matrix component production (41, 89) and initiation of apoptosis (24). Fibronectin (a glycoprotein involved in cell adhesion, cell movement, cell differentiation, and wound healing) is also degraded into fragments by proteinases, which is verified by the

observation of enhanced levels of fibronectin fragments found in cartilage and synovial fluids of patients with OA and Rheumatoid arthritis (97, 242). Fibronectin, when intact does not perform any catabolic activity, however the fibronectin fragments induce cartilage destruction by initiating the expression of metalloproteinases (63, 96, 213, 240, 245, 251, 252) thus signifying the importance of fibronectin fragments importance in the pathophysiology of osteoarthritis. Metalloproteases participate in the degradation of aggrecans early in the cartilage damage and are also capable to process bioactive molecules that participate in cartilage degeneration. Collagenases in the MMP family are significant in articular cartilage, because of their ability to degenerate collagen fibrillar network (MMP -1, 8 and 13) (67, 104, 113, 241). MMP-13 has the highest activity against Collagen type II, while it has good substrate specificity for collagen type I, III, IV, IX, X, XIV, fibronectin and aggrecan, among others (66, 114, 115, 241).

Enhanced biosynthesis in the initial stages of osteoarthritis is reported (59, 127, 140, 152, 196, 198). In spite of the increase in the anabolic activity, there is net loss of proteoglycan in all the stages of osteoarthritis (139). There is not only a change in proteoglycan content in quantitative terms, but also in its chemical composition that changes as the disease progresses. For example the CS/KS ratio changes (194, 221, 225), which suggest changes in transcriptional, translational and post-translational levels of gene expressions of chondrocytes.

New molecules, which are not seen in healthy articular cartilage, are observed in OA cartilage such as types IIA and III collagen (3, 7), the roles and function of which in the disease process are not yet determined. Alteration in chondrocyte phenotype have been observed in *in vitro* cell culture experiments (20, 21). These chondrocytes though

are very active cells, stop expressing cartilage-specific genes such as collagen II and aggrecan (20, 21). Thus alterations in the chondrocyte phenotype might play an important role in reduction of anabolic activity in OA cartilage.

Research has been done on the pericellular matrix in OA cartilage, as this is the immediate surrounding matrix of the chondrocyte. An upregulation of type VI collagen in middle and deep zones of OA cartilage is reported, while there is a decrease in the superficial zone (80, 182). Type VI collagen is found in the interterritorial matrix in OA cartilage (107) unlike healthy cartilage, where type VI is predominantly observed in pericellular matrix. There is excessive synthesis as well as damage of type VI collagen in OA cartilage (150, 179, 180, 193). In damaged cartilage, the tissue is exposed to the cell proliferating factors in synovial fluid. Proliferation is observed to be one of the chondrocytes responses to the tissue damage in OA cartilage (103, 139), this might be due to the exposure of the chondrocytes to proliferating factors in synovial fluid, following fissuring or damage. However, it seems to be a futile attempt by the cells, as the anabolic activity is not much improved by the increased cellular clusters in the upper zones of the OA cartilage (6, 103). Empty lacunae and cell debris are observed in the OA cartilage (35, 232). Thus cell death seems to have an important role in OA disease process.

Failure of the chondrocyte to resist the onslaught of degeneration effects and restoring normalcy results in the third stage of OA: progressive loss of articular cartilage and decline in the anabolic and proliferate response of the chondrocytes (34, 43, 137, 172). This may result for various reasons that include mechanical damage and death of the chondrocytes that are no longer protected by the matrix, down regulation of

chondrocyte anabolic response to cytokines; and accumulation and synthesis of molecules that bind to the cytokines and change their configuration such as decorin, insulin-dependent growth-factor binding protein.

Alterations in the subchondral bone accompany the loss of articular cartilage. Alterations include increase in the subchondral bone density, cyst-like cavities on the surface containing fibrous tissue, and appearance of regenerating cartilage within and on the bone surface (123, 186). At the end stage, articular cartilage is completely lost leaving only the subchondral bone with osteophytes and regenerating cartilaginous tissue on its surface. The bone remodeling and loss of cartilages changes the shape of the joint, which leads to shortening of the limb, deformity and instability.

There is a striking correlation between primary osteoarthritis and age (34, 122, 178), however role of age in OA disease is still an ongoing debate. There are some studies showing life long use of the cartilage or normal wear and tear of the articular cartilage, did not necessarily lead to OA (31, 32, 143).

4. Cell Death

There are two types of cell death – programmed cell death (apoptosis) and necrosis. Necrosis is often referred to as "cell murder". It occurs as a result of cell injury or exposure to cytotoxic chemicals (136). It is characterized by leaky cell membrane and loss of control of ion flow (55, 136). Apoptosis is often referred to as programmed cell death or "cell suicide". It is a normal physiological process which maintains the tissue homeostasis (136). It plays an important role in the degenerative diseases of the nervous system and other pathological processes. Apoptosis is characterized by condensation of cytoplasm and nucleus and by internucleosomal cleavage of DNA. The final stage is characterized by formation of apoptotic bodies for phagocytosis. But as the apoptosis progresses, all membranes are also compromised, and the distinction between apoptosis and necrosis can sometimes appear blurred (49, 55). Cell death is observed as a response to wounding and is considered to be a combination of necrosis and apoptosis (224). Chondrocyte loss had long been considered as one of the features of OA cartilage (135) with loss of as much as 25-30% of the cells (38, 39). Large number of empty lacunae are observed in calcified cartilage zone (214, 231) Cell death research is important in pathology of OA, because of the inability of the cartilage to replace the lost cells. However no consensus has been reached regarding the role and function of cell death in the progression of the disease.

5. Apoptosis

Apoptosis has a prominent role in tissue remodeling during growth and development, tissue homeostasis and in the immune systems (95, 174). For example central nervous system development involves apoptosis, where the neurons that are unable to make proper synaptic connection, undergo programmed cell death (174). Apoptosis plays central role in the formation of a frog from a tadpole and a butterfly from a caterpillar. It is interesting that the tadpole and frog are made up of the same genome but have a different phenotypes. Without apoptosis, humans would be born with webbed hands and with a lot of abnormalities. Apoptosis is a conserved complicated process in cells, as any imbalance in apoptosis (cell death) and proliferation leads to cancer, degenerative diseases such as Alzheimer's disease, neurodegenerative diseases, ischemia reperfusion injury, graft-versus-host disease, and autoimmune disorders (181, 209). Apoptosis is both a physiologic and pathologic process. Apoptosis can occur in response to causes such as physical stimuli and toxic exposures, cytokines, viral infection, immunologically, and physiologically relevant mechanisms (95). Apoptosis is triggered by a multitude of extracellular and intracellular signals, however these different pathways of apoptosis lead to similar morphological changes in the dying cells. There are some subtle but important differences in how different cell types respond to these multitudes of factors (237). Some of the hallmarks of the apoptotic process in general are the upregulation of caspase activities (apoptosis might occur in caspase independent fashion too), chromatin condensation, DNA fragmentation, cell shrinkage, cell isolation or withdrawal and formation of apoptotic bodies. DNA degradation is the standout feature of apoptosis. DNA is cleaved within the histone H1-bound spacer regions between

nucleosomes by deoxyribonucleases. This creates multiples of a 180 base pairs of DNA fragments.

Apoptosis is a well orchestrated mechanism that is initiated by proapoptotic signals acting on the cell, which trigger various intracellular signal cascades, autocrine mediators, positive feedback loops that finally lead to the cellular and nuclear disassembly, and finally to the formation of apoptotic bodies that are digested by phagocytosis.

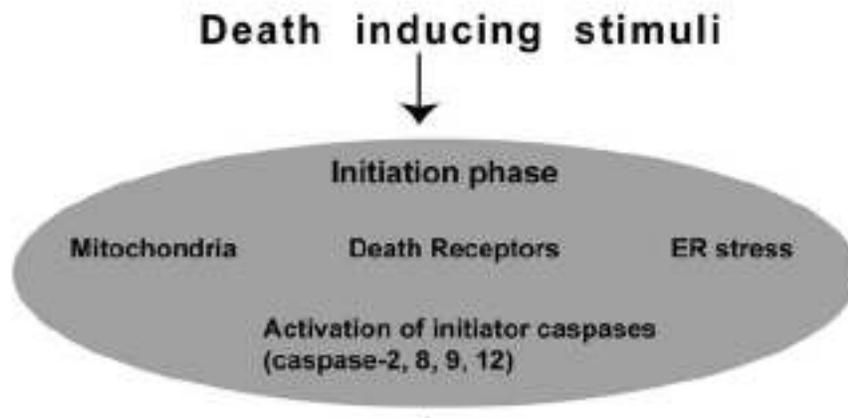


Figure 7. Activation of Programmed Cell Death - Different Pathways (death inducing stimuli via various pathways initiates initiator caspases which further initiate plethora of mediator and effector caspases) (117)

5.1 Death Receptors Pathway

Death receptors and ligands play a central role in apoptosis mechanisms in the immune systems and many other cell types. Receptors upon engaging ligands activate initiator procaspases and ultimately effector caspases to disassemble the cell. Death receptors are intermembrane receptors with intracellular death domain (DD) (15, 165, 223), and belong to tumor necrosis factor (TNF) receptor gene family which are characterized by cysteine rich extracellular domains (27, 130, 210). The death domains

of death receptors act as interface between the receptors and the apoptotic machinery. The various death receptors and the respective ligands that have been identified are summarized in Table 2.

Table 2. Death Receptors and Ligands

| Death Receptors | Death receptor ligands |
|---|-------------------------------------|
| CD95/Fas/Apo1 (10) | CD95L (self) |
| TNFR1 (12) | TNF and Lymphotoxin α (self) |
| Avian CAR1 (13) | Unknown |
| DR3 (Apo3) (14) | Apo3 ligand (TWEAK) (23, 24) |
| DR4 (15), DR5 (Apo2, TRAIL-R2, TRICK-2 or KILLER) (16-21) | Apo2L (TRAIL) (16-21, 25, 26) |

5.2 Mitochondria Pathway

The mitochondria intermembrane consists of many proapoptotic proteins such as procaspases 2,3, and 9; cytochrome C, and apoptosis inducing factor (AIF) that are leaked out in response to various death stimuli (217, 257). Chemicals and radiations that induce DNA damage, dexamethasone, staurosporin etc induce the release of cytochrome C from mitochondria. Cytochrome C is released from mitochondria during apoptosis (112, 250). Cytochrome C interacts with Apaf-1 (Apoptosis protease activating factor-1) and together they form a complex with procaspase-9 called apoptosome that leads to the activation of caspase 9 (101, 126, 236). Caspase 8 can act on one of the substrates Bid (Bcl-2 protein) to cleave it to truncated Bid (t-Bid) that can induce release of cytochrome C. (125, 134, 148, 219) (Figure 8). Death receptor activation of caspase 8, and

mitochondria activation of caspase 9 might be acting simultaneously to converge and ameliorate the apoptotic signal (44, 79, 120, 254) .

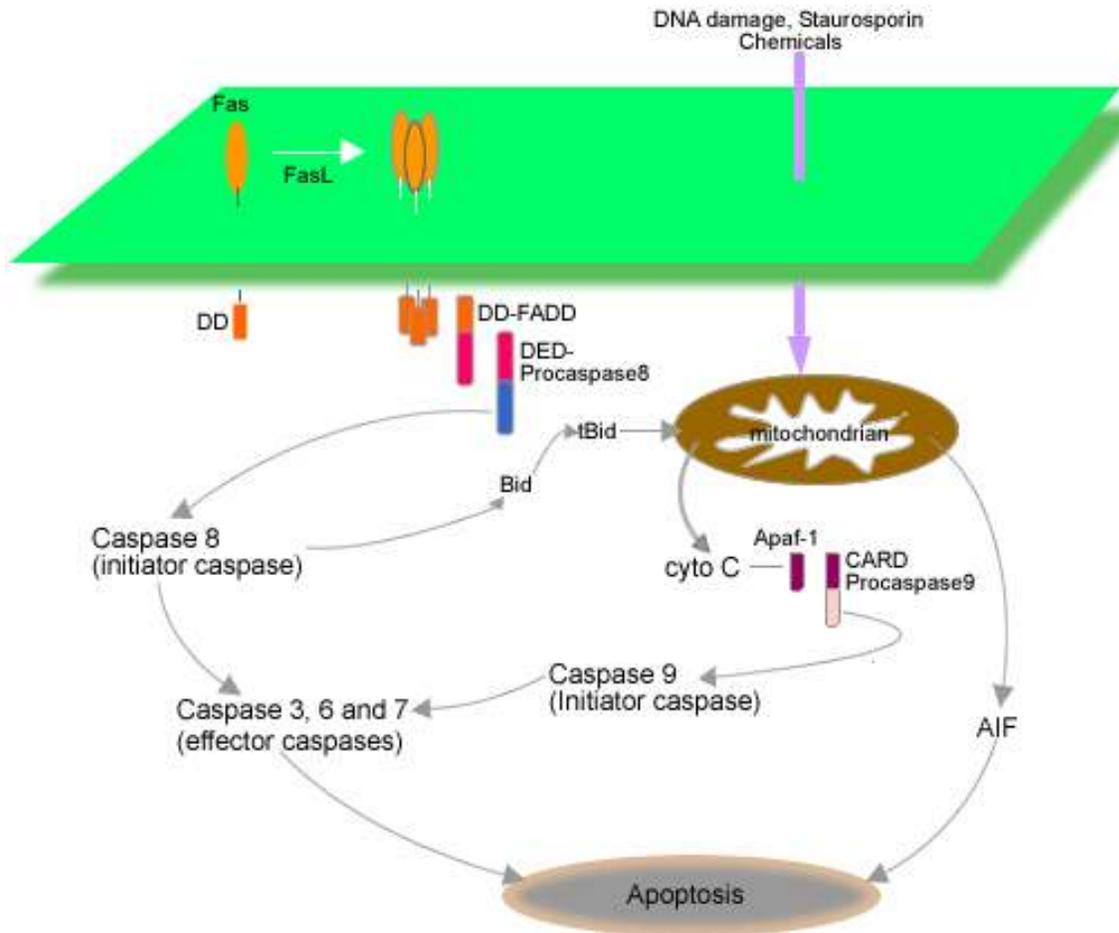


Figure 8. Death Receptor and Mitochondria Pathways of Apoptosis

Unlike Fas dependent apoptosis where caspase 8 and 10 are implicated, caspase 9 is the only known initiator caspase in mitochondria-dependent apoptosis (262). The other protein that is released from mitochondria is apoptosis inducing factor (AIF), which induces apoptosis or cell death in caspase independent manner (53, 220, 256). It is not yet

been elucidated, exactly how the intermembrane proteins leak from mitochondria into the cytoplasm. Outermembrane lysis (177, 230), and opening of nonspecific membrane channels on the outer membrane (56, 105, 112, 205, 250) are thought to cause intermembrane protein leakage.

The Bcl-2 gene family consists of pro and anti-apoptotic proteins and the family exercises control over mitochondria mediated apoptosis (2, 76). Cell death signals cause translocation of Bax from the cytoplasm to the nucleus and mitochondria (62). How Bax accomplishes mitochondrial membrane permeabilization and release of apoptogenic factors is not yet clearly explained. Bcl-2 and other anti-apoptotic proteins directly or indirectly inhibit the release of cytochrome C from mitochondria, which is essential for the activation of initiator procaspase-9 (116, 250, 261). Bcl-2 opposes the mitochondrial permeabilization (112, 148, 220, 230, 250, 256).

Abundance of proteins and disruption of calcium homeostasis in the endoplasmic reticulum (ER) may lead to ER stress which may also lead to apoptosis (166, 167). ER stress induced apoptosis seem to be mediated by caspase 12, as caspase 12 deficient mice are resistant to this pathway of apoptosis. The downstream effector molecules of this pathway are not yet identified.

5.3 Apoptotic Machinery: Caspases and Other Molecules

Caspases are an important component of apoptotic machinery. Caspase activity is one of the hallmarks of apoptosis (although it need not be true for all the apoptosis induced cells, as caspase independent pathways are also identified). Caspase activity distinguishes between necrosis and apoptosis, as caspases are not activated as part of necrosis (28). They are involved in the disassembly of the cell both as initiators as well as

effectors. Studies in the control or regulation of caspase activity forms potential research in therapeutic studies in controlling apoptosis.

Caspases were identified in an attempt to find the mammalian counterparts of *C. elegans*'s cysteine proteases (ceds). About 14 caspases have been characterized so far (170) that are expressed as inactive proenzymes. Caspases disintegrate DNA, induces the cell to display signals for phagocytosis, cleave proteins and are also instrumental in deactivating the inhibitors of apoptosis. Caspases activate DNase, cleave Bcl-2 proteins (2, 246) that are inhibitors of apoptosis, and activate other Bcl-2 members which are apoptotic and in this way participate in the positive feedback loop amplifying the proapoptotic signals. Caspases also engage in direct disassembly of the cell as illustrated by their catalytic action on the nuclear lamina (175, 222) that underlies nuclear membrane and is involved in chromatin condensation. Caspases deregulate proteins that are involved in DNA repair (DNA-PK), mRNA splicing (U1-70K), and DNA replication (replication factor C) (50, 190). It is not yet clear how deregulation of these factors mediate cell death.

Most of the caspases are synthesized in the form of precursors, which are activated by proteolytic processing. Some proteases or enzymes are capable of self-activation, and some can activate their own precursors ensuring the amplification of the protease activation and the speed with which they accomplish their action in important biological processes like the cell cycle and cell death. The binding of the death effector domain (DED) of procaspase8 and the death domain (DD) of FADD in DISC and likewise the binding of caspase recruitment domain (CARD) of procaspase9 to the apaf-1 and cytochrome C might be responsible for oligomerization of the initiator procaspases.

The effector caspases are proteolytically activated by the upstream initiator and mediator caspases. Caspase-8, 9, and 10 are initiator caspases; while caspase-3, 6, and 7 are effector caspases (208). A small amount of initiator caspases is sufficient to activate a plethora of effector caspases. Caspase-10 is similar in structure and has a large death effector domain like caspase-8 (235), its recruitment and involvement in the death receptor pathway is not yet identified (26, 212). Caspase activated DNase (CAD) forms a complex with the ICAD inhibitor of caspase activated DNase (ICAD) in its inactive form. Caspase-3 cleaves ICAD-CAD complex to activate CAD (129, 245) that cleaves DNA to produce DNA fragments. The caspases however cannot always be equated to apoptosis. Cell death is the subset of the many cellular changes induced by caspases (169). Caspases 1, 5 and 11 activate inflammatory cytokines (146).

It was observed that cells treated with Z-VAD.fmk (caspase inhibitor) and apoptotic stimuli did not show substrate cleavage, chromatin condensation or nucleosomal laddering, but rather showed other apoptotic features like cell shrinkage, membrane blebbing and ultimately cell death - suggesting that apoptosis like morphological changes can be taking place in a cell in a caspase independent pathway too (94). Various other studies (191, 201, 259) also showed that caspase inhibition did not stop the cells from eventually undergoing cell death. Although there is good evidence showing caspase independent pathways, the molecular mechanisms of caspase independent death remains poorly explained.

Apoptosis inducing factor (AIF) is the principal mitochondrial apoptosis factor in the caspase independent pathway, as in cell cultures depletion of AIF from intermembrane proteins ceased the nuclear apoptosis. AIF is a flavoprotein that is

confined to mitochondria in normal circumstances (218), but translocates to the nucleus on apoptotic stimuli. AIF on translocation to the nucleus causes chromatin condensation and high molecular wt DNA degradation (54, 216, 218).

The Bcl-2 family of proteins are another important molecules involved in the cellular response to apoptosis. At least 15 members of Bcl-2 family have been identified so far (249). All members contain at least one of the four domains BH1 to BH4 and those most similar to Bcl-2 have all four BH domains. Some of these are pro-apoptotic (e.g. Bcl-2 (92) which is a mammalian homolog for ced-9, Bcl-x_L (98)) and some are anti apoptotic (Bax, Bik (81, 173)).

Bcl-2 protects the cells from apoptosis due to gamma and ultraviolet-irradiation, cytokine withdrawal, dexamethasone, staurosporine, and cytotoxic drugs. Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and nuclear envelope (74, 255). Bcl-2 and other prosurvival proteins may bind to the cofactors of the initiator procaspases to deactivate or abrogate the ability of the cofactors to activate initiator caspases (102, 176). Bcl-2 interacts with mitochondrial permeability transitional (PT) pore complex (147, 168). It was observed that over expression of Bcl-2 and Bcl-x_L prevents the release of cytochrome C and Bcl-2 seems to inhibit apoptosis by inhibiting the release of cytochrome C (57, 72, 106, 154) from mitochondria rather than inactivating Apaf-1 (112, 250). Also, over expression of Bcl-2 stops the release of Apoptosis inducing factor (AIF) from mitochondria and is observed to stabilize transmembrane potentials (220, 230, 256),.

Nitric oxide (NO) is an important signalling molecule that regulates a wide range of physiological processes in cells. Nitric oxide modulates the chondrocyte metabolic

action and it is found to be produced by OA cartilage (9). So far, a lot of research has been done to understand the effects of endogenous NO (IL-1 β induced) and exogenous NO (from donors like sodium nitro prusside (SNP)) (25, 118, 119, 171). NO is observed to have dual mechanisms - i.e. it is proapoptotic and antiapoptotic as well. This dual mechanism of NO and its signal pathway is poorly explained. IL-1beta, a stimulus of inducible nitric oxide synthase (iNOS) produces NO (25, 118). However endogenous NO could not be correlated with proapoptotic activities, but produced apoptosis in the presence of oxygen radicals (25).

6. Apoptosis and Osteoarthritis

The Reduction in chondrocyte numbers or increase in empty lacunae in articular cartilage tissue are reported in many studies looking at OA (19, 153, 238) Chondrocyte death is proposed to have a key role in the pathogenesis of osteoarthritis. Aigner T *et al.* (4) observed that the rate of apoptosis and the number of empty lacunae did not match suggesting other forms of cell death in OA cartilage. Chondrocyte apoptosis was initially observed in endochondral ossification and hypertrophic region of growth plates (71, 86). Apoptotic chondrocytes are observed in C57/blackmice and wistar rats (1). Blanco *et al* were the first to report apoptotic cells in human OA cartilage (23) and subsequently many researchers observed apoptosis to be the primary type of cell death in OA pathology and implicated its importance in the progression of the disease (23, 83, 85, 93, 109, 133, 204). Excessive loading or unloading induces articular cartilage degeneration. Many research studies have been done to determine the effect of the various forces on chondrocyte viability, as the impact forces produce changes in cartilage akin to osteoarthritis. Different experiments with varying peak forces, number of cycles and amount of stresses during cyclic loading provides insight into the physiological tolerances of the tissue *in vivo*. Peak stress at the tibial articular surface - 4MPa (189), suggests tolerance levels of physiological range of stresses. 6.9 MPa (250 cycles) produced rapid cartilage damage, while less than 3.5 MPa did not produce damage even after 120000 cycles of compression (260). Canine cartilage explants loaded by 5 MPa for 2 mins showed 8% TUNEL positive chondrocytes, mostly localized to cell surface. This increased to 22% in explants loaded for 20 min extending to other layers. This increased further to 74% when loaded for 2h (46). Chen *et al.* observed that necrosis, rather than apoptosis was the

earliest event. Higher occurrence of necrosis might be due to the use of higher loads, which may cause traumatic or near traumatic injuries. Loads above 3.5 MPa produced significant cell death in human petallar explants (260). A strong correlation was observed between chondrocyte death and stress levels above 6 MPa (48). The rate of the mechanical stress also influences the amount of damage and chondrocyte death. Mechanical stress applied at higher rate inflicted cracks and chondrocyte death around the cracks, while stress at lower rates distributed chondrocyte death without significant cracks (183). Loening *et al.* reported apoptosis in calf cartilage explants at stress levels of 4.5 MPa (six cycles of 25 minute compression), and this correlation between TUNEL stained cells and stress levels is observed in other studies (51, 52).

Apart from the stress levels, studies were done to trace different apoptotic pathways at the molecular level in Osteoarthritic cartilage. CD95 (death receptor) and CD95L (ligand) are observed to be increased in aged rabbit articular cartilage, which showed decreased cellularity (226). Increased amounts of CD95L is found in the synovial fluid of OA joints (82). CD95 is observed to be expressed more in chondrocytes closer to lesions than those farther from lesions (110). These findings suggest the role for CD95 in the hypocellularity observations, though the precise mechanism is yet to be elucidated. CD95 receptor expression was observed in human OA articular cartilage in superficial and middle zones (239). Cell death via CD95 pathway can be initiated by CD95L derived from synovium (84). However, how CD95L crosses the barrier of the extracellular matrix, is not yet clearly understood. The CD95 activated apoptosis pathway is a positive feedback loop, with chondrocytes providing the amplifying signal by producing more CD95L (239). Cell adhesion is a survival factor for anchorage-dependent cell types.

Apoptosis resulting from lack of cell adhesion may be mediated by the CD95/CD95 ligand death receptor system (12, 68, 195). A gene regulator, p53 in apoptosis in many cell types is also found to correlate with apoptosis in OA cartilage (253). The Loss of collagen type IIA also seem to result in a decrease in the cell density, suggesting the importance of collagen type IIA and chondrocyte interactions for the survival of chondrocytes (248). Integrin receptors on the chondrocyte membrane provide the necessary interface between the cells and the matrix. Alpha1beta1 integrin is the major receptor expressed in chondrocytes. Inactivation of alpha1 gene in mice results in cartilage degradation, cell loss, proteoglycan depletion, and an increase in the number of TUNEL stained cells (258). This further confirms the fact that cell-matrix interactions are essential for cell viability and that extracellular matrix damage depletes survival signals to the chondrocytes. OA conditions such as matrix damage thus correlate very much with apoptotic favorable conditions.

7. Tissue Staining

Gross features of an apoptotic cell include chromatin condensation, cell shrinkage, membrane blebbing, appearance of protuberance on cell surface and division into apoptotic bodies to be phagocytosed (70). Apoptotic bodies are seen only for a few hours before they are phagocytised (36). The characteristic apoptotic bodies are short-lived and are difficult to distinguish from other cells when viewed by light microscopy. The number of cells undergoing apoptosis in a tissue undergoing 25% regression can be as low as 2-3% (37). In general it is difficult to distinguish and identify apoptotic cells based on their morphology alone.

Apoptosis, unlike necrosis is accompanied by water loss, cell shrinkage and nuclear fragmentation. The change in the cell size can be examined by flow cytometry. Apoptotic, necrotic and viable cells differ in their light scattering patterns (42). It is a rapid way of enumerating apoptotic cells; however these changes are not consistent in all cell types. Endogenous endonuclease activity and chromatin cleavage are characteristic biochemical feature in apoptosis (70). DNA is fragmented into large fragments by initial endonuclease activity (30, 234). Activation of Ca^{2+} - and Mg^{2+} - dependent endonuclease activity further cleaves the DNA at linker sites between nucleosomes (13). The DNA fragments are multiples of 180bp units. This phenomenon has been examined by agarose gel electrophoresis which measures DNA fragmentation in nuclear extracts showing DNA ladder configuration (13, 36).

One of the earlier methods used to identify apoptosis was gel electrophoresis of the DNA extract. Appearance of the ladder of nucleosomal DNA fragments became a hallmark of apoptosis. An improved method to analyze DNA fragmentation is to visualize DNA fragments after Southern blotting them with radiolabelled probe which increases the sensitivity. These methods can be effective in finding the presence of apoptosis in a tissue, however, are ineffective to mark apoptotic cells in a tissue. DNA strand breaks can be examined *in situ* by *in situ* nick translation (ISNT) (70, 203) or by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique (70). Both techniques work by labeling the free 3' – hydroxyl (OH) termini of the DNA strand breaks with modified nucleotides. These free ends that are generated upon DNA fragmentation are localized in apoptotic bodies. Normal or proliferative nuclei, which contain insignificant numbers of 3'- OH ends, don't stain. In comparison to staining by ISNT, TUNEL has higher sensitivity (73). The major advantage of 3' –OH end labeling include the ability to detect early-stage apoptosis where chromatin condensation has begun and there are fewer free 3' – OH ends (45), and the ability to mark apoptotic cells within heterogeneous populations. In the TUNEL assay, chemically labeled and unlabeled nucleotides, that are contained in the reaction buffer, are enzymatically added to free 3' – OH ends of DNA strand breaks by terminal deoxynucleotidyl transferase (TdT) (70). The added nucleotides form an oligomer composed of digoxigenin-conjugated nucleotide and unlabeled nucleotides in random sequence. Anti-digoxigenin antibody, conjugated to a peroxidase reporter molecule, is allowed to bind to DNA fragments that have been labeled with the digoxigenin-nucleotide.

8. Purpose of Study

Osteoarthritis is a severe degenerative joint disease and. The symptoms associated with osteoarthritis are well documented, but the bone matrix changes that trigger onset of osteoarthritis disease have yet to be fully understood. Reduction in chondrocyte numbers or an increase in empty lacunae in articular cartilage tissue are reported in studies looking at osteoarthritis (19, 153, 238). Chondrocyte apoptosis and apoptotic pathways in the pathology of osteoarthritis has yet to be elucidated, however apoptosis is understood to have prominent role in the osteoarthritis (23, 83, 85, 93, 109, 133, 204). Impact injury can lead to cellular and matrix changes similar to that seen in osteoarthritis (47, 60). It is important to understand the time-dependent cellular changes in response to mechanical injury that is known to cause Osteoarthritic-like changes in the cartilage. Very few studies have examined the development of chondrocyte apoptosis with respect to time, zones within the cartilage, and distance from the site of injury. It is also still unclear as to how these factors effect the early articular cartilage degeneration. The purpose of the current study was to investigate chondrocyte apoptosis in relation to distance from the site of injury, the magnitude of impaction forces, its time dependent development and the interaction of these factors.

8.1 Hypotheses

Impaction injury of patella articular cartilage causes cellular and matrix changes similar to that found in osteoarthritis. The cellular behavior is dependent on the mechanical loads, days in culture and distance from the site of injury.

Chondrocyte apoptosis will be high for impacted porcine knees compared to non-impacted controls. Chondrocyte apoptosis will increase for medium loads on the surface

zone and increase for high loads on surface and deep zones. Chondrocyte apoptosis will be higher at the site of impaction and decrease with distance from the site of impaction.

9. Methodologies

Paired porcine knees were brought fresh from the slaughter house (City Packing Inc, Burlington, NC) within three hours of slaughter. Patellae were removed from knee joint using sterile techniques. Controlled impactions to the cartilage surface (control = no load, medium = 1000 N or high = 2000 N) were made on both the facets of cartilage of patellae. Lateral facets were used for the study and medial facets were stained with MTT for separate study on chondrocyte viability. The patellae were put in organ culture for 0, 3, 7 or 14 days. Apoptosis analysis was performed through the depth of tissue using light microscopy.

9.1 Randomization of Specimens

A completely randomized split plot design was used to set up the experiment. The samples were randomized using Excel software. For each load condition, each culture time was paired up with other three culture times using paired left and right patellae, giving 12 treatments for each load, 9 for each culture time and 18 for each leg (Table 3, Table 4).

Table 3. Impaction Groupings - left and right pairs from same animal

| Control | | Medium | | High | |
|---------|-------|--------|-------|------|-------|
| Left | Right | Left | Right | Left | Right |
| 3 | 0 | 3 | 0 | 3 | 0 |
| 7 | 0 | 7 | 0 | 7 | 0 |
| 14 | 0 | 14 | 0 | 14 | 0 |
| 7 | 3 | 7 | 3 | 7 | 3 |
| 14 | 3 | 14 | 3 | 14 | 3 |
| 14 | 7 | 14 | 7 | 14 | 7 |

Table 4. Randomization of Specimens

| Treatment | | Randomization number |
|-----------|-------|----------------------|
| Left | Right | |
| M3 | M14 | 0.012737 |
| H0 | H3 | 0.06598 |
| C7 | C14 | 0.075959 |
| H0 | H14 | 0.196122 |
| M0 | M3 | 0.205526 |
| C0 | C7 | 0.213661 |
| C3 | C7 | 0.254995 |
| M7 | M14 | 0.298631 |
| H7 | H0 | 0.326353 |
| H3 | H7 | 0.328928 |
| C3 | C14 | 0.521827 |
| C14 | C0 | 0.572349 |
| H3 | H14 | 0.612532 |
| C0 | C3 | 0.622903 |
| M0 | M7 | 0.681366 |
| M3 | M7 | 0.855188 |
| H7 | H14 | 0.885797 |
| M14 | M0 | 0.90603 |

Specimens naming convention was based on Load (C = Controls, M = Mediums, H = Highs), Culture time (0, 3, 7, 14 days), leg type (L = Left, R = Right) as shown in Table 5.

Table 5. Order of Performance of Experiments

| Order | Load | Culture time | Leg | Specimen Name |
|-------|---------|--------------|-------|---------------|
| 1 | Medium | 7 | Right | M7R |
| 2 | Medium | 14 | Left | M14L |
| 3 | High | 7 | Right | H7R |
| 4 | High | 0 | Left | H0L |
| 5 | High | 3 | Right | H3R(a) |
| 6 | High | 7 | Left | H7L |
| 7 | Control | 3 | Right | C3R |
| 8 | Control | 14 | Left | C14L |
| 9 | Control | 14 | Right | C14R |
| 10 | Control | 0 | Left | C0L |
| 11 | High | 3 | Right | H3R(b) |
| 12 | High | 14 | Left | H14L |
| 13 | Control | 0 | Right | C0R |
| 14 | Control | 3 | Left | C3L |
| 15 | Medium | 0 | Right | M0R |
| 16 | Medium | 7 | Left | M7L |
| 17 | Medium | 3 | Right | M3R |
| 18 | Medium | 7 | Left | M7L |
| 19 | High | 7 | Right | H7R |
| 20 | High | 14 | Left | H14L |
| 21 | Medium | 14 | Right | M14R |
| 22 | Medium | 0 | Left | M0L |
| 23 | Medium | 3 | Right | M3R |
| 24 | Medium | 14 | Left | M14L |
| 25 | High | 0 | Right | H0R |
| 26 | High | 3 | Left | H3L |
| 27 | Control | 7 | Right | C7R |
| 28 | Control | 14 | Left | C14L |
| 29 | High | 0 | Right | H0R |
| 30 | High | 14 | Left | H14L |
| 31 | Medium | 0 | Right | M0R |
| 32 | Medium | 3 | Left | M3L |
| 33 | Control | 0 | Right | C0R |
| 34 | Control | 7 | Left | C7L(a) |
| 35 | Control | 3 | Right | C3R |
| 36 | Control | 7 | Left | C7L(b) |

9.2 Patella Removal

All the instruments and the laboratory equipment used for dissecting the patella were dry sterilized in an autoclave at 121⁰C for 55 min in a gravity cycle and 60 min dry time, prior to the day of the experiment. On the day of the experiment or the day of removal of the patella from the knee joint, sterile paper sheets were placed on a countertop cleaned with 70% ethanol. A knee obtained fresh from a slaughter house was placed on the sterile sheet. The knee was sprayed with butadiene and wiped with sterile cotton pad, followed by spraying with 70% ethanol. The knee was then transferred onto a fresh sterile paper sheet. The person extracting patella wore surgical mask, sterile surgical gown, sterile gloves and care was taken to follow aseptic techniques while dissecting the patella from the porcine leg. Before doning sterile gloves, all the required instruments for dissection were put on the paper sterile sheet. Connective tissue was shaved off the bony non articular surface of the patella using a scalpel blade (No.10) and single edge razor blades, before dissecting patella free from the joint. After the patella was dissected free of the knee joint, the cartilage surface of the patella was inspected for any surface damage. Only non-damage patellae were used and any cartilage with visual surface damage was discarded. Care was taken to keep the cartilage surface wet by spraying it with phosphate buffered saline (PBS, ph-7.4) intermittently using a plastic sterile squirt bottle. The connective tissue around the edges of cartilage surface was removed using sterile single edge razor blade with extra care. Once removed from the joint, the patella was sprayed with PBS thoroughly to wash off any surface particles. The dissected patella was then put

in PBS with 1% penicillin and streptomycin (Sigma, USA #P-4333) until they could be potted in polymethyl methacrylate (PMMA) mold for testing. Hemispherical shaped PMMA molds were made to hold the patella during impaction, as shown in figure 9.



Figure 9: Patella Placed in PMMA Mold

The PMMA mold was prepared immediately after patella extraction. It was prepared by mixing 80g sterile PMMA powder and sterile PMMA monomer (Osteobond Copolymer Bone Cement, Zimmer, Inc., USA #1101-08) at room temperature. The patella was pressed into the PMMA mold to create an imprint and was removed immediately to prevent any heat related damage to the patella. The PMMA mold was then allowed to set and the patella was placed in the mold, once it was set. The right and the left knees were dissected in pairs.

9.3 Patella Impactions

The stainless steel holder that holds the PMMA mold in the MTS Mini Bionix II hydraulic load frame (MTS Corp., Minneapolis MN, USA) (figure 10), and wooden

sticks (to apply dye on tissue) were steam sterilized in an Autoclave at 121⁰C for fifty five minute gravity cycle and sixty minute dry time, prior to the experiment day.



Figure 10. The MTS Mini Bionix Hydraulic Load Frame

Sterile paper sheets were placed around the sterile steel holder on the load frame. The PMMA mold-embedded patella was secured tightly in the matching spherically shaped cavity of the steel holder which allowed the articular surface of each facet to be aligned perpendicular to the impactor.

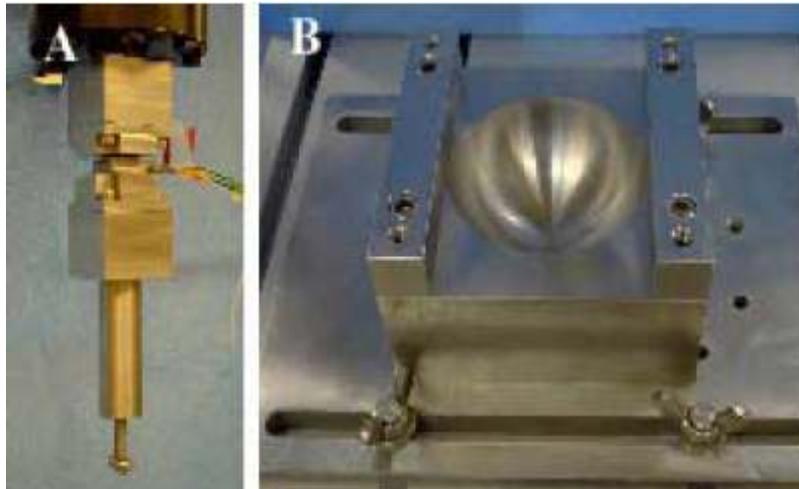


Figure 11. Impactation Apparatus A. Impactor and three-degree of freedom Piezo electric load cell B. Holder for potted patellae

The cylindrical, stainless steel, non porous impactor was 10 mm in diameter and 7.7 mm long. The impactor was in series with a piezoelectric load cell, which measured the applied radial, shear, longitudinal shear and axial forces (figure 12)

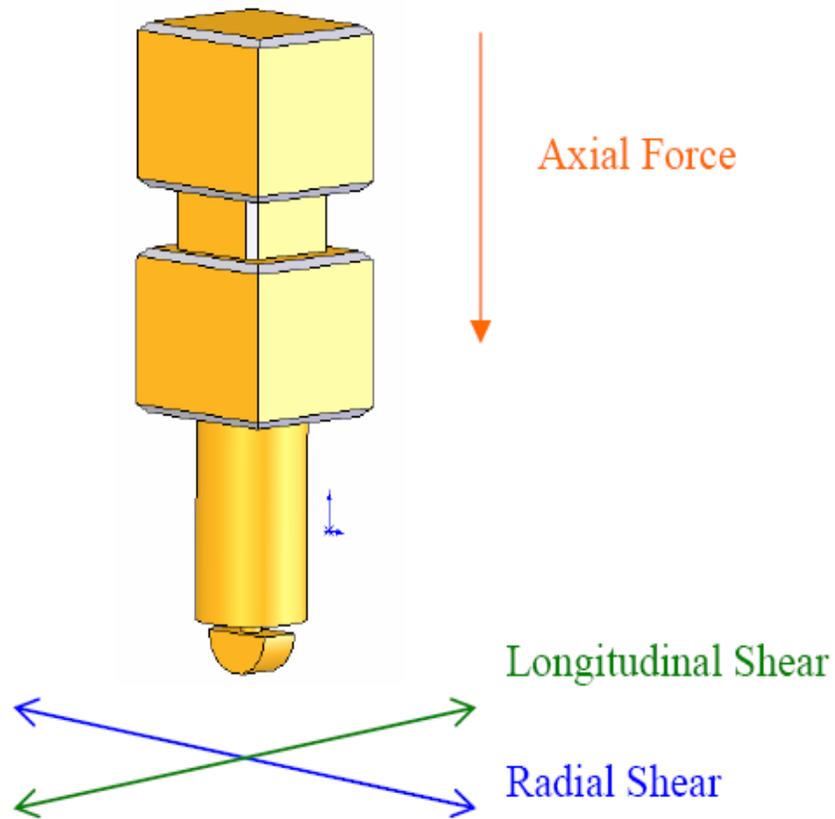


Figure 12. Piezoelectric Load Cell - measurement of force in three directions in series with the contoured impactor

Patellae were marked with two dots using green colored tissue marking dye (Polysciences, Inc. USA #24110), prior to the impaction, to facilitate the location of impaction during histological analysis and to act as a landmark for repeatable location of impaction site (Figure 12). The dye was filtered with sterile syringe filter before use. The dye was applied using a sterile wooden stick.



Figure 13. Location of Tissue Dye Marks (on one facet)

Care was taken to keep the patella hydrated by periodically spraying it with sterile PBS. Controlled mechanical impactions were made on each facet at 25 mm/s, using the MTS load frame, to pre selected force levels of 1000N (characterized as medium) and 2000N (characterized as high).



Figure 14. Mechanical Impactions on Patella A. Patella secured In steel holder B. Patella and impactor just before the impaction.

Non-impacted patellae were used as controls. Each Patella was impacted twice, once on the middle of each facet. Cartilage from the lateral facet was used for the apoptosis. analysis.

9.4 Patella Organ Culture

Following the impaction patellae were separated from the PMMA mold and each patellae was thoroughly rinsed with PBS with 1% penicillin and streptomycin. The patellae were then rinsed three times by placing them in three sequential beakers containing PBS (mixed with 1% Penn Strep) for five minutes each. After the last wash the patella was again thoroughly sprayed down with PBS. Each patella was then fitted on a steel holder in a deep Pyrex dish. The dish was then filled with enough culture media, to cover the patella. The culture media was prepared one day prior to the experiment day. Each bottle of culture media consisted of 500ml Dulbecco's MEM/Ham's F-12 (Sigma, USA #D8437) with 50ml of 10% Fetal Bovine Serum (Sigma, USA #F2442), 12.5 mg L-Ascorbic Acid 2-Phosphate (Sigma, USA #A8960) and 5ml Penicillin-Streptomycin (Sigma, USA #P-4333).

The Pyrex dishes were then put in a humidified incubator at 37⁰C with 5% CO₂. The patella in the medium and the incubator are shown in Figure 15.

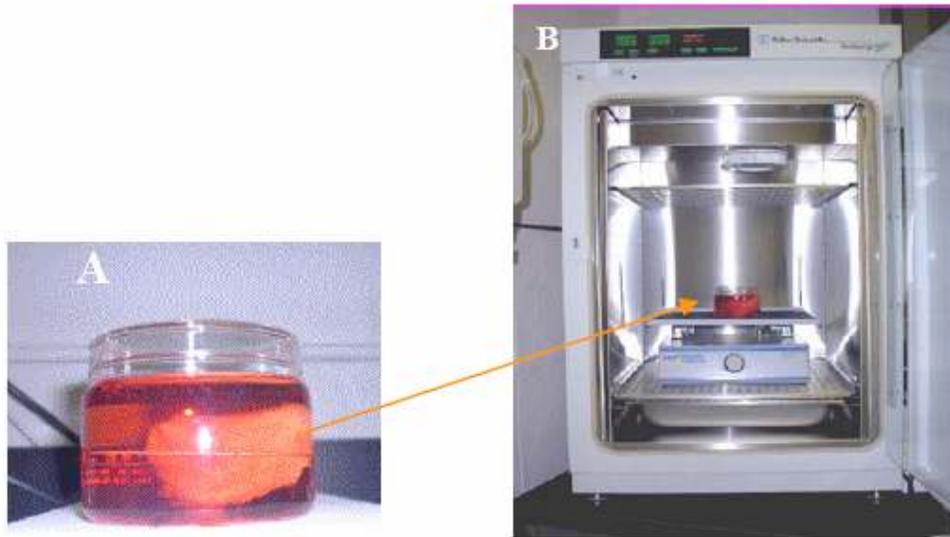


Figure 15. Patella in Culture Medium and Incubator (132)

The Pyrex dishes were placed on the rocking platform in the incubator, the speed of which was adjusted so that the media in the dish was constantly stirred so that it could be evenly distributed. The culture media was changed daily (after 24 hours) inside a laminar flow hood. The old media was removed using an aspirator and 10 ml sterile pipette. Fresh media at room temperature was poured in the Pyrex dishes aseptically. The patellae was again placed in the incubator for another 24 hours and the process was repeated until the patella was taken out of culture. The culture times chosen were 0, 3, 7 or 14 days. For the 0 days culture specimen, the patellae were not placed in culture medium.

9.5 Tissue Analysis

Once the patellae were removed from culture they were cut into two halves – lateral and the medial facets, using a band saw. The lateral facet was taken out and the center of impaction was remarked with tissue marking dye, joining the two points used to

identify the center impaction. After marking with dye, digital pictures were taken labeling the picture appropriately. Rectangular cross-sections (approximately 8x15 mm) were separated from the subchondral bone of each facet using a scalpel blade. The harvested cartilage section was cut in half perpendicular to the tissue dye line. The cartilage sections were placed in a mold filled with OCT compound, so that the dye line was perpendicular to the base as shown in Figure 16:

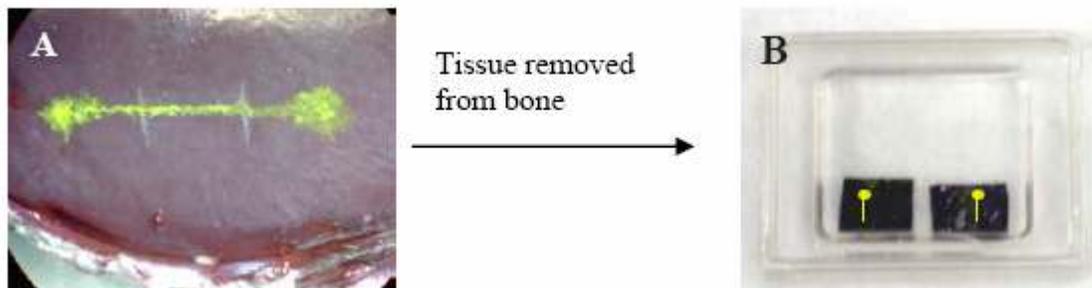


Figure16: Placement of Cartilage in OCT Compound in Mold

The OCT compound containing the facets was flash frozen by holding the mold in 100% ethanol containing dry ice pellets. Care was taken so that ethanol did not splatter into the mold. Frozen tissue samples were labeled and placed in a freezer at -80°C until they could be sectioned. Cryosections at $6\mu\text{m}$ were cut using a Leitz Cryostat. Sections were then put on aminoalkylsilane-coated microscope slides.

The slides were then stained using the TUNEL staining procedure. The Apop Tag apoptosis detection kit detects apoptotic cells *in situ* by specific-end labeling of the DNA fragments using the TUNEL method. For the TUNEL staining procedure, the cryosection was fixed in 1% paraformaldehyde (0.48g/3ml dH_2O dissolved, added to 45 ml PBS) for 10 min at room temperature in a coplin jar. After 10 minutes, the excess liquid was drained. The slide was then washed in 2 changes of PBS for 5 minutes each. The sections

were then fixed in glacial acetic acid + absolute ethanol (30 ml + 15 ml) in coplin jar for 5 minutes at -20°C for 5 minutes and washed in two changes of PBS for 5 minutes each. Endogenous peroxidase was quenched in working strength H_2O_2 (36 ml of PBS + 4 ml of 30% H_2O_2) for 5 minutes at room temperature in a coplin jar and then rinsed twice with dH_2O in a coplin jar for 5 minutes each. After two washes, the excess liquid was tapped off and the liquid around the section was carefully aspirated away using aspirator. Seventy five μL of equilibration buffer was applied directly on the specimen and incubated for 10 seconds at room temperature. After 10 seconds, the excess liquid was tapped off and the liquid around the section was carefully aspirated using an aspirator and pipette. Fifty five μL of TdT enzyme was applied directly onto the specimen which was then incubated in the humidified chamber for 1 hour. The specimens were placed in a coplin jar with working strength stop/wash buffer (1ml of stop/wash buffer + 34 ml of dH_2O) and agitated for 10 seconds. The coplin jar was then placed in the incubator (maintained at 37°C with 5% CO_2) for 10 minutes. After 10 minutes the specimens were washed with 3 changes of PBS in coplin jar for 1 minute each. Sixty-five μL of anti-dioxigenin peroxidase was then applied directly on the specimens and the slides were kept in a humidified incubator (maintained at 37°C with 5% CO_2) for 30 minutes. After 30 minutes, the specimens were washed in 4 changes of PBS for 2 minutes each. After that, the excess liquid was tapped off and the liquid around the section was carefully aspirated or blotted. Seventy five μL of working strength Peroxidase substrate (one hundred forty seven μL of diAmino benzyne (DAB) dilution buffer + three μL of DAB substrate) was applied directly onto the specimen for 5 minutes. The sections were counterstained using 0.5% methyl green in a coplin jar for 10 minutes. After 10 minutes,

the specimens were washed in 3 changes of dH₂O. In the first two changes, the specimen was dipped 10 times and the third change, the slides were allowed to sit in coplin jar for 30 seconds. The washing procedure was repeated with dH₂O replaced with 100% 1-butanol. The slides were then moved through three jars of xylene, 2 minutes for each jar. The excess moisture was drained without allowing the specimen to dry. Finally the sections were mounted under a cover slip using Permount (Fisher Scientific) mounting medium.

9.6 Data Collection

Previous experiments using ultra low load Fuji film on separate test patella determined that a value of 1640 μ m (or 1.64mm) contact radius (r), for a medium impaction and 1.795 mm was the contact radius for high force impactions. These values were used to photograph the stained tissue at various distances ($\pm 2r$, $\pm r$, $\pm r/2$) from the center of impaction using high resolution spot CCD camera attached to a NIKON Y-FL research microscope under light microscopy.

Starting at the center of impaction, pictures were taken at an increment of 173 μ m through the depth of the tissue from the surface to the deep zone. A journal was written using Metamorph (Molecular devices) to allow the microscope stage to move from surface zone to deep zone at increments of 173 μ m. Each picture at these increments was 223 μ m in width.

Once the pictures were taken from the surface to the deep zone, a montage was created that stacked the individual images as a single column. The process was repeated at radial distances of $\pm 2r$, $\pm r$, $\pm r/2$, from the center of impaction (Figure 17).

Each column was divided into 10 equal rectangular regions using Metamorph software to facilitate the analysis of apoptotic cells through the thickness of each cartilage layer (Figure 18).

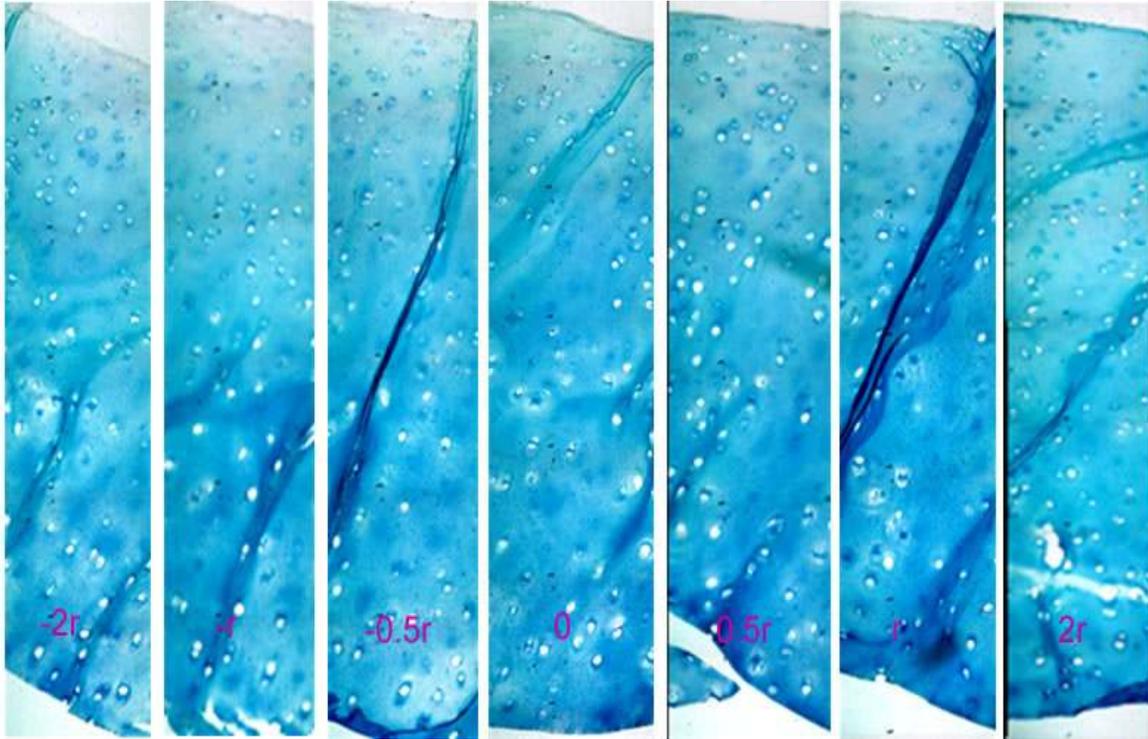


Figure 17. A Collective Montage (one for each radial position)



Figure 18. Tissue Column (divided into 10 regions)

9.7 Quantification of Apoptotic Cells

The number of apoptotic and live cells were counted in all the regions using Metamorph software. All the brown and dark colored cells were designated as apoptotic cells and all the unstained cells were designated as live. Cells were counted manually by labeling the apoptotic cells green and live cells red.

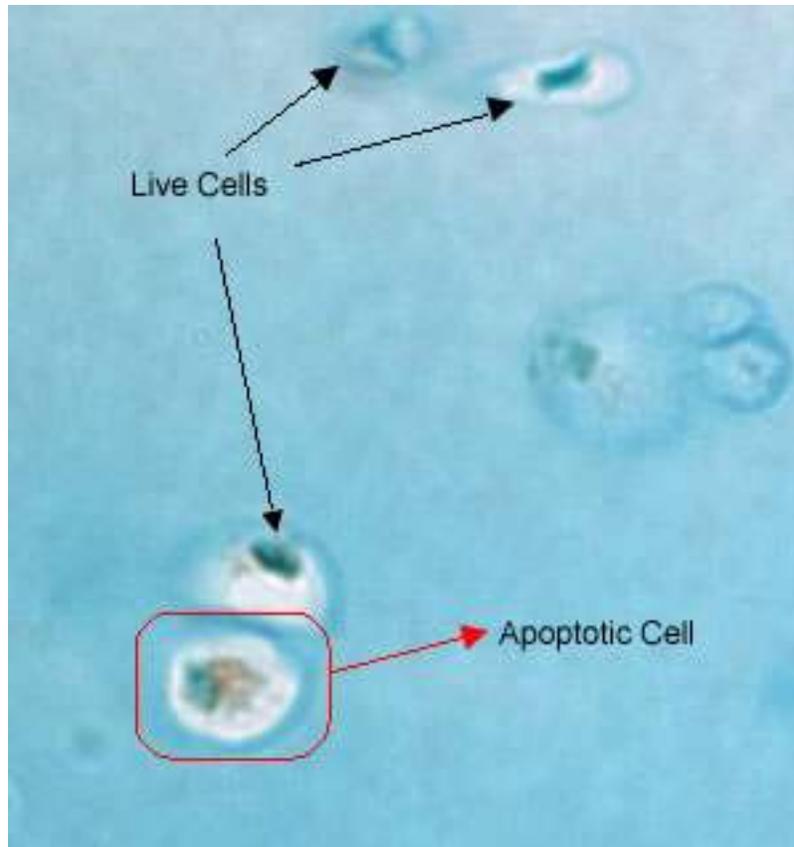


Figure 19. Tissue after TUNEL Stain - showing apoptotic and live cells (picture of medium-0days-center-depth1-2)

Cells at the borders of each region were counted only if they were at least halfway within the region. The number of live cells, apoptotic cells and total number of cells in each region and also total number of cells in the entire column were entered into an Excel spread sheet. Some of the samples did not span the entire radial distance to $\pm 2r$ from the center of impact. Hence not all the samples provided information at the larger radial distances. This was because the specimens were not cut wide enough to cover the entire array of radial positions. For a few samples, live cells and stained cells were not counted at smaller radial distances, if the specimen has very few cells in the section due to damage during specimen preparation.

9.8 Statistical Analysis

The above collected data were logged into text files and exported to Microsoft Excel sheet. The region with zero number of total cells was not included in the data set, as excel sheet because that did not truly represent a case of 0% of apoptotic cells. ANOVA was performed using significance level $\alpha=0.05$ using SAS software (Appendices E.1, E.2). Changes in the percentage of apoptotic cells were analyzed with experimental factors of impact level (control, medium, high), time in culture (0, 3, 7, 14), distance from center of impaction (center of impaction, $\pm r/2$, $\pm r$, $\pm 2r$), and depth in tissue (levels 1-10, 1 being the surface layer and 10 being the deep layer just above the calcified zone of articular cartilage). Load-culture, load-depth, load-radial, and radial-depth interaction effects were assessed using multiplicity correction.

10. Results

A total of 36 porcine patellae were used. The experiment was designed to have an equal number of left and right patellae (18 each), and 3 replicate patellae per load-by-treatment combination. Three patella were used for each of the culture times (0 days, 3 days, 7 days and 14 days), for each of the impaction forces. The impaction forces obtained for medium impactions were 1047 ± 122 N (mean \pm standard deviation) normal, 132 ± 77 N radial shear and 48 ± 29 N longitudinal shear. The impaction forces obtained for high impactions were 2239 ± 178 N normal, 233 ± 164 N radial shear, and 81 ± 61 N longitudinal shear. Apoptotic cells and live cells were counted and the percentage of apoptotic cells was determined at all the radial positions ($r = 0, \pm r/2, \pm r, \pm 2r$) through the depth of the tissue. Preliminary analysis showed no significant difference between the negative and positive radial positions ($p=0.369, 0.6789, 0.134$ for $\pm 2r, \pm r, \pm r/2$ respectively) . Further analysis was done using absolute value of the radial positions so that radial positions on either side of the center of impactions were considered the same.

Overall analysis showed load-culture ($p<0.0001$), load-culture-radial position ($p<0.0001$) and load-culture-depth ($p=0.0048$) interaction effects. Radial distance effect ($p=0.0507$) was nearly significant at level $\alpha=0.05$. The overall percentage of apoptotic cells were 8.55 ± 11.83 for controls, 8.55 ± 11.76 for mediums, and 9.34 ± 13.61 for highs. Significant difference was observed between 3 days culture samples and 7 days culture samples ($p=0.0209$), where the percentage of apoptotic cells were 9.66 ± 13.35 for 3 days culture time and 7.47 ± 12.56 of apoptotic cells for 7 days in culture. A slight increase in the percentage of apoptotic cells from 0 days culture time (8.88 ± 11.84) to 3

days culture time (9.65 ± 13.35) for all loads was noted but was not significant ($p=0.5857$). A significant difference was observed ($p=0.0321$) between depth 1 and depths 3-8, where depth 1 has highest number percentage of apoptotic cells (13.07 ± 12.98) for all loads and culture times.

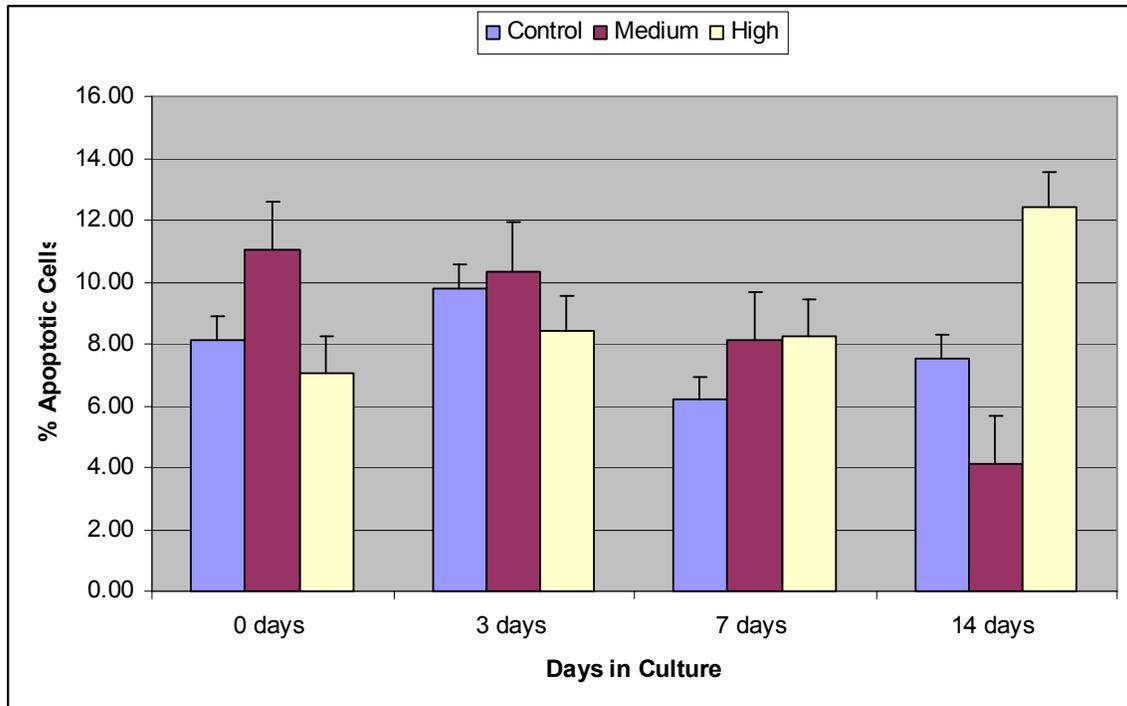


Figure 20. Percentage of Apoptotic Cells by Culture Time – for all loads (control, medium, high), Y error bar, standard error +1

The difference in controls over days of culture was not significant ($p=0.1427$) indicating no change in percentage of apoptotic cells in non-impacted cartilage placed in medium *in vitro*. A significant difference between controls and highs after 14 days of culture was observed, where percentage of apoptotic cells were 7.54 ± 13.8 for controls and 12.45 ± 15.23 for highs, indicating percentage apoptotic cells increased in highs only after 14 days. However a significant difference in mediums and highs was observed

immediately after the impaction, with mediums having higher percentage of apoptotic cells (11.03 ± 14.25) than highs (7.21 ± 9.44) after 0 days of culture. The percentage of apoptotic cells was higher for high-14 days culture time in the deeper zones and for medium-0days culture time in the surface zone. Simple effects of depth across all the levels of load-culture and simple effects of culture across all levels of load-depth were analyzed. An increase in the percentage of apoptotic cells was observed for medium-0 days at depth 2, 9 and 10 (15.79 ± 16.66 , 19.22 ± 23.46 , and 13.82 ± 15.65), and for medium-7 days at depth 1 (20.54 ± 8.14) compared to non-impacted controls. A decrease in percentage of apoptotic cells was observed for medium-14days in depths 9 and 10 (0.95 ± 3.68 , and 4.64 ± 7.69). An increase in percentage of apoptotic cells was observed for high-14 days at depths 8 and 9 (13.84 ± 13.75 and 19.99 ± 20.86). The percentage of apoptotic cells for high-14 days culture time at depths 8 and 9 were 13.84 ± 13.75 and 19.99 ± 20.86 respectively, indicating high forces may induce a higher percentage of apoptotic cells in deeper zones after 14 days of culture and medium forces may induce higher percentage of apoptotic cells in the surface and deeper zones immediately after impaction.

There was a significant load-culture-radial position effect ($p < 0.0001$). However since percentage of apoptotic cells for some samples could not be calculated at the 2r radial position, the load-culture-radial position interaction effect was analyzed only for radial distance upto a distance r from center of impaction. For the simple effect of radial position across all the combination levels of load-culture, medium-0 days ($p < 0.0001$) showed significant effect. There was a significant difference in the percentage of apoptotic cells in mediums over controls and highs immediately after impaction and at

the center of impaction. Medium force produced a higher percentage of apoptotic cells (24.09) after impaction (0 days of culture time) and at center of impaction.

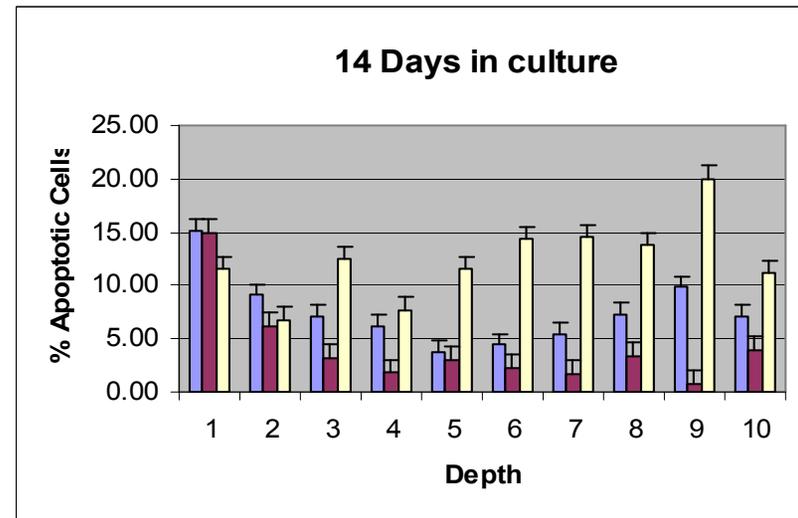
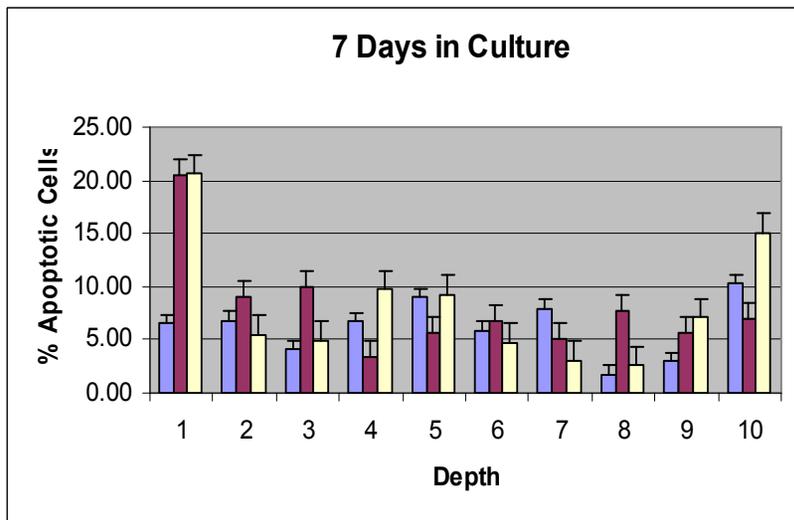
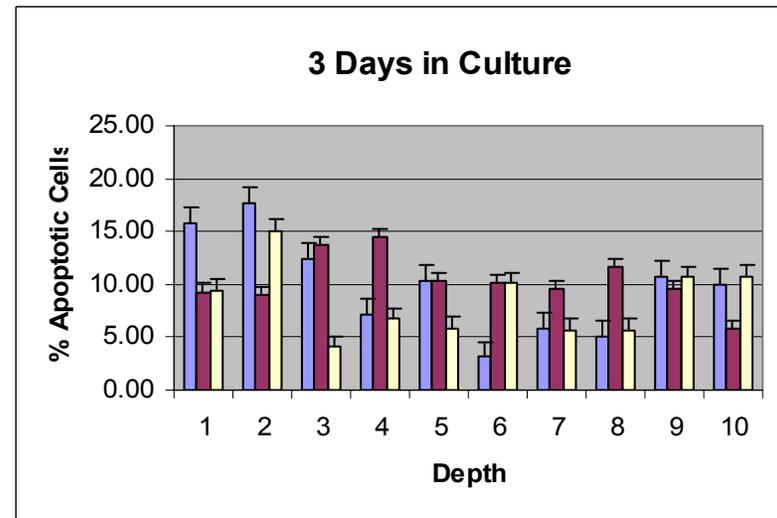
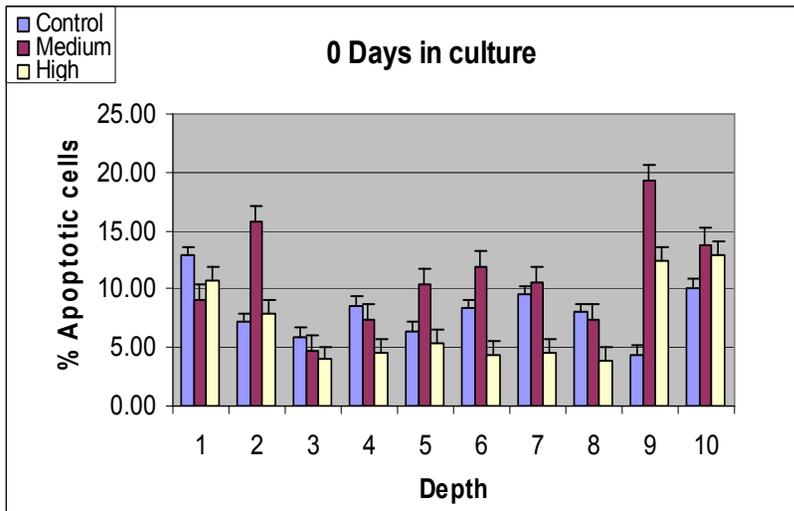


Figure 21. Percentage of Apoptotic Cells by Tissue Depth - for all loads (Control, Medium, High) and for all culture times (0,3,7,14 days). (Y error bars, standard error, +1)

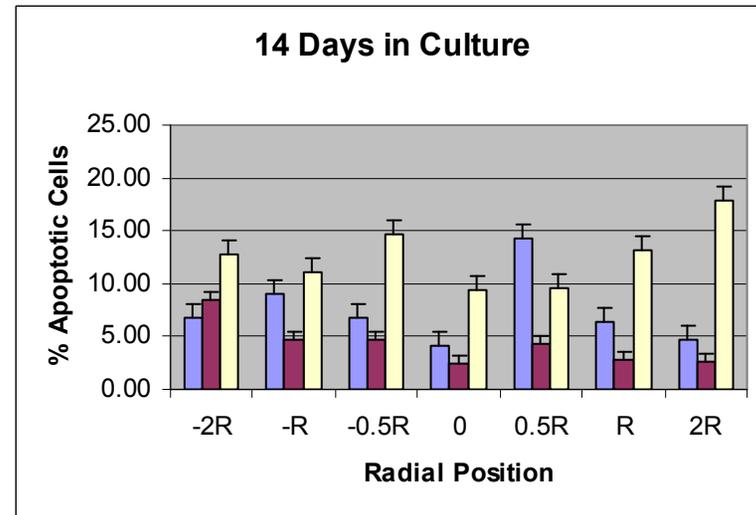
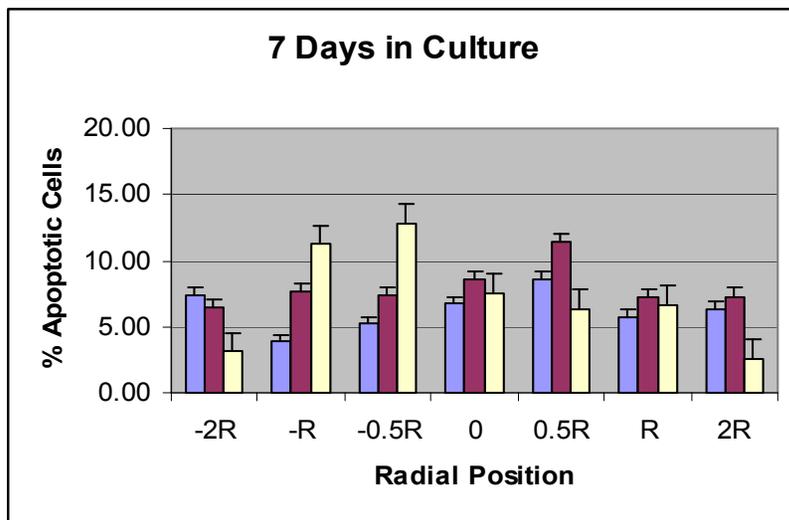
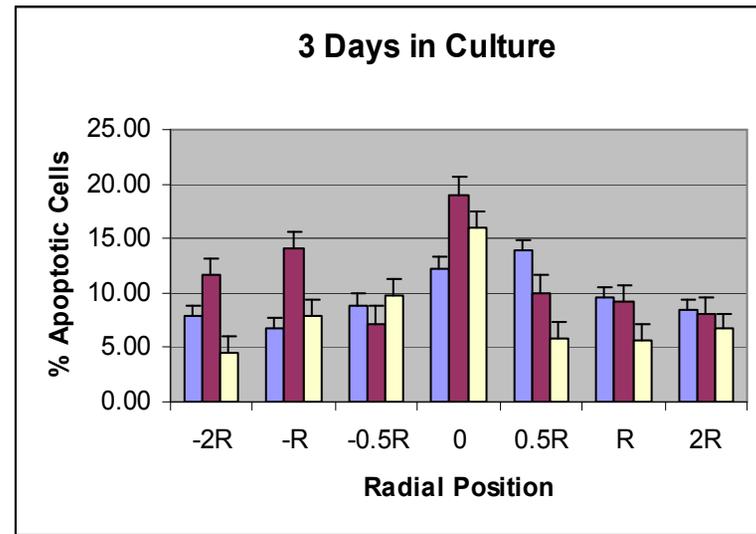
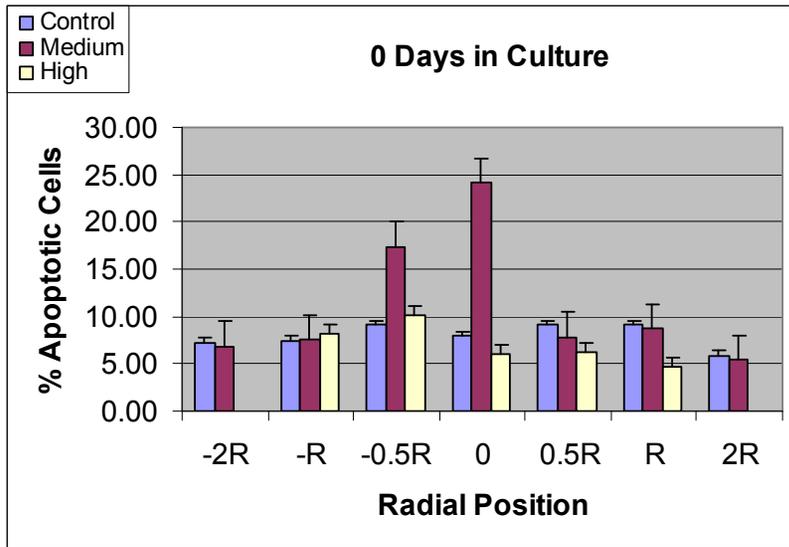


Figure 22. Percentage of Apoptotic Cells by Radial Position - for all loads (Control, Medium, High) and for all culture times (0,3,7,14 days). (Y error bar, standard error, +1).

11. Discussion

The main objective of the study was to investigate chondrocyte apoptosis caused by impactions of different force levels at various culture times after the impaction. Several studies have shown that impaction injuries on articular cartilage cause changes akin to OA cartilage (19, 29, 46, 51, 153, 238). A decrease in the chondrocyte numbers in articular cartilage is reported in studies looking at OA (19, 153, 238). However the mode of cell death in OA is not yet elucidated. Aigner T *et al.* (4) observed that the number of empty lacunae and the rate of apoptosis did not match suggesting necrosis was the mode of cell death in OA cartilage. Blanco et al (23) was first to report chondrocyte apoptosis in human OA cartilage and subsequently many researchers observed apoptosis to be the primary mode of cell death in OA (23, 83, 85, 93, 109, 133, 204). However it is still unclear if the degenerative changes in OA cartilage are cell-mediated or caused by matrix related changes. No known studies have investigated the location-specific and time related effect of impact loads on chondrocyte apoptosis. In this study, the effect of the loads on chondrocyte apoptosis was seen together with days in culture, distance from the center of impaction, and depth of the tissue.

The findings in this study showed significant correlation between load-culture-depth and load-culture-radial position. Tissue sections excluded from study due to irregularities reduced the power of statistical analysis. For example the H0 samples did not provide data at $\pm 2r$. In agreement with the first statement of hypothesis, it was observed that chondrocyte apoptosis depended on impact load and culture of time. The depth dependent changes in chondrocyte apoptosis depended on both load and culture

time. The radial positions dependent changes in chondrocyte apoptosis too depended on load and culture time.

In summary, medium impactions induced chondrocyte apoptosis at the center of impaction in surface and deep zones, immediately after impact (0 days culture time) and the presence of chondrocyte apoptosis continued to remain the same in the surface zone but decreased in the deeper zones after 3 days of culture time and were a minimum after 14 days of culture time. Jennifer Lossing (132), in a similar study observed that medium forces induced cell death (necrosis) in surface and deep layers and the presence of cell death continued to occur mostly in the middle to deep layers with increasing time in culture. Comparison of cell viability study (132) and this study suggests that mode of cell death induced by medium force impactions is a combination of necrosis and apoptosis after impaction and predominantly becomes necrotic with increasing time in culture. Triantafillopoulos *et al.* (228), in a study conducted on rabbit knees observed a combination of necrotic and apoptotic-like cell morphology after an impact injury.

The high impaction forces did not induce significant chondrocyte apoptosis after impact for all radial positions and depths in comparison to non-impacted controls. However there was an increase in the apoptotic chondrocytes with increasing time in culture for high impactions which showed a significant increase in apoptotic cells after 14 days in culture compared to non-impacted controls. In the study conducted by Jennifer Lossing (132), it was observed that high impactions (2000 N) induced cell death (necrosis) immediately after impact in surface zone and spread to middle and deep zones with increasing time in culture. Thus comparison of cell-viability reports and this study indicate necrosis to be the primary mode of cell death in surface zones after impact and

both apoptosis and necrosis to be the mode of cell death with increasing time in culture. This finding supports the theory of Chen *et al.* (46), that necrosis occurred before apoptosis in repeated cartilage impacts. However, it is necessary to investigate the mode of cell death within the same sample to confirm the theory. The loss of data at the larger radial positions reduced the power of the statistical analysis. Only the medium impactions were observed to show an increase in chondrocyte apoptosis in comparison to non impacted controls at center of impaction. More data at larger radial positions would be necessary to confirm the findings of radial position dependent effects for impact loads. This study indicated that chondrocyte apoptosis might be dependent on the load, radial position and depth. This study compared the depths at one radial position. The study can be improved by calculating the normalized chondrocyte apoptosis at one depth and radial position relative to the depth at different radial position. For example use depth 1 at center of impaction to calculate normalized intensity at depth 2 at radial position 2r. However this would complicate the study. Probable solution to facilitate such analysis is reducing the number of levels of each factor. For example use the findings of the study to eliminate certain depth position where there is no significant effect in the future studie. The analysis of the study can be improved by observing the matrix changes along with the cellular changes. Apoptotic pathways can be studied in different ways such as the identification apoptotic specific proteins, for example identification of expression of caspase-3 protein which is an effector caspase in apoptosis. This further analysis of apoptotic pathways could provide an insight of the chondrocyte-specific apoptotic pathways as well as confirm the chondrocyte apoptosis.

Generally speaking, articular cartilage subjected to medium (1000 N) impact forces develops apoptosis in the surface and deep zones, and experiences a decrease in deep zone apoptosis with increasing time in culture. Articular cartilage subjected to high impaction forces (2000 N) develops apoptosis in deep zones only after 14 days culture time.

Future studies will determine the specific mode of cell death within the same specimens to confirm the mode of cell death in articular cartilage after impact injury .

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APPENDICES

A. Porcine Patella Removal

Materials:

Paired porcine knee joints (with joint capsule intact)
Sterile table covers (Bio-shield Regular Sterilization Wrap, Allegiance # 4030)
No.22 sterile surgical blades (Feather, cat# 72044-22)
Single edged industrial razor blades (VWR Scientific, no.55411-005)
400ml plastic beakers
Sterile stainless steel large forceps
Sterile stainless steel small forceps
Sterile plastic squirt bottle (Nalgene, USA # 2405-0500)
Protective eyewear
Face mask
Sterile and non-sterile latex surgical gloves
Sterile cotton pads (VWR Scientific, cat# 21902-985)
Sterile stainless steel bowl
Sterile surgical gowns
Dulbecco's Phosphate Buffered Saline (Sigma, no.D-8862, Lot 70K2343)
Pennicilin with Streptomycin (Sigma, no. P-4333, Lot 100K2405)
70% EtOH
Betadine solution (The Purdue Frederick Co., CT, USA, Lot #4D91)

Procedure:

- 1) Lay down sterile table covers onto a clean countertop. Put on non-sterile gloves and protective eyewear.
- 2) Before proceeding, make sure that there are no holes in the synovial capsule of the leg.
- 3) Thoroughly spray fresh porcine leg with betadine and wipe down with a cotton pad.
- 4) Spray leg with 70% EtOH prior to cutting.
- 5) Transfer leg onto a fresh sterile table cover. Dispose non-sterile gloves and put on sterile gloves.
- 6) Using no.22 blade and tweezers, cut away tissue surrounding patella (careful not to puncture joint capsule).
- 7) With razor blade, gently carve away small tissue fibers that are closest to the bone (keeping patella attached to knee joint as long as possible).
- 8) Remove remaining connective tissue surrounding the outer edges of the cartilage surface of the patella using the razor blade. Gently cut along outer edge, careful not to injure the cartilage surface, until patella is completely detached from the joint.
- 9) Once removed from the joint, place the patella on saline soaked sterile cotton pads (which are kept in the steel bowl) and finish cleaning soft tissue off bony surface.
- 10) Spray patella with PBS using a sterile squirt bottle to wash away any surface bacteria or particles.

11) Keep patella soaked in a plastic beaker filled with PBS + antibiotics until ready for impaction.

B. Articular Cartilage Impaction

Materials:

Sterile razor blade
Sterile porcine patellae
MTS Mini-Bionix II hydraulic load frame (MTS Corp., Minneapolis MN, USA)
Dulbecco's Phosphate Buffered Saline (Sigma, no.D-8862, Lot 70K2343)
Spray bottle Stainless steel
cylindrical impactor
Wooden applicator sticks (Puritan, USA # 807)
PMMA (Osteobond Copolymer Bone Cement, Zimmer Inc., USA #1101-08)
Spherically bottomed mold
X-Y positioning jig
Tissue marking dye (Polysciences, Inc. USA #24110)
Sterile gloves

Procedure:

- 1) Keep patellae moist throughout patellar impaction procedure by spraying them periodically with saline solution.
- 2) Press the bony non-articular surface into partially set PMMA, in a spherically bottomed mold to create an imprint. Remove patella immediately to prevent any heat related damage.
- 3) When PMMA is completely set, embed the patella in the cement.
- 4) Place specimen on x-y positioning jig. Rotate the patella and align the facets as needed.
- 5) Prior to impaction, mark patellae with tissue marking dye (to be used as reference markers) with the wooden applicator stick.
- 6) With hydraulic load frame (using the long axis of the cylinder) impact loading at a rate of 25 mm/sec to pre-selected force levels: 1000 N and 2000 N. Use a non-impacted level for a control.

C. Patella Incubation

Materials:

For 10 bottles of culture:

(10) 500 ml bottle Delbeco's MEM/Ham's F12 (Sigma, no.D8437, Lot 100K2316)
(1) 500 ml bottle of 10% Fetal Bovine Serum (Sigma, no.F-2442, Lot 110K8403)
(1) bottle of Penicillin with Streptomycin (Sigma, no.P4333, Lot 100K2405)
125mg Ascorbic acid (Sigma, no.F2442, Lot 50K0688)
50ml Distilled H₂O
100x80mm Pyrex Dishes (Corning, no.3250-DO)

Fischer Scientific CO2 Water-Jacketed Incubator
2 copper pennies (prevents fungus growth in incubator pan)
Rocking platform (VWR Scientific Products, Model 200)
Sterile dH2O
Laminar Flow hood
Sterile stainless steel large forceps
400ml plastic beakers
Sterile plastic squirt bottle (Nalgene, USA # 2405-0500)
Electric stirrer
Electronic scale
Sterile gloves
Sterile 5 ml pipettes
Sterile 10ml pipettes
Sterile 50 ml pipettes
Sterile 100ml pipettes
Pipet-aid
Sterile 60 ml syringe and needle
Sterile syringe filter
Sterile stainless steel patella stands
Plastic tube
Aspirating Pipette
Aspirating flask
Aspirator
Vacuum pump

Procedure:

Preparation of 1 bottle of Media:

- 1) Pipet 50ml of FBS into each of 10 bottles of MEM/Ham's F12 using a 50ml sterile pipette.
- 2) Transfer 5ml of Penn-Strep to each bottle of the MEM/Ham's F12 solution using a 10ml sterile pipette.
- 3) Weigh 125 mg Ascorbic acid and dissolve in 50ml of deionized water. Add 5ml of this solution to each bottle of MEM/F12 mixture using a sterile 60ml syringe with a sterile filter.

Incubating the Patella:

- 1) Separate each patella from the PMMA.
- 2) Using 3 plastic beakers, wash the first patella three times in PBS with antibiotics in beaker #1. Let patella soak in PBS for about 5 minutes before transferring it to the next beaker (#2 then #3). For the second patella, dispose of beaker #1 and use beaker #2 to replace it. Fill another beaker with fresh PBS and use it as beaker #3. Repeat for the 3rd patella. Irrigate the patella with PBS and antibiotics using a sterile squirt bottle.
- 3) Carefully (avoiding fingers from touching the rim) remove the lid of a Pyrex dish and place it rim side up on the counter. Place a patella holder inside the dish

using a pair of sterile tongs.

- 4) Fill the dish with the media mixture, enough to completely immerse the patella.
- 5) Place the dish on the rocking shaker (speed=1.5) in the humidified incubator.
- 6) Keep the culture dish at 37°C in the incubator with 5% CO₂.
- 7) Change the media daily. Do all steps under a fume hood, careful not to place hands (or other non-sterile objects) on or over the specimen and media.

Changing the Media:

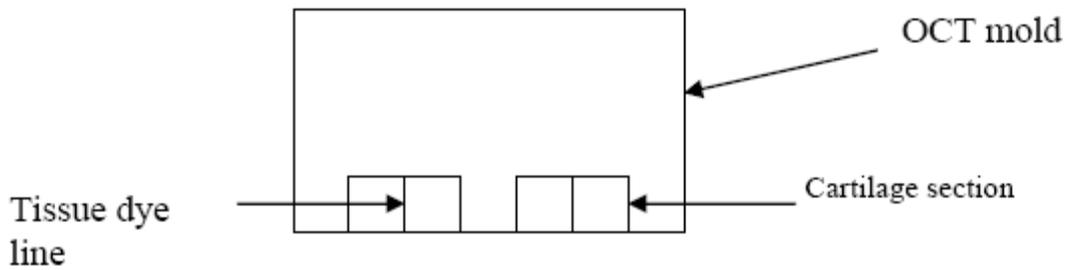
- 1) After spraying gloves with EtOH, remove the dish from the incubator and place under the fume hood.
- 2) Attach a sterile 5ml aspirating pipette to the tube on the aspirator.
- 3) Remove the cover of the dish and place it rim side up on the counter.
- 4) Turn on the aspirator and hold the dish on an angle (to avoid putting hands over it).
- 5) Using the pipette attached to the aspirator, suck out all of the old media. If a significant amount of solid debris is present on the bottom of the dish, (if a significant amount of solid debris is present in the dish, wash patella once with PBS with antibiotics).
- 6) Quickly pour enough fresh media mixture to completely cover the patella.
- 7) Carefully put the cover back on the dish and place it in the incubator.
- 8) Change the media once daily for 3, 7 or 14 days, depending on each patella.

D. Histology

D.1 Cutting Sections

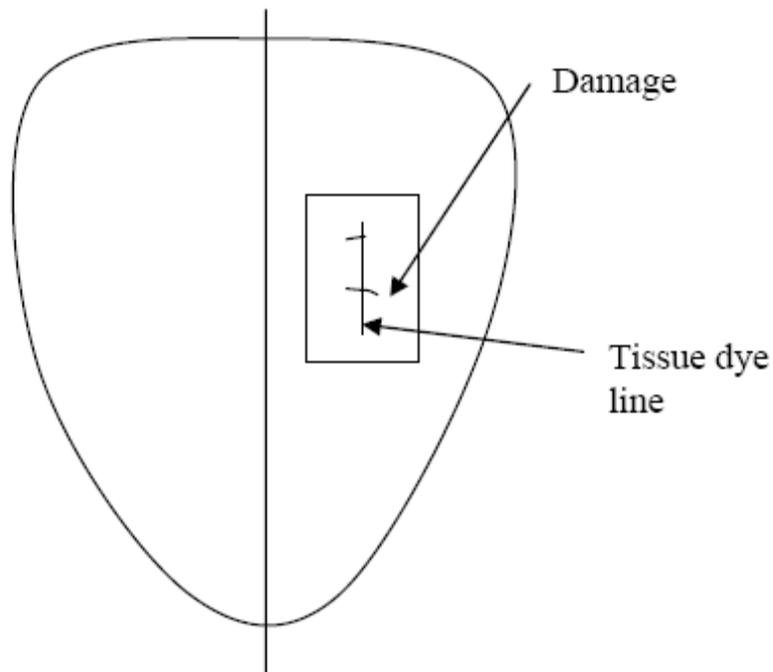
Procedure:

1. Place table cover on counter and fill a 400ml plastic beaker with PBS (enough to use for rinsing the patella).
2. Using an autopsy saw, cut the cartilage+bone surface from the patella. Cut the specimen in half, separating the medial and lateral facets.
3. Make sure the line drawn between the two dots of tissue dye are visible. If not, re-apply the dye to the cartilage surface using a wooden applicator stick.
4. Place the medial facet in a beaker containing 0.1ml MTT solution per 1ml Ham's F-12 in a 150ml beaker.
5. Let the sample sit overnight in the humidified incubator.
6. Cut a rectangular section of cartilage (see #3 below) from the lateral facet and freeze in OCT. See Tissue Freezing protocol.
7. Note any damage seen on either facet. If there is damage present, cut through the length of the "crack" and mount this section of tissue in OCT with the damaged edge up against the edge of the OCT mold. For example:



Harvesting Sections:

1. After 24hrs, remove the samples from the MTT solution and soak them 3X with PBS.
2. Put the samples in a vial filled with Formalin until ready to cut cartilage sections (see next step).
3. Using a scalpel, cut the cartilage off the bone in a rectangular shape, cutting around the tissue dye line. Make sure to include any damaged areas in the section (see #7). For example:



4. Put the section in a plastic vial containing 60% OCT with PBS for 24 hrs.
5. After 24 hrs, transfer the section to 80% OCT with PBS for 24 hrs.
6. After 24 hrs, transfer the section to 90% OCT with PBS until ready for freezing.

D.2 Tissue Freezing

Materials:

Liquid nitrogen or Dry Ice + 100% EtOH

Tissue-Tek O.C.T. Compound, Sakura Finetek (VWR Scientific Products, no.25608-930)
Tissue-Tek Cryomolds, Sakura Finetek (VWR Scientific Products, no. 4557)
Stainless steel small forceps
Stainless steel large forceps
Stainless steel container
Latex gloves
Protective eyewear
Aluminum foil

Procedure:

1. Pour the liquid nitrogen in the steel container (to about ½ inch high) **OR** if using dry ice, fill about 1/3 of the steel container with 100% EtOH and drop 5-6 pellets of dry ice into it. When the liquid ceases to fizz, it is cold enough for freezing.
2. Squeeze the OCT gel into the embedding container until it reaches the top of the container.
3. Cut the rectangular cartilage section in half, perpendicular to the tissue dye line.
4. Using the small forceps, place the two tissue halves side-by-side into the gel.
5. Using the large forceps, place the embedding container (with the gel and tissue specimen) in the liquid nitrogen or EtOH. Be careful not to let the nitrogen touch the OCT gel because it will cause it to boil.
6. When all of the gel turns white, quickly remove it from the liquid. Leaving the gel in too long will cause it to crack.
7. Pop the frozen block out of the embedding container and wrap it in aluminum foil. Keep the specimen in the freezer until ready for use.

E. Statistical Analysis

E.1 Statistical Analysis Software Program

```
options ls =90 pagesize=49;
options pageno=1;

data cells;
infile "F:\SAS data\data.csv" dlm="," firstobs=2;
INPUT name $ load $ leg $ culture rp abs_rp depth d_cells l_cells
t_cells p_live p_apop;
run;

proc freq;
  tables load*culture*abs_rp / list sparse;
  /*
  tables load culture abs_rp depth;
  tables load*culture*abs_rp*depth / list sparse;
  tables load*culture*abs_rp / list sparse;
  tables load*culture*depth / list sparse;
  tables load*culture / list sparse;
run;
*/
data new; set cells;
```

```
proc glm;
class load culture rp depth;
model p_apop = load culture load*culture
           rp rp*load rp*culture   rp*load*culture
           depth depth*load depth*culture depth*load*culture
           depth*rp  depth*rp*load /* depth*rp*culture */;

lsmeans load culture rp depth /adjust=tukey pdiff=all;
lsmeans load*culture load*depth load*rp /adjust=tukey pdiff=all;
lsmeans culture*rp culture*depth /adjust=tukey pdiff=all;
lsmeans rp*depth /adjust=tukey pdiff=all;
*lsmeans culture*rp*depth /adjust=tukey pdiff=all;
run;
```

E.2 Analysis of Variance

Fixed effects

The GLM Procedure

Class Level Information

| Class | Levels | Values |
|---------|--------|----------------------|
| load | 3 | Control High Medium |
| culture | 4 | 0 14 3 7 |
| rp | 7 | -2 -1 -0.5 0 0.5 1 2 |
| depth | 10 | 1 2 3 4 5 6 7 8 9 10 |

The GLM Procedure

Dependent Variable: p_apop

| Source | DF | Squares | Sum of Mean Square | F Value | Pr > F |
|-----------------|------|-------------|--------------------|---------|--------|
| Model | 819 | 141658.3513 | 172.9650 | 1.13 | 0.0285 |
| Error | 1269 | 194696.4311 | 153.4251 | | |
| Corrected Total | 2088 | 336354.7824 | | | |

| R-Square | Coeff Var | Root MSE | p_apop Mean |
|----------|-----------|----------|-------------|
| 0.421158 | 144.5281 | 12.38649 | 8.570297 |

| Source | DF | Type I SS | Mean Square | F Value | Pr > F |
|-----------------------|-----|-------------|-------------|---------|--------|
| load | 2 | 705.95713 | 352.97856 | 2.30 | 0.1006 |
| culture | 3 | 1334.00028 | 444.66676 | 2.90 | 0.0340 |
| load*culture | 6 | 7435.12181 | 1239.18697 | 8.08 | <.0001 |
| rp | 6 | 1188.78889 | 198.13148 | 1.29 | 0.2580 |
| load*rp | 12 | 3448.33879 | 287.36157 | 1.87 | 0.0336 |
| culture*rp | 18 | 3854.53807 | 214.14100 | 1.40 | 0.1240 |
| load*culture*rp | 34 | 9940.34249 | 292.36301 | 1.91 | 0.0014 |
| depth | 9 | 7669.28694 | 852.14299 | 5.55 | <.0001 |
| load*depth | 18 | 2285.02971 | 126.94610 | 0.83 | 0.6688 |
| culture*depth | 27 | 5468.55819 | 202.53919 | 1.32 | 0.1267 |
| load*culture*depth | 54 | 12791.20490 | 236.87416 | 1.54 | 0.0077 |
| rp*depth | 54 | 7604.14713 | 140.81754 | 0.92 | 0.6443 |
| load*rp*depth | 108 | 17544.26006 | 162.44685 | 1.06 | 0.3278 |
| culture*rp*depth | 162 | 16922.82971 | 104.46191 | 0.68 | 0.9989 |
| load*culture*rp*depth | 306 | 43465.94722 | 142.04558 | 0.93 | 0.7968 |

The GLM Procedure

Dependent Variable: p_apop

| Source | DF | Type III SS | Mean Square | F Value | Pr > F |
|--------------------------|-----|-------------|-------------|---------|--------|
| load | 2 | 318.80529 | 159.40265 | 1.04 | 0.3541 |
| culture | 3 | 1323.02999 | 441.01000 | 2.87 | 0.0351 |
| load*culture | 6 | 8810.74694 | 1468.45782 | 9.57 | <.0001 |
| rp | 6 | 1637.04641 | 272.84107 | 1.78 | 0.1001 |
| load*rp | 12 | 3620.92441 | 301.74370 | 1.97 | 0.0239 |
| culture*rp | 18 | 4725.26782 | 262.51488 | 1.71 | 0.0318 |
| load*culture*rp | 34 | 9975.48805 | 293.39671 | 1.91 | 0.0013 |
| depth | 9 | 6759.46344 | 751.05149 | 4.90 | <.0001 |
| load*depth | 18 | 2360.36507 | 131.13139 | 0.85 | 0.6352 |
| culture*depth | 27 | 5020.90286 | 185.95937 | 1.21 | 0.2096 |
| load*culture*depth | 54 | 12726.09174 | 235.66837 | 1.54 | 0.0084 |
| rp*depth | 54 | 7647.96387 | 141.62896 | 0.92 | 0.6336 |
| load*rp*depth | 108 | 16200.45347 | 150.00420 | 0.98 | 0.5472 |
| culture*rp*depth | 162 | 17074.66945 | 105.39919 | 0.69 | 0.9986 |
| load*cultur*rp* depth | 306 | 43465.94722 | 142.04558 | 0.93 | 0.7968 |