

Chapter 1:

Oscillatory Fluid Flow Regulates Glycosaminoglycan Production via an Intracellular Calcium Pathway in Meniscal Cells.

ABSTRACT

Meniscal alteration caused by injury or degeneration affects the homeostasis of the knee joint and has been shown to lead directly to osteoarthritis (OA). A further understand of degenerative knee joint ailments, and the biosynthetic processes for tissue matrix protein production in meniscal cells will aid in the advancement of tissue engineering efforts to create a suitable meniscal implant, prescribing rehabilitation regiments for injury, and pharmaceutical intervention efforts. Mechanical loading in the form of fluid flow at the cellular level has been shown to cause protein production via an increase in intracellular calcium ($[Ca^{2+}]_i$) in bone and cartilage. The focus of this research was to determine if mechanical loading in the form of oscillatory fluid flow (OFF) induced shear stress causes protein production by meniscal cells and to identify if a biochemical pathway involving an increase in $[Ca^{2+}]_i$ is responsible for protein production. Rabbit meniscal cells were isolated, cultured onto microscope slides, and placed in a parallel plate flow chamber and exposed to OFF that induced a shear stress of 4 Pa for protein studies and a range of shear stresses from 0-6.5 Pa for calcium studies. A fluorescent probe and imaging techniques were used to identify the immediate calcium response of the cells to OFF; this response was effectively blocked by the addition of thapsigargin to the flow media. Thapsigargin was used to determine if $[Ca^{2+}]_i$ is necessary in the biochemical pathway responsible for proteoglycan production by cells exposed to OFF. Proteoglycans were measured using the modified 1,9-dimethylmethylene blue colorimetric assay that is specific to the sulfated glycosaminoglycan side chains of proteoglycans. Significant differences were not found in the percentage of cells responding with an increase in $[Ca^{2+}]_i$ at 6.5 Pa ($72\pm 17\%$), 5 Pa ($62\pm 17\%$), 4 Pa ($65\pm 21\%$), and 2.5 Pa ($57\pm 33\%$). The

percentage of cells responding was significantly reduced at 1.5 Pa to only $24\pm 12\%$, and to $2\pm 1\%$ for baseline no flow as compared to shear stress levels greater than 2.5 Pa. There was no significant difference concerning the amplitude of the calcium response between different shear stress levels giving an average amplitude ratio between all specimens and shear stress levels of 2.10 ± 0.30 ($n=5$, $p=0.96$). The amount of sulfated GAG's produced due to OFF induced shear stresses of 1.5 and 4 Pa, the no flow control, and no flow and 4 Pa with the inclusion of thapsigargin were, 197.29 ± 85.10 ($n=3$), 253 ± 95 ($n=9$) 158 ± 86 ($n=10$), 155 ± 59 ($n=3$) and 170 ± 72 ($n=5$) ng GAG/ μ g cell protein respectively. OFF that induced a shear stress of 4 Pa produced a significantly greater amount of sulfated GAG compared to the no flow control. The addition of thapsigargin inhibited the increase in GAG content due to OFF to no flow control levels. The findings of this study suggest that OFF induced shear stress increases production of proteoglycans with sulfated GAG side chains, and that calcium is necessary in the biochemical pathway that leads to sulfated GAG production.

Introduction

The tibiofemoral (knee) joint contains two C-shaped disks called menisci that are wedged between the tibia and femur. The menisci are attached to the proximal surface of the tibia and during knee flexion and extension, the femoral condyles at the distal end of the femur slide along the menisci. The main functions of the menisci are to distribute the load from the femur to the tibia by increasing the contact area providing protection for the underlying articular cartilage that coats the ends of the long bones, and to increase joint congruency, stability, and lubricity.

The meniscus is composed primarily of water (~75%), and structural proteins (20% type I collagen and 1-3% proteoglycans).⁷² Type I collagen is the most abundant structural component and contributes to the circumferential tensile strength of meniscal tissue. The major proteoglycan of meniscal tissue is aggrecan. Aggrecan is a hydrophilic molecule composed of chondroitin and keratin sulfate glycosaminoglycans (GAG) aggregated with hyaluronic acid that assist in maintaining tissue hydration, firmness (resistance to compressive loading), and elasticity via osmotic pressure. The negative charge held at the branched ends of the GAG side chains draw and hold water within the tissue. A balance of collagen and proteoglycans gives the meniscus its structural rigidity and enables it to withstand the large loads occurring in the knee joint.

Injury and/or degeneration of the meniscus can have profound effects on tibiofemoral joint function and homeostasis.^{4,29,53,60} Surgical repair of the meniscus after injury has included meniscectomy, partial or full removal of the meniscus, and suture or other fixation techniques used to repair meniscal tears (Arrows, Fasteners, Staples, T-Fix, etc...). Suturing has been found to be the optimal fixation technique that aids in repairing

the meniscus, in closing the tear gap, over the use of Arrows, Fasteners, T-fix, and staples by measure of peak failure load and the occurrence and severity of gapping after loading.^{12,66,86,114} A comparative study of suturing techniques found failure rates of 100% and 50% for vertical and horizontal sutures under a 60N static load (equivalent to less than ½ of physiological loading), and a significant gap appeared between the suture margins for all suture and material types tested after the first test cycle during cyclic loading.⁹⁶ Therefore, as of this date, it appears that tear fixation techniques are inadequate to fully rejuvenate meniscal function. Clotting techniques,^{76,111} and partial implantation of scaffolds¹¹¹ into meniscal lesion areas have been used experimentally without success in the hope that they will reduce articular cartilage damage and facilitate self healing while maintaining meniscal tissue integrity. Articular cartilage damage including OA is characterized by altered mechanical properties of the tissue (reduced stiffness), tissue degradation (loss of collagen crosslinking, proteoglycan release, and tissue thinning), and chondrocyte biosynthetic modifications (reduced proteoglycan and collagen synthesis). Full restoration of meniscal tissue either structurally or functionally has not been possible by use of the aforementioned techniques. Furthermore, meniscectomy and fixation procedures^{91,96} disturb the loading pattern of the knee joint and ultimately cause or fail to reduce chondropathy, articular cartilage damage, possibly leading to OA.^{19,30,49,54,80,81,117,119} Wyland et al., 2002 has shown that chondropathy becomes more severe with more extensive meniscal alteration (tear, partial, and full meniscectomy) and that damage occurs with any type of meniscal modification.¹¹⁹ Meniscectomy and meniscal tears have been documented to cause OA as far back as 1977,^{19,30} and this procedure is now used to create an experimental model of OA within

the knee joint.^{49,80,117} The fact that meniscectomy leads to OA emphasizes the importance in maximizing the preservation of the meniscus after injury or degeneration. Only the outer 1/3 of the meniscus is vascularized, which limits the ability of self-repair. This has led to the task of creating a suitable replacement for meniscal tissue when adequate preservation is not possible because of the location, orientation, or severity of tissue damage.

Efforts to replace removed or damaged meniscal tissue has included the use of autografts, allografts, artificial implants, and tissue engineered constructs.^{2,21,41,59,68,70,89,90,99,104,113} Allograft implantation of freshly frozen menisci only provide partial protection against articular cartilage damage at best,^{70,89} and does not restore meniscal function in the knee joint mainly due to geometrical mismatch, altering contact mechanics.^{2,99} Thus far, all efforts aimed at creating an artificial implant have fallen short due to problems with implant fixation,^{21,59} insufficient mechanical properties,⁶⁸ or by inflicting an immune response that becomes destructive to the body and/or the implant.⁶⁸ Polyvinyl alcohol-hydrogels (PVAH) with a 90% water content have been used in 1 year experimental studies in rabbits, and have been found to exhibit similar material properties to the native meniscus.⁵⁹ Regressive changes in articular cartilage are curbed with time using PVAH as compared to cases involving meniscectomy. However, implant fixation remains to be a problem, and further fatigue and biocompatibility testing need to be performed on PVAH implants before clinical applications are pursued.⁵⁹ Recent developments in tissue engineering using scaffold technology may provide a likely avenue where a suitable implant could be constructed using native meniscal cells.^{41,90,113} Polymeric¹⁰⁴ and tissue matrix protein⁹⁰ bioresorbable

scaffolds that were implanted exhibited regrowth of meniscal tissue as the scaffold material was resorbed and provided significant protection of the underlying articular cartilage as compared to menisectomized control groups.

Mechanical loading has been found to be regulatory of the biochemical pathways responsible for tissue matrix protein production in musculoskeletal tissues including the meniscus.^{20,22,25,51,52,97,98,108,110,118} The expression of the two main constituent proteins of meniscal tissue, collagen and proteoglycans, are increased by exercise¹¹⁰ while conversely, immobilization of the knee joint decreases the expression and production of aggrecan in the intact menisci²² as well as decreasing collagen production in the healing meniscus²⁵. Dynamic explant compression studies (0.1 MPa at 0.5 Hz for 24 hrs) have also been shown to increase proteoglycan production in the meniscus.⁹⁸ In cartilage explant studies both collagen^{52,93,108} and proteoglycan^{20,52,56,108,118} production increased due to dynamic compression (1-15% strain at 0.1 and 1 Hz for 24 and 48 hrs). Cartilage explant studies have also revealed that dynamic shear deformation causes an increase in both collagen and proteoglycan synthesis without the mediation of fluid flow.⁵²

Specific loading conditions have resulted in various biosynthetic responses among different cell types. Dynamic mechanical loading as compared to static loading has shown to differentially affect both meniscus and articular cartilage. In cartilage, static compression has been found to be inhibitory while dynamic mechanical loading is stimulatory of chondrocyte biosynthesis.^{56,62,108} Upton et al., 2003, found that type I collagen mRNA expression was decreased and collagenase mRNA increased due to static meniscal explant compression studies (0.1 MPa for 24 hrs) while no change was found with dynamic compression (0.8-1.6 MPa at 0.5 Hz for 24 hrs).¹⁰⁹

Ultimately, it is the cell response to tissue level loading that causes protein production. For this reason it is important to determine how tissue level loading may be perceived at the cellular level. The cellular or subcellular stimulus that causes meniscal cells to produce tissue matrix proteins due to mechanical compression of the tissue are not known. Biosynthesis of proteins in other musculoskeletal cells have been attributed to mechanical stress, and more specifically due to fluid mediated responses to mechanical compression including intermittent hydrostatic pressure,^{48,62} molecular transport,^{3,87} streaming electrical potentials,^{45,55,87} and shear stress^{9,101}. In bone it has been suggested that fluid flow induced shear stress^{10,23,88} has a greater effect on cellular biosynthesis than streaming potentials,^{10,45} chemical or molecular transport,^{10,23,88} or cellular deformation.^{100,123}

Physiological cyclic loading, such as during walking, of biphasic tissues including the meniscus and cartilage¹⁰⁶ causes fluid flow to occur where fluid in the tissue matrix is redistributed from areas of high pressure to areas of low pressure. It is hypothesized that during tissue unloading the fluid movement is reversed as the tissue reorganizes to its unloaded state creating an oscillatory, or back and forth, fluid flow. Dynamic loading as opposed to static loading also has been found to have a greater biosynthetic response in cartilage and the meniscus^{62,109} suggesting the importance of using fluid flow that is more than unidirectional. Similar to bone^{23,122} and cartilage,^{27,46,101} this oscillatory fluid flow (OFF) induces a shear stress on the cells that may be responsible for the maintenance and synthesis of biochemical and biomechanical healthy meniscal tissue. The effects of cyclic shear stress applied to tissue engineered scaffold constructs, with the occurrence of fluid flow, on chondrocyte biosynthesis was found to increase tissue thickness through the

accumulation of a greater amount of extracellular matrix containing a higher percentage of collagen and proteoglycans than static controls.¹¹³ Dynamic shear deformation of cells caused by fluid flow induced shear stress across the cells surface is stimulatory to produce proteoglycans and collagen in bone⁴⁶ and articular cartilage^{14,101}. OFF induced shear stress has been shown to regulate osteopontin gene expression,¹²² an important bone matrix protein, the intracellular signaling molecule PGE₂,^{23,95} and more immediately an increase in intracellular calcium ($[Ca^{2+}]_i$)^{23,95,122}.

In bone^{20,88,122} and cartilage, protein production has been proven to be facilitated by $[Ca^{2+}]_i$ including tissue matrix protein production of GAG's⁷⁸ and collagen by chondrocytes⁵⁶. The application of OFF has been shown to elicit an immediate biochemical response by increasing $[Ca^{2+}]_i$ levels in chondrocytes similar to what has been found in bone cells.²⁷ Preliminary data obtained by our research group concerning the meniscus has indicated that an increase in $[Ca^{2+}]_i$ is an immediate response to fluid flow.^{28,73}

The focus of this research was to determine if mechanical loading in the form of OFF induced shear stress altered proteoglycan production levels via the calcium second messenger pathway in meniscal cells. An understanding of the remodeling characteristics of meniscal tissue, and determining the stimulus and biochemical pathways that cause meniscal cells to produce tissue matrix proteins, will aid in the advancement of tissue engineering efforts to create a suitable tissue construct for replacement, as well as further the understanding of degenerative knee joint ailments such as OA.

Methods

Cell Culture:

New Zealand white rabbits were obtained from the lab directed by either Dr. O'Driscoll or Dr. Lewis at Mayo Clinic, Rochester, MN. The legs of the rabbit were transected, using sterile techniques, approximately 1" above the knee joint, packaged on ice, and delivered overnight within 17 hours of sacrifice. (Biological Material Transfer Agreement, Appendix D) Lateral and medial menisci were excised (Figure 1, Appendix A) under sterile conditions, cut into 1 mm³ sections, washed with phosphate buffered saline (PBS), and digested in 0.5% collagenase (Type 2, Worthington Biochemical Corp, Lakewood, NJ) for 6-8 hours at 37 °C. The cells were then isolated by passing the supernatant through a 70 µm nylon filter, centrifuged at 1000g, resuspended, and plated onto 50 mm diameter Petri dishes. This method has been used successfully to yield approximately 5x10⁶ cells isolated from each wet weight gram of tissue.⁷³ The cultures were maintained in growth media consisting of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 (F12) with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin (P/S) at 37 °C in an atmosphere consisting of 5% CO₂/95% air. The cells achieved 100% confluency after 7 days in culture upon which they were transferred to two larger 100 mm diameter Petri dishes. Previous studies using similar isolation techniques have shown that the cells isolated are indeed fibrochondrocytes.^{15,75,116} Once the cells from the primary isolation achieved confluency, the cells were trypsinized and subcultured onto quartz slides (75x25x1.75mm) at 100,000 cells/slide for calcium studies and glass slides (75x25x1mm) for protein studies at

150,000 cells/slide. Cells were cultured for 3 days prior to fluid flow experiments to achieve 80% and 100% confluency for calcium and protein studies respectively.⁴²

Calcium Imaging:

Prior to flow, preconfluent cells cultured onto quartz slides were washed with phenol red free DMEM, and incubated with 5 μ M Fura-2 acetoxymethyl (Fura-2AM) ester fluorescent indicator solution for 20 min at 37°C. The AM ester modification creates an uncharged molecule that is cell permeable. Once inside the cell the AM esters are cleaved by nonspecific esterases allowing the fluorescent probe to bind to Ca^{2+} ions. The slides were again washed with phenol red free DMEM to remove excess Fura-2AM, and then mounted on a single parallel plate flow chamber. The dimensions of the flow chamber were 8mm by 45mm with the use of a silastic gasket to set the height of the chamber at 0.27mm. The flow chamber was attached to a custom designed flow delivery device containing a 250 μ l glass syringe mounted in a custom clamping frame. The syringe was in line with a linear actuator (LinMot Linear Actuators model P01-37x240/260x460) that was controlled through PC software (WinDaq, LinMOT). The outlet of the syringe was connected to rigid tubing connected to the inlet of the flow chamber. To verify the flow rate output of the syringe an ultrasonic flow meter (Model 106, Transonic Systems, Ithaca, NY) was attached to the flow chamber inlet.

A vacuum pump and associated tubing were used to hold the slide firmly against the gasket in the flow chamber. The chamber was placed on an inverted epifluorescence microscope and illuminated with ultraviolet excitation light at 340 (excitation wavelength for $[\text{Ca}^{+2}]_i$ bound Fura-2) and 380nm (excitation wavelength for unbound Fura-2) alternately every second, under computer control. Emitted light was filtered at 510nm,

detected by an intensified charge coupled device (CCD) camera, sampled and recorded as a ratio of bound to unbound Fura using image acquisition and analysis software (Photon Technology International, Lawrenceville, NJ) every 2 seconds. The field of view contained 30-60 cells for each experiment. Each cell was manually outlined using the analysis software, and individually analyzed for transient changes in $[Ca^{2+}]_i$. The 340:380 ratio is proportional to $[Ca^{2+}]_i$ levels.

Following a 1 min no flow baseline period the cells were exposed to OFF resulting in a shear stress of 1.5, 2.5, 4, 5, or 6.5 Pa at 1 Hz for 3 min while calcium levels were recorded. The shear stress was determined from the measured flow rate using parallel plate fluid dynamics theory,

$$\tau = \frac{6\mu Q}{bh^2},$$

where τ is the shear stress (dynes/cm²; 10 dynes/cm² = 1 Pa), μ is the viscosity of the fluid ($0.921E^{-3}$ N•s/m², water at 24°C) Q is the flow rate (ml/min), b is the width (8 mm), and h is the height (0.27 mm) of the flow chamber.³² Flow media consisted of phenol red free DMEM supplemented with 2% FBS. One field from each slide at a confluency of approximately 80% (containing between 20-60 cells) was used to analyze the calcium response for each shear stress level tested. A cell was considered responsive if the excitation amplitude ratio increased by more than 25% above baseline. Figures and diagrams depicting the calcium imaging setup as well as the flow chamber are located in Figure 5 of Appendix A.

Calcium Blocking:

Thapsigargin was used to investigate the role of calcium signaling for meniscal cell protein production in response to OFF induced shear stress. For calcium blocking studies, cells were incubated in flow media containing 50 nM Thapsigargin (Sigma-Aldrich) for 30 min prior to as well as during testing.^{17,78,122} Pilot studies were performed to confirm the efficacy of calcium blocking using the calcium imaging technique described previously with OFF rates capable of producing shear stresses of 4 and 5 Pa at 1Hz with 50 nM thapsigargin present in the flow media.

Protein Production Studies:

Cells cultured onto glass slides were placed in a parallel plate flow chamber where six slides could be tested simultaneously (Streamer, Flexcell) attached to a custom designed flow delivery device similar to that used in calcium studies except for the use of a 60 ml syringe (Figure 6, Appendix A). An ultrasonic flow meter (Model 110, Transonic Systems, Ithaca, NY) placed in the inlet tube was used to verify the flow rate during testing. The cells were exposed to OFF at 1 Hz and a peak shear stress of 4.0 Pa for either 1 or 2 hours. Control groups were placed in the flow chamber but not exposed to fluid flow for 1 or 2 hours. No differences were found between the data for 1 and 2 hours of either flow or no flow groups allowing the tests to be analyzed together. Following fluid flow, slides were post-incubated for 1,2, and 3 days to allow protein production and secretion.^{23,63} Pilot data using 2 specimens with 2 samples/day for each specimen showed maximal sulfated GAG expression at 3 days post incubation, therefore, remaining studies were done using the 3 day post incubation period. Flow and post flow culture media contained DMEM/F12 with 2% FBS, and 1% P/S. Slides were removed

from the chamber following fluid flow and placed in new Petri dishes with 10 ml of fresh media for post-incubation. Approximately 1/4 of the samples were treated with thapsigargin prior to and during OFF to block $[Ca^{+2}]_i$ in order to investigate the role of $[Ca^{+2}]_i$ in the production of sulfated GAG's.

Total Protein Isolation

Total cell protein content was used to normalize the concentration of sulfated GAG's contained in the post flow media of each sample allowing comparisons to be made between samples. To prepare the samples, post flow media was removed and placed in a 15 ml conical tube and frozen at $-80^{\circ}C$ for later analysis, and the monolayer of cells on the glass slides were washed with PBS (x2) to remove the phenol red from the dish. In order to isolate total cell protein 1 or 0.5 ml of extraction buffer (3.8 M Urea, 50 mM Tris pH 8.5, 0.1mM Phenylmethylsulfonyl fluoride (PMSF)) was placed on the glass slides for each sample, the cells were scraped off of the slide using a cell scraper, pooled, and passed through a 21-gauge needle to lyse the cells. The lysate mixture was placed in a microcentrifuge tube, incubated on ice for 30 min, and centrifuged at $4^{\circ}C$ for 20 min at 13,000 rpm (16,000 g). After separation of the soluble and insoluble portions of the sample, the supernatant was placed into a new microcentrifuge tube and stored at $-80^{\circ}C$ for later analysis. The pellet, or insoluble portion was discarded. Total protein was quantified using the Lowry Method (Bio-Rad DC Protein Assay II). GAG concentration was determined for all samples following flow.

GAG Content:

50 μ l aliquots of post flow media were placed in duplicate on a 96 well microplate to determine the amount of total sulfated GAG produced by the meniscal cells using the

modified 1,9-dimethylmethylene blue (DMMB) method with shark chondroitin 6-sulfate as a standard.^{31,74} 200 μ l of DMMB dye (3.04g of glycine, 2.37g NaCl, 95 ml of 0.1 M HCl, and 16 mg DMMB brought to a final volume of 1 L with deionized distilled water) was added to 50 μ l of sample and the absorbance was measured by a microplate reader at 525nm immediately.

Statistical Analysis:

5 rabbit specimens were used with at least 1 slide per shear stress level for calcium imaging studies. Three specimens with at least 4 samples per specimen were used at a single shear stress level for calcium imaging studies with thapsigargin included in the flow media. At least 5 specimens with 2 samples per condition were used to determine the levels of GAG production in the flow, no flow, and flow with calcium blocked with thapsigargin studies. Three specimens with 3 samples per specimen were used as a vehicle control in the calcium blocked no flow control studies with the inclusion of thapsigargin. The number of specimens used for both calcium and protein studies, along with the overall results of the experiments, are given in Appendix B. Single specimens were used for multiple studies. GAG concentration in μ g of media was gained by multiplying the concentration of GAG's/ml of media by the volume of media remaining in culture when the incubation period was terminated. Average culture media volumes were obtained and used in the analysis for 1 (8.75 ml), 2 (8.15 ml), and 3 (7.75 ml) day incubation periods. Total cell protein levels in μ g were calculated by multiplying the amount of buffer added to each slide during protein isolation to the total protein concentration in μ g/ml as measured by the microplate reader during total protein quantification. Total GAG in ng was normalized against μ g of total cell protein.

One way analysis of variance (ANOVA) with a completely randomized design was used to determine if differences existed between any pair of treatment means for the calcium meniscal cell percent response data. Duncan's post hoc paired comparison testing was used to identify differences between various shear stress levels for calcium response data.⁹⁸ The relative peak amplitude calcium response data failed to meet the assumption of normality, thus, Kruskal-Wallis nonparametric one way analysis was used. A one tailed t-test assuming equal variances was used to determine if one condition produced a greater amount of sulfated GAG's than another using Excel. One way ANOVA was used for sulfated GAG production studies with a completely randomized design to evaluate whether differences in treatment means among the 0, 1.5, and 4 Pa OFF condition existed. A significance level of $\alpha=0.05$ was used for all tests. All analysis was performed using SAS 8.2 (1999-2001 SAS Institute Inc., Cary, NC) unless stated otherwise.

Results

Calcium Imaging:

Approximately 60% of the representative population of meniscal cells responded with a robust and transient increase in $[Ca^{2+}]_i$ to a threshold value OFF induced shear stress. A typical response profile exhibiting the characteristic calcium response was observed (Figure 1. A), and the illumination of the cells loaded with Fura-2AM fluorescent indicator during calcium imaging was easily recorded (Figure 1. B). Significant differences were not found in the percentage of cells responding with an increase in $[Ca^{2+}]_i$ at 6.5 Pa ($72\pm 17\%$), 5 Pa ($62\pm 17\%$), 4 Pa ($65\pm 21\%$), and 2.5 Pa ($57\pm 33\%$) ($p>0.05$, $n=5$) (Figure 2. A). The percentage of cells responding was significantly

reduced at 1.5 Pa to only $24\pm 12\%$ (n=5), and to $2\pm 1\%$ (n=5) for baseline no flow as compared to shear stress levels greater than 2.5 Pa ($p<0.05$). There was no significant difference concerning the amplitude of the calcium response between different shear stress levels (average amplitude ratio was 2.10 ± 0.30 , n=5, $p=0.96$) (Figure 2. B).

Similar to the no flow baseline response, less than 1.5% of cells responded to shear stresses of 4 or 5 Pa while $[Ca^{2+}]_i$ was blocked by the addition of thapsigargin to the flow media (n=3), indicating that thapsigargin did indeed effectively block $[Ca^{2+}]_i$ oscillations from occurring in meniscal cells exposed to OFF (Figure 3). The relative $[Ca^{2+}]_i$ amplitude for studies where $[Ca^{2+}]_i$ was blocked (1.33 ± 0.15) were not statistically different from studies where $[Ca^{2+}]_i$ was not blocked. The percentage of cells responding, and relative calcium peak amplitudes for varying conditions are given in Table 1.

GAG Production:

Sulfated GAG production was found to be significantly increased due to an OFF induced shear stress of 4 Pa (253 ± 95 ng GAG/ μ g cell protein, n=9) over the no flow control (158 ± 86 ng GAG/ μ g cell protein, n=10) using a one tailed t-test ($p<0.02$). A one tailed t-test also determined that the inclusion of thapsigargin not only effectively blocked the immediate increase in $[Ca^{2+}]_i$, but also blocked the increase in sulfated GAG production due to a shear stress of 4 Pa (170 ± 72 ng GAG/ μ g cell protein, n=5) to no flow control levels with the inclusion of thapsigargin (155 ± 59 ng GAG/ μ g cell protein n=3) ($p=0.4$). Comparing the no flow group where thapsigargin was included to the no flow control group using a one tailed t-test indicated that thapsigargin did not adversely affect the meniscal cells during the study ($p=0.5$). Significant differences were not found between

treatment means concerning the no flow control, 1.5 Pa (197 ± 85 ng GAG/ μ g cell protein $n=3$), and 4 Pa OFF groups using a one way ANOVA ($p=0.1$).

Discussion

The aim of this study was to discover if OFF induced shear stress is a potent stimulator of meniscal cells to produce tissue matrix proteins in the meniscus, and to identify a biochemical pathway associated with proteoglycan production. To accomplish this, a parallel plate flow chamber was used and OFF was applied to cells isolated from meniscal tissue in two independent tests that concentrated on either the beginning or the end of the biochemical pathway of interest. Calcium imaging studies were used to indicate whether an increase in $[Ca^{2+}]_i$ was an immediate response, and long term flow studies evaluated the amount of sulfated GAG's secreted into the media to indicate proteoglycan production levels due to OFF induced shear stress. The findings of this study suggest that OFF induced shear stress increases the production of proteoglycans with sulfated GAG side chains, and that calcium is involved in the biochemical pathway leading to sulfated GAG production in meniscal cells due to OFF.

Mechanical Loading (Tissue Mechanotransduction)

It is well known that mechanical loading has been recognized as a mechanism to aid in the maintenance and synthesis of musculoskeletal tissues including cartilage and the meniscus.^{20,22,25,51,52,97,98,108,110,118} Both overloading and immobilization studies have contributed to the understanding of how mechanical loading regulates tissue homeostasis. Knee immobilization (3-12 weeks) studies have been shown to cause cell apoptosis and p53 expression, which leads to arrested cell growth, in rabbit and mouse cartilage.⁷⁷ Progressive thinning of articular cartilage due to knee immobilization in spinal cord

injured patients has also been observed.¹¹² Hind leg suspension studies (4 weeks) have shown a 2 fold decrease in aggrecan expression²² and collagen formation in the meniscus,²⁵ and in cartilage, knee casting (11 weeks) provoked a 50% decrease in GAG concentration in dogs.⁵⁷ Remobilization (immobilized 11 weeks, remobilized 15-50 weeks) of the knee joint after prolonged disuse was not able to fully restore cartilage atrophy as indicated by reduced GAG content,^{40,58} and collagen crosslinking,⁴⁰ and visible thinning of the cartilage.⁵⁸ Even after early stage meniscal formation, meniscal maturation does not occur without mechanical compression, ultimately causing meniscal tissue degeneration in chick embryos.⁶⁹

When overloaded ($4.5 \leq \text{MPa} \leq 20$), cartilage tissue develops early stage OA symptoms^{16,105} including decreased proteoglycan^{50,61,107} and total protein production,^{50,61,82} reduced mechanical properties represented by decreases in compressive and shear stiffness,^{61,64,84} with an associated increase in water content,^{16,64,107} nitrite levels,⁶⁴ cell apoptosis,^{50,61,64,84} and proteoglycan^{64,82,84} and collagen degradation^{16,107} or release, analyzed less than 1 day after injurious compression. After a longer period of incubation time following cartilage injury, 10 days to 6 weeks, increased water content, degenerative effects of the collagen matrix and associated tissue material properties persisted, and proteoglycan synthesis⁸⁵ was found to increase along with fibronectin synthesis,¹⁶ possibly suggesting a reparative effort being conducted by the cells. Patwari et al., 2003 also observed the initial increase in proteoglycan degradation peaking at 1 day after injurious compression, and then leveling to control uninjured tissue levels after 7 days in culture.⁸² Unloading or overloading of tissue can have adverse effects that alter the material properties of the tissue, the functionality of the cells, and ultimately cause cell death and tissue

degradation that cannot be fully recovered even when physiological loading levels are again applied to the tissue. In order to further understand how mechanical loading of the tissue affects tissue health, it is important to look at how tissue level loading is transduced and perceived at the cellular level. The current study applied fluid flow induced shear stress to meniscal cells in culture.

Cellular Level Mechanical Loading (Cell Mechanotransduction)

The current study showed that fluid flow induced shear stress was a potent biophysical signal, resulting in $[Ca^{2+}]_i$ mobilization and proteoglycan production. *In situ* loading of biphasic (containing both solid and liquid composition) materials can result in a complex mechanical environment at the cellular level. Simple compression of the tissue can result in fluid flow induced shear stress, direct deformation, and fluid pressure.³⁹

Numerous mechanical loading mechanisms that directly act on the cells of biphasic materials as a result of dynamic tissue compression have been proposed including fluid flow induced effects. The meniscus itself is 70% water, and the pattern of dynamic loading and unloading of biphasic tissues located in the knee joint, such as during walking, are ideal to provoke interstitial OFF across the surface of the cells. However, due to the small amount of time (<200 ms) associated with impact loading Eberhardt et al., 1990 has shown through modeling, that biphasic tissues essentially behave linearly elastic suggesting that water does not contribute to the material behavior of the tissue, and that ultimately fluid flow does not occur.²⁶ A biphasic model used to investigate the importance of fluid load support for a rolling contact cartilage theoretical design found a similar result. In this model it was found that for physiological load conditions (normal gait) the interstitial fluid resistance to flow is large with little fluid

transport occurring except for near the tissue surface layers. This creates a high fluid pressure within the tissue which would support up to 90% of the total load in the tissue.^{7,8,65,115} During normal gait analysis at 1 Hz frequency peak loads are achieved at heel strike and toe push off, the first of which occurs after 200 ms of loading with a total load duration time of 600 ms.⁸³

At time points greater than 200 ms, the intrinsic viscoelastic effects including fluid movement become more pronounced and are found to contribute significantly to the load behavior of biphasic materials as fluid pressure decreases and tissue creep occurs.^{8,26} Biphasic models of cartilage predict that with increased load duration more fluid movement occurs at greater depths in the tissue as indicated by fluid velocity profiles.⁷¹ During loading it has been found that between 70-90% of the applied load is supported by hydrostatic pressure in the tissue at the point of contact, and that this pressure decreases to zero near the edges of contact during load times of 200 ms.²⁴ The greatest amount of fluid flow has been shown to occur where the pressure gradients are the highest in the tissue during loading¹¹⁵ suggesting that as the contact area shifts fluid flow, exists in cartilage. The biphasic conewise linearly elastic quasi-linear viscoelastic model that accounts for both the flow dependent and independent viscoelastic effects as well as tension compression nonlinearity, indicated a strong agreement with experimental dynamic cartilage explant compression studies using a sinusoidal 5% strain amplitude including a 1 Hz frequency for a duration of 10 cycles ($r^2=0.91$).⁴³ The improved models suggest that fluid contained in the tissue is an important factor in dictating material behavior during dynamic compressive loading. Furthermore, interstitial fluid flow was found to exist in cartilage explant cyclic compression studies at 1 Hz using physiological

loading conditions by the use of tracer molecules in the surrounding fluid.¹⁰⁶ Based on these findings, others have studied the response of musculoskeletal tissues to fluid flow induced shear stress, and found it to be a potent stimulant for bone, cartilage, and endothelial cells when applied to monolayer cultures, resulting in cellular biochemical cascades affecting tissue matrix composition.^{23,27,46,101,122}

When fluid flow is applied to monolayer cultures, such as in this study, an array of proposed mechanisms of cellular stimulation can coexist including streaming potentials, molecular transport, and shear stress. Previous studies suggest that fluid flow induced shear stress is the most potent stimulator of osteoblasts by using alterations in fluid viscosity while maintaining flow rate to differentially separate the effects of fluid flow on the cells.^{11,45,87} Blocking of voltage gated channels⁴⁵ and the application of electrical fields⁴⁵ to bone cells indicate that streaming potentials do not take part in $[Ca^{2+}]_i$ oscillations in bone cells. ATP was thought to aid in the calcium response of chondrocytes via molecular transport, but blocking the release of ATP by chondrocytes using 100 μ M suramin did not inhibit $[Ca^{2+}]_i$ when applied to chondrocytes.¹²⁰ Mechanotransduction studies involving molecular transport and electrical streaming potentials have added molecules such as ATP and charged ions to the culture media to invoke a cellular response. The culture media (DMEM/Ham's F12 with 2% FBS and 1% P/S) (Donahue et al., 2003 and Edlich et al., 2001 only used 2% FBS and verified that the addition of the serum was enough to claim that chemotransport was sufficient to cause a response)^{23,27} was not supplemented with additional molecules in the present study, thus reducing the contribution that streaming potentials or molecular transport may have on

the meniscal cells. One of the most understood and investigated responses to fluid flow is that of intracellular calcium mobilization.

Intracellular Calcium

An immediate response to fluid flow in chondrocytes, osteoblasts, and the meniscus has been found to be an increase in $[Ca^{2+}]_i$.^{17,23,27,28,47,73,120-122} The current study showed that approximately 60% of the cell population responded with a robust and transient increase in intracellular calcium levels in meniscal cells with the onset of OFF. This response was transient in nature, and not dependent on shear stress level, as long as a specific shear stress threshold level was reached. The transient increase in intracellular calcium was blocked with pharmacological agents. Calcium increases within the cell can be caused by either an influx of calcium into the cell from the extracellular space, or from the release of $[Ca^{2+}]_i$ stores. By tracing the source supplying the increase in $[Ca^{2+}]_i$ the mechanotransduction (cellular sensory) mechanisms responding to fluid flow can be further identified.

Extracellular calcium has the ability to enter the cell via voltage gated and calcium ion channels including those that are stretch activated. Application of L type voltage operated calcium channel blockers nifedipine and verapamil either reduced or had no effect on the percentage of cells responding and the mean peak $[Ca^{2+}]_i$ amplitude of the response of chondrocytes to fluid flow.^{44,122} Gadolinium, a pharmacological agent responsible for blocking stretch activated channels on the cell membrane, was reported to have no effect on the percentage of cells responding with an increase in $[Ca^{2+}]_i$ in bone by You et al, 2001,¹²² and only partially inhibited the flow response in bone according to Hung et al, 1996.⁴⁴ In cartilage, gadolinium reduced the percentage of cells responding

as well as the amplitude of the calcium response, but did not result in a complete inhibition.¹²¹ A similar response was found when chelating extracellular calcium with EGTA in chondrocytes.¹²¹ Therefore, extracellular calcium has the ability to at least partially influence the level of increase in $[Ca^{2+}]_i$, but because the signal was only partially inhibited when extracellular calcium was blocked, another source must exist.

Intracellular calcium stores are contained within the endoplasmic reticulum (ER) of the cell. The ER possesses two known calcium pools that are activated by different mechanisms and gated by different receptors, ryanodine and inositol-1,4,5-trisphosphate (IP₃) receptors. Ryanodine at low concentrations (<10 μ M) blocks the release of $[Ca^{2+}]_i$, and at high concentrations (>10 μ M) release the ryanodine receptor $[Ca^{2+}]_i$ stores located in the ER. The application of ryanodine at either concentration had no effect on $[Ca^{2+}]_i$ oscillations in chondrocytes.¹²⁰ Ryanodine receptors located on the ER are not influenced by intracellular IP₃ levels, and may participate in a protective function for the cell, aiding protein folding and vesicle transport between the ER and the golgi apparatus, explaining why these stores are not used as a result of mechanical stimulation.^{79,92} Agents that directly block the IP₃ pathway, or chelate $[Ca^{2+}]_i$ including thapsigargin, 2 APB, neomycin, and BAPT-AM were not only able to completely block the immediate increase in $[Ca^{2+}]_i$, but also protein production in chondrocytes¹²⁰ and osteoblasts^{44,122} due to fluid flow. This data suggests that the IP₃ pathway, as regulated by G-protein activation, is the most potent stimulus of protein synthesis via intracellular calcium oscillations in bone and cartilage, and most probably, the meniscus.

Fluid flow induced shear stress is a known stimulus of the membrane bound heterotrimeric G-proteins. The suggested mechanism of G-protein modulation include

the activation of a protein receptor or directly by cell membrane lipid bilayer conformational changes.³⁷ The stimulatory effect of fluid flow induced shear stress has been shown to be mediated by both pertussin toxin-sensitive (G_i) and insensitive (G_q) cell membrane bound G-proteins in endothelial cells.³⁸ G_i blocked with pertussin toxin significantly reduced the percentage of cells responding to fluid flow with an increase in $[Ca^{2+}]_i$ as well as the amplitude of the calcium response,¹²⁰ and inhibited the synthesis of sulfated GAG's in chondrocytes.²⁰ Reich et al., 1997 indicated that blocking G_i with pertussin toxin and chelating intracellular calcium with quin 2AM have the same inhibitory affect on PGE_2 production in osteoblasts indicating that G-protein activation as well as an increase in $[Ca^{2+}]_i$ are responsible for protein production.⁸⁸ Pertussin toxin blocking of G_i was also shown to completely inhibit the calcium response of osteoblasts to fluid flow linking G-protein activation to $[Ca^{2+}]_i$ oscillations.⁶⁷ Therefore, it may be the G-protein receptor that is stimulated by the fluid flow induced shear stress that causes a global calcium response in meniscal cells.

The relative amount of calcium within the cell designated by the excitation amplitude ratio obtained from calcium imaging is not dependent on shear stress level, indicating that the calcium response is either all or none in meniscal cells. This all or nothing effect has been found by investigators using other cell types including bone²³ and articular cartilage¹²¹. There is evidence showing that the threshold value indicative of an all or none response for calcium excitation is controlled by the concentration of IP_3 in the cell; once a specific concentration is achieved, a global calcium wave is spread throughout the cell.⁹²

Calcium imaging studies were also used to indicate the effectiveness of thapsigargin to block $[Ca^{2+}]_i$ oscillations from occurring within meniscal cells. Thapsigargin is a pharmacological agent that releases calcium from the IP_3 receptor stores (85% of total calcium stores within the ER) into the cytosol and inhibits the Ca^{2+} -ATPase pump in the ER.⁷⁹ Through calcium imaging techniques thapsigargin was found to effectively block the $[Ca^{2+}]_i$ oscillations in meniscal cells when added to the flow media prior to OFF application. The $[Ca^{2+}]_i$ response that occurred without the initiation of fluid flow (baseline response), with the depletion of $[Ca^{2+}]_i$ by the addition of thapsigargin, and the $[Ca^{2+}]_i$ “spikes” that occurred a finite time after the initiation of fluid flow may be attributed to the release of calcium by a source outside of the ER. It has been suggested that this mechanism is brought out by the uptake of intracellular calcium by ER localized mitochondria. Once the cytosol returns to basal $[Ca^{2+}]_i$ levels, the permeability transition pores of the mitochondria open releasing calcium into the cytosol creating a second $[Ca^{2+}]_i$ oscillation in the cell.⁹²

Calcium as a pathway to PG production

When $[Ca^{2+}]_i$ pathway blockers thapsigargin and neomycin were applied to bone and cartilage cells exposed to fluid flow they not only blocked the immediate intracellular calcium second messenger response, but also inhibited the production of matrix protein, suggesting that an increase in $[Ca^{2+}]_i$ is a necessary step to protein production in bone and cartilage cells.^{20,122} Likewise, in meniscal cells, the amount of sulfated GAG's produced due to OFF with the application of thapsigargin were reduced to no flow control levels. The fact that sulfated GAG production levels after 3 days in culture were maintained with the no flow control group after the application of thapsigargin indicates that the meniscal

cells remained viable throughout the tests. For this reason it could be hypothesized that cell membrane bound G-proteins that are directly stimulated by mechanical shear stress, and ultimately cause intracellular calcium release into the cytosol, may exist in meniscal cells. Once G proteins are activated, a biochemical pathway results that causes the hydrolyzation of IP₃ ions that bind to IP₃ receptors located in the ER responsible for calcium release from the ER lumen.⁹²

In this study meniscal cells responded to fluid flow with an increase in sulfated GAG production over control no flow levels suggesting that OFF induced shear stress is able to stimulate tissue matrix protein production. The amount of sulfated GAG's produced in this study was consistent with other studies using the modified 1,9-dimethylmethylene blue method involving both cartilage and the meniscus.^{22,36} The increase in proteoglycan production to OFF is reinforced by studies involving exercise and physiological loading conditions that showed an increase in proteoglycan production, and conversely in osteoarthritic conditions, created by injurious compression and knee joint immobilization, that initially cause a decrease in proteoglycan production and an increased release or degradation of existing extracellular matrix proteoglycans. However, after an extended period of time after cartilage injury (>10 days) this trend is reversed, and proteoglycan production is increased along with water content, quite possibly as a reparative effort by the cells that instead causes further degradation of the tissue shown by decreased compressive and tensile stiffness. For this reason, future studies will be performed using OFF induced shear stress that look at collagen synthesis by meniscal cells to determine whether the increased proteoglycan levels are indeed aiding to sustain

tissue homeostasis, or if they are contributing to a negative degrading spiral effect in the tissue.

Based on protein synthesis studies presented in the literature and results from the current study, it is hypothesized that OFF induced shear stress caused a conformational change in the cell membrane lipid bilayer modulating membrane bound G proteins that activate the IP₃ pathway to release calcium stores from the ER causing an [Ca²⁺]_i oscillation responsible for proteoglycan production in meniscal cells.

Limitations of the study

The real time calcium response of meniscal cells was monitored by using the Fura-2AM fluorescent calcium probe while OFF was applied to induce varying shear stress levels. Calcium imaging in response to OFF indicated that shear stresses greater than 2.5 Pa were stimulatory of approximately 60% of the cell population, suggesting that the percentage of cells responding is not dependent on the level of shear stress applied as long as it is greater than 2.5 Pa. A linear trend also existed from 0 to 2.5 Pa for the % of cells responding versus shear stress level, after which the % of cells responding remains fairly constant from 2.5 Pa to 6.5 Pa. Additionally, the calcium data provided motivation for studying the GAG response following fluid flow induced shear stresses of 4.0 Pa, which is similar to the physiological shear stresses estimated to exist in cartilage (3.7 Pa).¹²¹ In bone,^{23,47} cartilage,^{14,27} and endothelial³⁴ cells the opposite has been found to be true, that the percentage of cells responding was dependent upon the flow rate with a greater percentage responding at higher rates. However, when comparing the shear stress range of 1 to 4 Pa caused by OFF with other studies, there was a similar shear stress dependent percent cell response in the meniscus and articular cartilage. At a frequency of

1 Hz there was a 30, 60, and 95% cell response corresponding to a shear stress of 1.1, 2.2, and 4.2 Pa in cartilage,²⁷ and in the meniscus there was an average of 24, 57, and 65% cell response due to a shear stress of 1.5, 2.5, and 4 Pa.

The comparison between chondrocytes and meniscal cells signify that there is a difference in the percentage of cells responding at higher shear stress levels. In fact, the average percentage of cells responding in the meniscus did not exceed 72%, even though there were individual cases at 6.5 Pa that exceeded a 95% cell response for the respective cell populations. The cells themselves may have the ability to shield neighboring cells from the full OFF induced shear stress profile, resulting in a lower percentage of cells responding. The cells may also communicate with one another in vivo through gap junctions possibly enhancing the shear stress dependent response, and the 80% confluent population used in calcium studies may not allow gap junctions to occur throughout the representative population for each sample tested.

Researchers have noted the existence of two distinctly shaped cells within the meniscus based on their location in the tissue, those that are elongated and fibroblast like in the superficial layer, and those that are polygonal or chondrocyte like in the deeper layers.³⁵ It has not been determined whether or not these are two distinct cell phenotypes, but culture media composition, and extended primary cultures suggest that one type of cell can be selectively chosen over that of another.^{15,75,103,116} DMEM and F12 alone have been suggested to enhance the growth of only one cell shape, where an equal volume of DMEM and F12 has been shown to maintain both cell shapes in monolayer cultures.^{15,75,103,116} Growth media consisting of equal volumes of F12 and DMEM was used in this study to eliminate the selective growth of one of the cell shapes over the

other. The references detailing such phenomena contradict one another as to which is selectively chosen over the other due to specific growth media and culture period. In this study both cell shapes were observed under a phase contrast microscope throughout the number of passages where tests were conducted, (passage 1-4) and for this reason it may be that only one of the distinctly shaped cells are responsive to OFF induced shear stress explaining the incomplete percentage of cells responding in almost all of the individual test cases.

Since the physiological shear stress occurring in the meniscus due to fluid flow is unknown, a range of shear stresses were used to cover the physiological range for bone (0.8-3.8 Pa),¹⁸ and the level estimated to be physiological in cartilage (3.7 Pa)⁴⁴. The shear stresses realized in the meniscus may be higher or lower than the prescribed levels in this study. In either case, the correct range may not have been covered. If a range of shear stresses above 6.5 Pa were used a greater percentage of cells may have responded. In the future, modeling and research performed on the meniscus to identify the physiological shear stress occurring in the meniscus will be compared to the levels used in this study.

In this study the measure of GAG production by meniscal cells due to shear stress was only performed at one shear stress level. Additional tests were performed in hindsight at 1.5 Pa, chosen because of the statistically significant reduction of the percentage of cells responding with an increase in $[Ca^{2+}]_i$ from higher shear stress levels, to discover if the concentration of GAG was also decreased respectively. The three rabbit specimens tested at 1.5 Pa indicated that a trend may exist depicting a reduced concentration of GAG produced from 4 Pa to 1.5 Pa to the no flow control case.

However, sulfated GAG concentrations achieved at 1.5 Pa were not found to be significantly different from no flow control levels using a one tailed t-test ($p=0.25$). A larger sample size may show an increased response over the control levels. The results from the different shear stress levels tested indicate that altering the shear stress may effect the sulfated GAG production level in meniscal cells, and that this change in production may be related to the percentage of cells responding with an increase in $[Ca^{2+}]_i$ levels at the specific shear stress.

Fluid flow itself is capable of causing an array of cellular stimuli. In this study only shear stress was investigated. Intermittent hydrostatic pressure applied to articular cartilage has been shown to cause an increase in proteoglycan production,^{48,62} and biphasic models have indicated that during normal physiological loading (gait) hydrostatic pressure may account for up to 90% of the load support in cartilage.^{8,24,65} However, articular cartilage and the meniscus are different tissues with different tissue porosities,^{6,72} and therefore, may respond differently under similar loading conditions. Modeling and dynamic explant compression studies of the meniscus under normal gait will aid in distinguishing between the importance of fluid in the tissue matrix during loading, and to discover whether fluid flow or hydrostatic pressure are significant factors under load conditions to promote biosynthesis in meniscal cells.

The relationship between flow rate and shear stress was determined by parallel plate fluid dynamics theory with a set viscosity of $0.921E^{-3} \text{ N}\cdot\text{sec}/\text{m}^2$ at 24 °C. Calcium studies performed on different specimens did not occur on the same day, and from day to day the temperature of the room where the studies were performed could have fluctuated ± 4 °C altering the shear stress pertaining to any flow rate by as much as ± 0.5 Pa.

Complete randomization of the data was achieved by testing each shear stress level on the same day for each specimen eliminating temperature as a necessary factor in the statistical analysis. However, in the future, it may be desirable to monitor the room temperature and/or fluid temperature during flow tests to accurately represent the viscosity used in theoretical calculations.

Using a cell monolayer model to represent the physiological response of cells to fluid flow may not be accurate compared to the conditions that exist *in situ*. In healthy menisci the mechanical environment of the cells is manipulated by the surrounding extracellular matrix. It has been suggested that proteoglycans may act as a sieve regulating flow patterns because of their strong interaction with water molecules.¹ The deformation of the extracellular matrix due to loading may also occlude fluid flow by narrowing and closing the interstitial space through which it travels. In future studies the measurement of GAG production by meniscal cells due to fluid flow embedded in a 3D structure representative of the physiological state of the meniscus could be compared to the monolayer culture results for verification. Tissue explant compression studies with the explant immersed in culture media is one such method that could be used to achieve this.

A rabbit model was used in this study because of the limited ability to obtain healthy human menisci for testing. The loading pattern of human to rabbit menisci is not the same due to postural differences resulting in gross anatomical difference between the rabbit and human menisci as well as the number of attachments occurring between the meniscus and the femur. However, histological examination of rabbit meniscal attachments, and inspection of rabbit medial attachments sites, with the inclusion of a

transverse ligament, are comparable to human.^{13,33} Similarities between rabbit and human menisci during characteristic OA models of degeneration also exist including increased swelling and decreased GAG production.⁹⁴ In an effort to curb the effects of OA, human protease nexin-1 was used in a rabbit model to inhibit the plasmin/plasminogen cascade responsible for matrix metalloproteinase production, a mediator of GAG loss in cartilage, with positive results.¹⁰² Antibodies for collagen used in Western blotting techniques for human are also cross reactive with the rabbit species. (Calbiochem) This data suggests that cells obtained from soft cartilage of the rabbit closely resemble that of human biosynthetically. Although the rabbit is not a perfect anatomical model of the meniscus as compared to human, trends gained from cellular activity of rabbits to fluid flow may be transferable to what would occur in human meniscal cells.

During excision of the menisci, the transition zone and ligamentous portion as described by Gao et al., 1994,³³ of the menisci were removed before tissue digestion occurred. In the event that not all of the transition zone and ligament like portion were adequately removed from the menisci, there may have existed fibroblast cells in the cell population under study.³³ Fibrochondrocytes themselves are a differentiated form of fibroblasts derived from mesenchymal stem cells, and the composition of both ligaments and menisci are similar in the percentage of proteoglycans (1-3%) making up the tissue matrix. Mesenchymal stem cells have been shown to differentiate into chondrocytes with the application of hydrostatic pressure⁵ and fibroblasts have been found to change reversibly into cartilage cells based on their environment¹. This suggests that fluid flow

may transform fibroblasts into a different connective tissue cell and that the effects of primary culture contamination with fibroblasts may be minimal.

Summary

Meniscal cells cultured in monolayer respond to OFF induced shear stress with an increase in $[Ca^{2+}]_i$, and an increased production of proteoglycans with sulfated GAG side chains. When $[Ca^{2+}]_i$ was blocked it was evident that proteoglycan production was inhibited suggesting that $[Ca^{2+}]_i$ oscillations are necessary in the biochemical pathway responsible for proteoglycan production. From a clinical perspective, understanding the biochemical pathways necessary to produce healthy meniscal tissue will have widespread applications that may be used to further understand and prevent/combat degenerative knee joint ailments such as OA, the leading cause of disability afflicting more than 40 million people nationwide, with drug therapy. From this data it is evident that tissue engineering methods employed to create a suitable tissue construct used to replace damaged or removed meniscal tissue will benefit from the application of OFF induced shear stress when using native meniscal cells embedded in a 3D scaffold or suspension.

Acknowledgements

I would like to thank Rose Riemer, an MTU graduate student performing research at Mayo Clinic, and the lab of Dr. Lewis at Mayo Clinic for the rabbit specimens, and Dr. Seth Donahue for his insight and the use of laboratory space and equipment. The guidance provided by Dr. Eric Blough to detect proteoglycan content was also appreciated.

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Figure Legend

Figure 1. (A) Real time $[Ca^{2+}]_i$ response of meniscal cells to OFF induced shear stress of 6.5 Pa at 1Hz frequency. Flow was initiated at 60 sec and continued for 3 min. Each cell is represented by a line, with a representative population of 37 cells. It is evident that the majority of the cells responded with a transient increase in $[Ca^{2+}]_i$ shortly after the onset of OFF at 60 sec, with only a few cells maintaining a relative amount of calcium at baseline levels. (B) Meniscal cells from specimen 10 loaded with Fura-2AM fluorescent probe excited at 380 nm and imaged at 510 nm using calcium imaging techniques with an OFF induced shear stress of 6.5 Pa at 1 Hz. This image was captured directly at the onset of OFF or at 60 sec.

Figure 2. (A) Mean percentage of cells responding with an increase in $[Ca^{2+}]_i$ at the various shear stress levels (n=5). The baseline response is depicted as zero shear stress where 2% of the representative cell population indicated an increase in $[Ca^{2+}]_i$ occurring. *Statistically significant difference from shear stresses greater than or equal to 2.5 Pa. (B) Mean 340:380 excitation ratio peak amplitude cell response at various shear stress levels. The 340:380 excitation ratio is proportional to the concentration of $[Ca^{2+}]_i$.

Figure 3. Similar to Figure 1. A, but with the inclusion of the $[Ca^{2+}]_i$ blocker thapsigargin in the flow media depicting the meniscal cell response to OFF induced shear stress of 4 Pa at a frequency of 1 Hz.

Figure 4. Amount of total sulfated GAG (ng)/total cell protein (μ g) produced by meniscal cells in response to an OFF induced shear stress of 4 Pa at 1 Hz for 1-2 hours, 1.5 Pa at 1 Hz for 1 hour, no flow for 1-2 hours, OFF induced shear stress of 4 Pa at 1 Hz for 1 hr with the inclusion of the $[Ca^{2+}]_i$ blocker thapsigargin, and no flow for 1 hr with

the inclusion of thapsigargin. *Statistically significant difference between an OFF induced shear stress of 4 Pa to the no flow control sulfated GAG production level. A post flow incubation period of 3 days was used to allow protein production and secretion from the cells for all conditions.

Figure 1. A

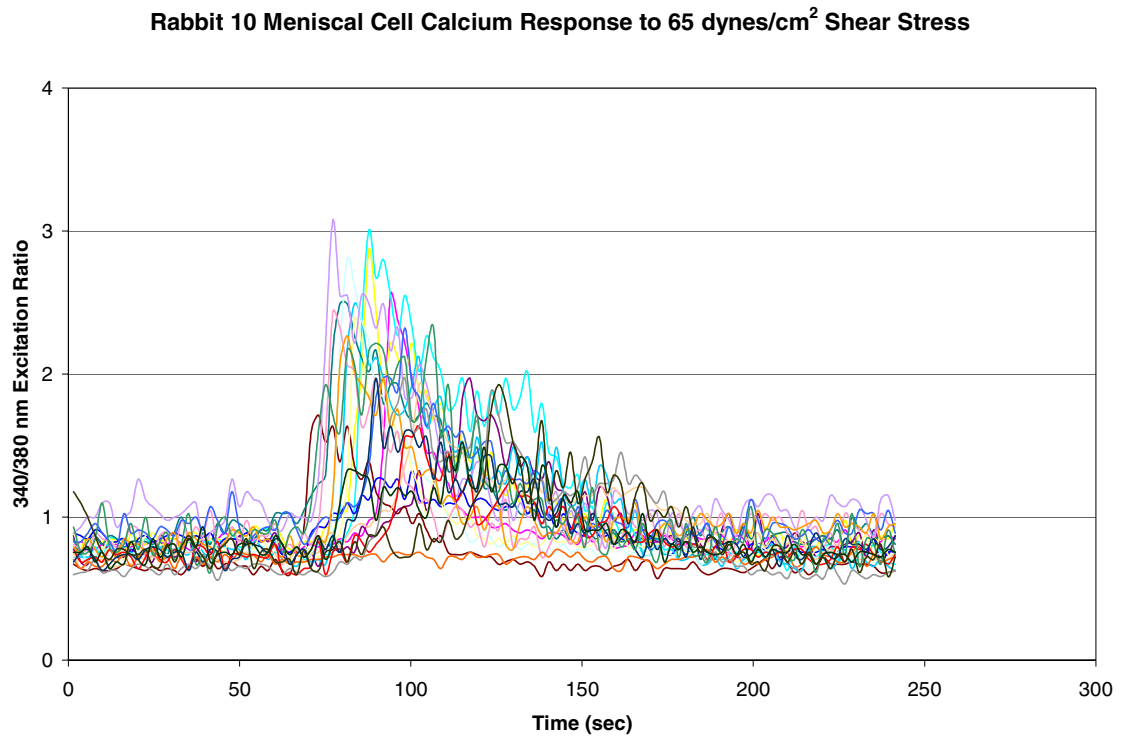


Figure 1. B

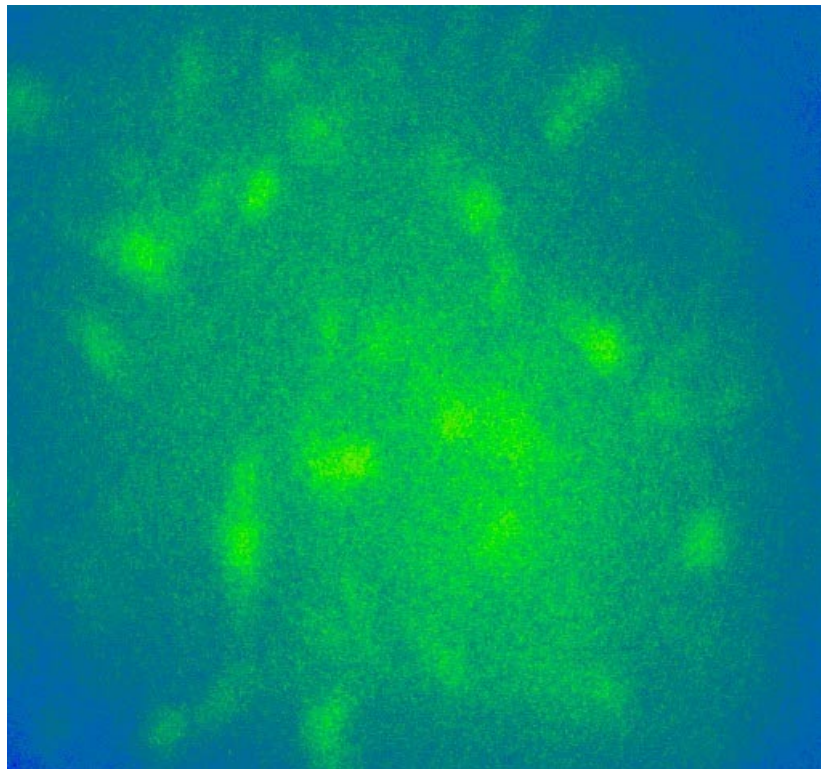


Figure 2. A

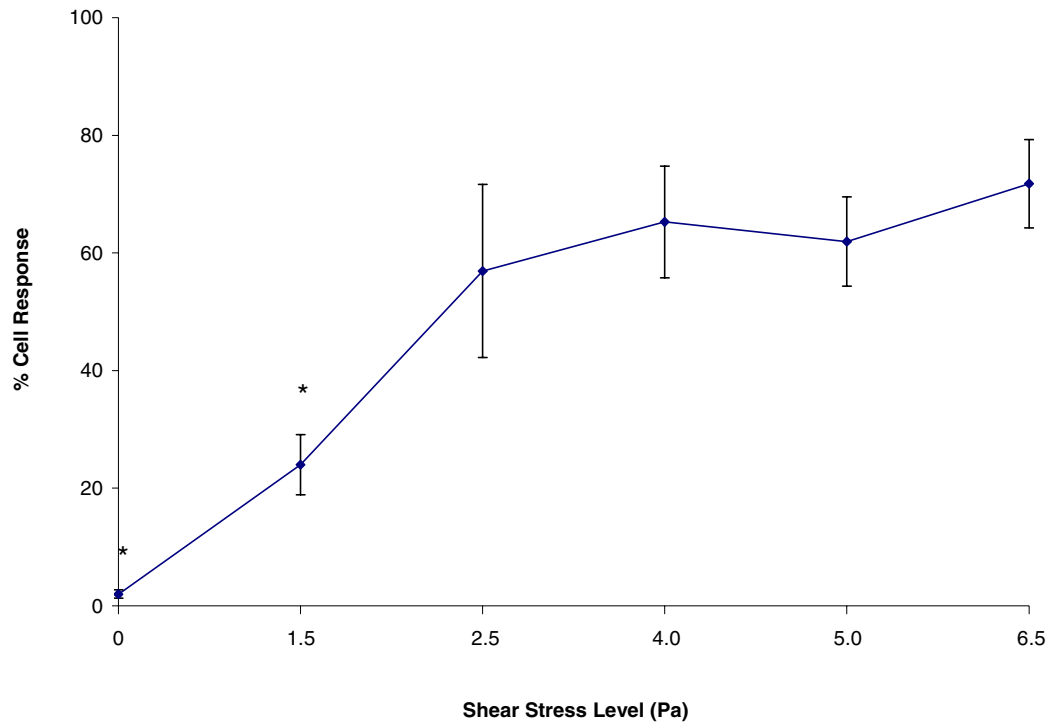


Figure 2.B

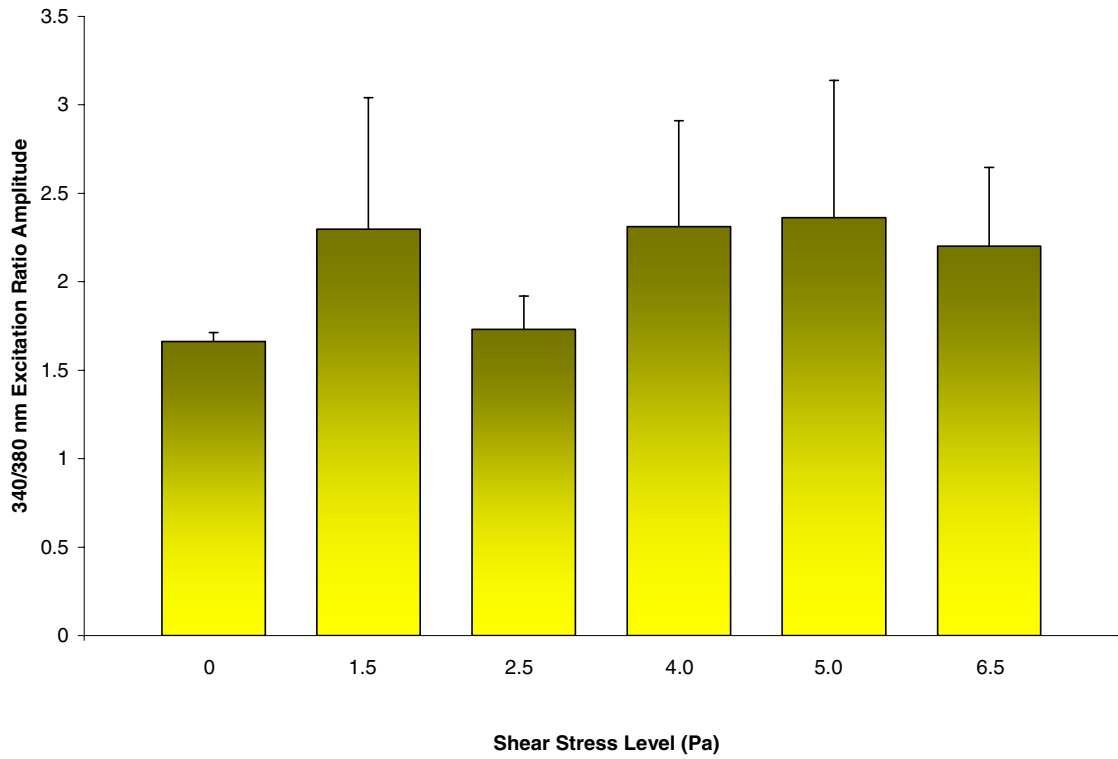


Figure 3.

Rabbit 14 Meniscal Cell Calcium Response to 40 dynes/cm² Shear Stress w/ Tg Calcium Blocker

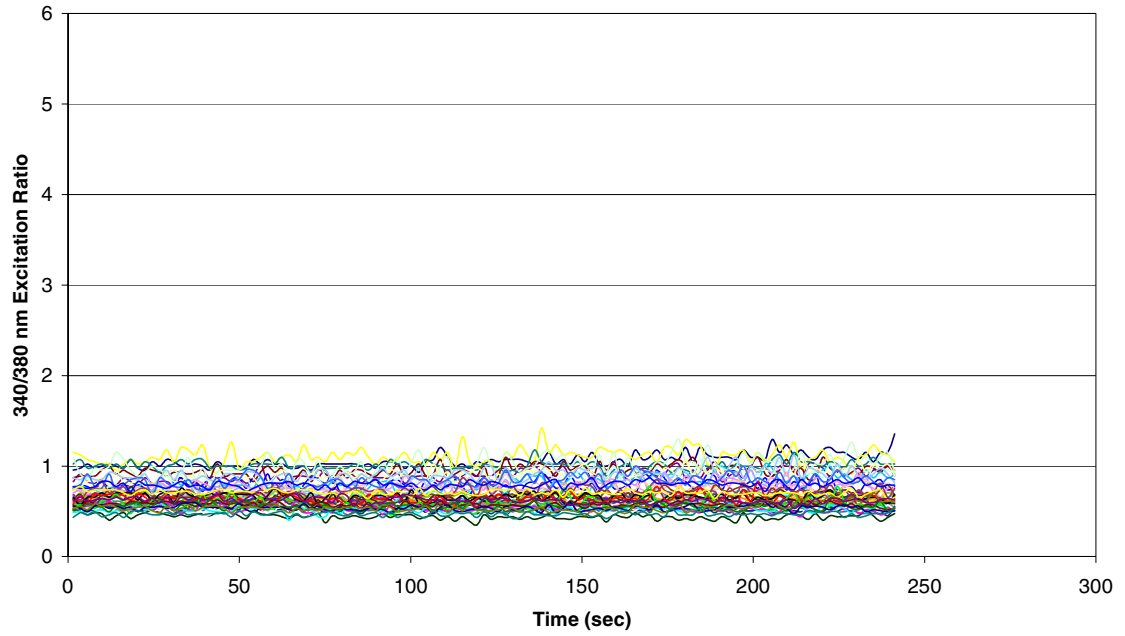


Figure 4.

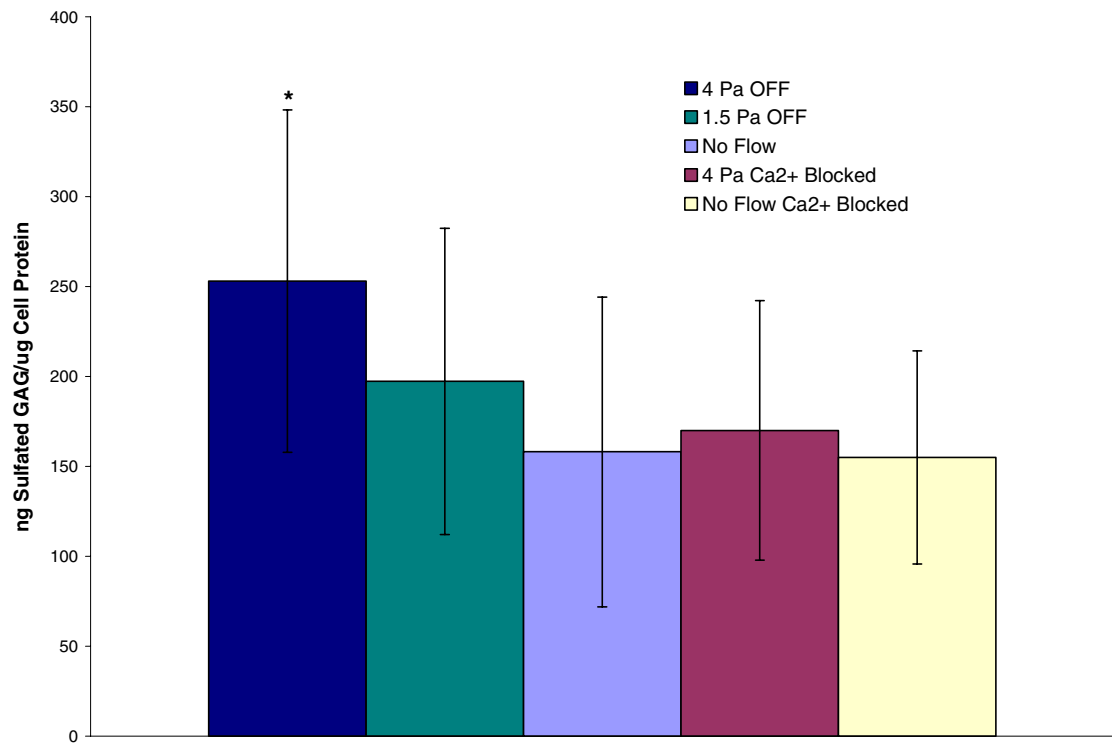


Table 1. Summary of the $[Ca^{2+}]_i$ response of meniscal cells to OFF induced shear stress with the application of fluid flow at various shear stress levels, the no flow control baseline level, and OFF induced shear stress at various shear stress levels with $[Ca^{2+}]_i$ blocked by the pharmacological agent thapsigargin.

Experimental Condition	Shear Stress (Pa)	peak 340:380 ratio relative peak amplitude increase in $[Ca^{2+}]_i$	% Cell Response
OFF	6.5	2.20±0.98	71.8±16.9
OFF	5	2.36±0.74	61.9±17.0
OFF	4	2.31±1.34	65.3±21.1
OFF	2.5	1.73±0.42	56.9±32.9
OFF	1.5	2.29±1.67	24.0±11.5
Control	Baseline	1.69±0.11	2.0±1.5
OFF + Thapsigargin	5	1.23±0.00	1.3±0.0
OFF + Thapsigargin	4	1.44±0.35	1.7±1.5