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Effects of Mechanical Stress on Human Trabecular Meshwork Cells

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Short title: Mechanical Stress on Trabecular Meshwork

Abstract

BACKGROUND: High intraocular pressure (IOP) is a major risk factor for glaucoma. Resistance to outflow of aqueous humor through the trabecular meshwork cells (HTMCs) is believed to cause high IOP. However, the exact mechanism is unknown. IOP is known to fluctuate throughout the day, with much greater fluctuations in glaucomatous compared to normal eyes. These fluctuations cause a continual stretching of the trabecular meshwork. The aim of the study is to develop a dose- and time-response relationship between degree of stretch and HTMCs viability as well as to study specific downstream effects of mechanical stretch on changes in gap junction Connexin43 expression. Gap junctions are known to be important structural and functional intercellular channels in HTMCs.

METHODS: HTMCs from various donors were obtained and grown in primary culture. Upon reaching near-confluency, HTMCs were stretched at 5%, 10%, and 15%, each for 24hr, 48hr, 72hr. Cell health was then measured using vital Trypan blue stain, lactate dehydrogenase (LDH) assay, and ELISA apoptosis assay. Expression of connexin43 was measured using real time qPCR and western blotting.

RESULTS: No significant changes in cell viability were noted upon stretching of HTMCs as measured by Trypan blue stain. LDH levels increased in a dose- and time-response manner with increasing % stretch. There was a significant decrease in apoptosis after 10% stretch. An up regulation of connexin43 protein concentration was observed with a slight decrease in RNA concentration under high % stretch conditions.

CONCLUSIONS: To date, the cause of glaucoma remains elusive and there is scarce information on ocular cell response to mechanical stress. This report demonstrates that mechanical stretch of HTMCs does affect the cellular health and that gap junction protein expression may be a possible mechanism. Understanding sources of cellular stress and mechanisms involved may provide some insights into potential new therapeutic targets.

Keywords: Trabecular meshwork, glaucoma, mechanical stress, intraocular pressure, gap junction, cyclical stretch

Introduction:

Glaucoma is currently the second leading cause of blindness globally according to the World Health Organization and it has affected up to 60 million people in 2010[1]. It is a chronic disease that is on the rise with the aging population yet currently has no cure. The exact cause remains unknown. Glaucoma can be congenital, but a majority of cases are acquired, and falls under the category of open-angle glaucoma[2] with high intraocular pressure (IOP) being the most important risk factor. Recently, however, IOP fluctuations has been thought to be an independent risk factor for glaucoma and have been associated with poor compliance with medication[3]. Physiological IOP fluctuations can range from 4-9 mmHg each day, whereas in glaucoma, IOP fluctuations range as much as 15-20 mmHg over a 24 hour period. Hong et al. have demonstrated from their 13 year follow-up study that patients with higher IOP fluctuations had greater visual field deterioration even despite surgical treatment[4].

The current first line of treatment is eye drops, which includes several categories: prostaglandin analogs, beta blockers, alpha agonists, and carbonic anhydrase

inhibitors.[5] These drugs help maintain an acceptable level of IOP by either decreasing production of aqueous humor or increasing its outflow. Despite the fact that the trabecular meshwork is responsible for 80% of the outflow, no current eye drop targets specifically the trabecular meshwork cells. A new drug class is high in demand as 1996 was the last time that a new class of glaucoma eye drops was introduced.[6]

Both high IOP and IOP fluctuation can potentially act as mechanical stressors by inducing cyclical stretch on the trabecular meshwork, the functional outflow pathway in the eye. Previous work in our lab has demonstrated increase in lactate dehydrogenase (LDH) release and apoptosis of HTMCs under acute stretch conditions, but results were variable and the stretch parameters used were empirical obtained from other cell types in the literature. This variability establishes the need to demonstrate a dose response curve to better understand the physiological and pathological effects of stretch on human trabecular meshwork cells.

There is currently little known about the exact mechanism as to how trabecular meshwork responds to mechanical stress[7]. Gap junctions are a group of intercellular channels that allow for both structural and functional roles such as cell-cell communication, particularly in cells that are physically connected to each other such as in the trabecular meshwork. Gap junctions are known to be involved in signaling between cells and play a critical role in homeostasis. In particular, connexin43 is the most ubiquitous gap junction protein, is known to be expressed in the trabecular meshwork with very little knowledge of its role in this cell. It is for these reasons that connexin43will be examined for its potential role in mechanical stress in HTMCs.

Hypothesis:

Mechanical stress causes a decrease in cell health and an increase in apoptosis of primary human trabecular meshwork cells with an effect on connexin43 expression.

Specific aims:

The objectives of the study are:

1. Examine the effects of % stretch, frequency and duration of stretch on HTMCs. Endpoints of cell necrosis, apoptosis and viability will be measured.
2. Develop a clear dose- and time-response curve between degree of stretch and HTMC viability.
3. Determine the effect of mechanical stretch on gap junction connexin43 expression in HTMC.

Materials and Methods:

Experiment 1: Effects of % stretch, duration of stretch and frequency of stretch on HTMC viability.

Primary human trabecular meshwork cells (HTMCs; ScienCell, Carlsbad, CA) were cultured in cell culture medium containing 89% DMEM, 1% penicillin, and 10% fetal bovine serum. After the cells reach 80-90% confluency, they were plated from 3 different donors (made available commercially), onto collagen-coated 6-well stretch plates (Flexcell, Hillsborough, ON) at a cell density of $5x10^5$ cells/well. This cell density is approximately 45,000 cells/cm^2, which would typically yield an almost confluent layer. For each % stretch, we will prepare 3 different plates for the 3 time points, 24hr, 48hr, 72hr. After the cells in the 6-well plates reach ~90% confluency, we changed the medium to serum-free medium to prevent further proliferation during stretch to control

the degree of confluency. After one hour, we placed the experimental plates in the stretch machine (Flexcell, Hillsborough, ON), and control plates in the sae incubator without subjection to any stretch. We then used the FX-5000 software to create a regimen for each % stretch, setting the frequency to be 1Hz and 72hr, the software was paused temporarily after 24hr and 48hr to take out the plates for that time point. After 24hr, 48hr, and 72hr respectively, we removed supernatant for LDH assay (Sigma, Oakville, ON) and the cells were trypsinized and stained with 10uL of Trypan blue mixed with 10uL cell suspension and counted using Countess Cell Counter (Life Tech, Burlington, ON). The experiment is repeated to generate cells available to use for ELISA apoptosis assay (Roche, Missisauga, ON). A 96 well plate was fixed with antibodies, and the cell lysate were prepared according to the protocol provided by Roche[8]. The data from LDH assay, Trypan blue vital stain, and ELISA apoptosis assay were used to generate dose and time response curves using Microsoft Excel.

Experiment 2: Expression of Connexin43 using real time quantitative PCR.

HTMCs were cultured and stretched as previously mentioned in Experiment 1 for 15% stretch and 72hrs. After the stretch was complete, total RNA was extracted from experimental and control plates using Trizol™ (Invitrogen, Burlington, Canada) according to the manufacturer's protocol. RNA concentrations were quantified using the Nanodrop Spectrophotometer ND-1000 (Nanodrop technologies Inc., Wilmington, DE, USA). manufacturer's guidelines (Life Tech, Burlington ON). Primers against the connexin43 coding strand were obtained from Sigma. The specificity of these primers were tested by running a standard curve from HTMCs cDNA.

The Connexin43 mRNA was quantified by running 100ng of cDNA from control and experimental plates in duplicate with iQ™ SYBR green Supermix (Biorad, Mississauga, Ontario) according to the manufacturer's instructions. CFX384 Touch™ was used for qPCR and Precision Melt Analysis v1.2 software was used for quantification of cycle threshold. The reaction will be carried out at temperatures and time as previously described by Belrose et al[9].

Experiment 3: Protein expression of Connexin43 using western blot.

Using samples obtained from experiment 2, western blots for connexin43 were performed in HTMCs following the protocol as previously described[10]. We isolated connexin43 protein from HTMCs after stretch by washing in PBS at 4°C and then adding lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.02% N_3Na, 100 µg/mL phenylmethylsulfonyl fluoride, 1% NP-40, 50 mM NaF, 2 mM EDTA, and protease inhibitor [Roche Diagnostics, Laval, Quebec, QC]). Cell lysates were collected and reduced SDS-PAGE and Western blotting performed as described by Laird et al[11]. The blots were labeled with anti-Cx43 antibody (Sigma-Aldrich, Oakville, ON) at a dilution of 1:10,000. We then washed the membranes and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000). We quantified protein expression with the enhanced chemiluminescence method (Pierce Biotechnology, Rockford, IL). To ensure equal protein loading, nitrocellulose membranes were stripped (REblot; Millipore, Etobicoke, ON) and reprobed with anti–GAPDH antibody at a dilution of 1:10,000 (Cedarlane Laboratories, Hornby, ON, Canada).

Statistical Analysis

Data were analyzed using Prism software and statistical significance will be analyzed through the use of 2-tailed t-test at 0.05% significance looking at multiple comparisons. $P < 0.05$ is considered statistically significant.

Results:

Cell Viability Dose-Response:

Cell viability was measured using the Countess Cell Counter machine after cells were stretched at increasing doses of 5%, 10% and 15% for the same duration of 48hr and frequency of 1Hz. Viability was expressed as a percentage of the ratio of live cells to dead cells determined by the machine. The red dotted line resembles the mean for the control. Results in Figure 1 show that there is no discernible difference in terms of viability between stretched cells and control. Furthermore, there appears to be no significant difference as the degree of stretch increased from 5% to 15% (n=3, P>0.05).

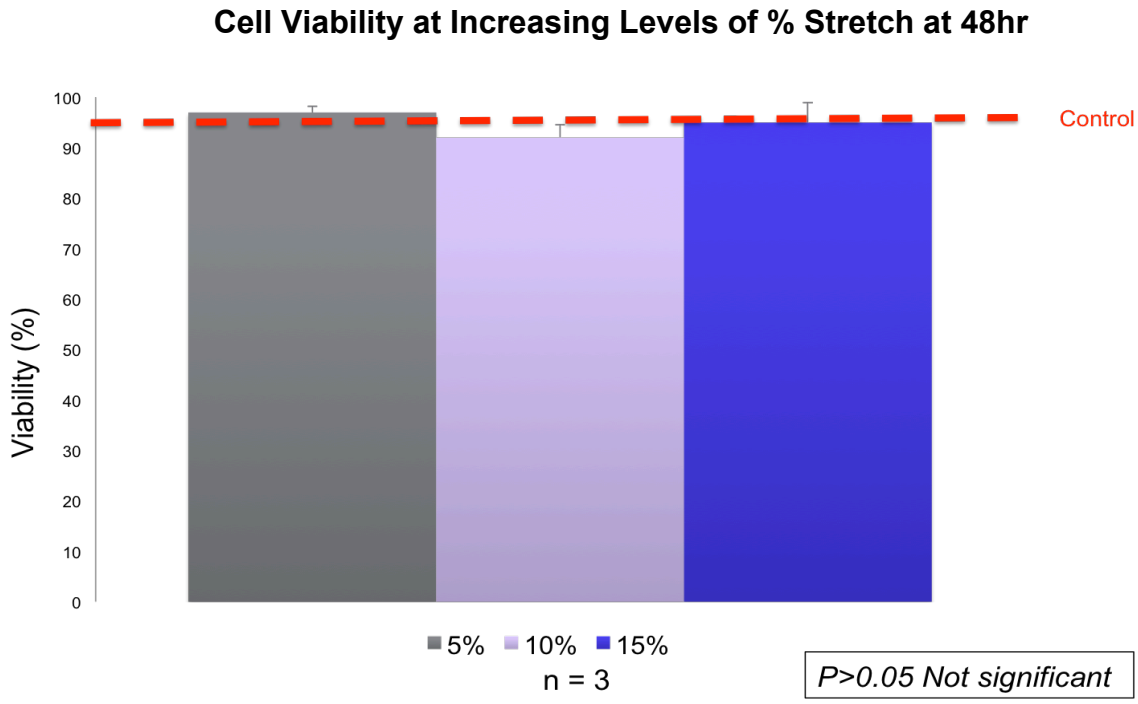


Figure 1. HTMCs viability in dose-response stretch conditions.

Cell viability was investigated by Trypan Blue vital stain using Invitrogen Cell Counter machine. No significant change is observed among the different degree of dose and the control.

Lactate Dehydrogenase Release Dose-Response:

LDH assay was performed using Roche Apoptosis kit to determine cell death. LDH concentration of samples were measured at increasing percent stretch at 5%, 10% and 15% subjected to the same duration of 48hr and frequency of 1Hz. Results in Figure 2 indicate that there appears to be a trend of increasing cell death as cells are stretched at progressively higher degree as shown by increasing levels of LDH. There is no significant change between stretched cells and control (n=3, P>0.05). The red dotted line resembles the mean for the control.

Cell Death in Increasing Levels of % Stretch at 48hr

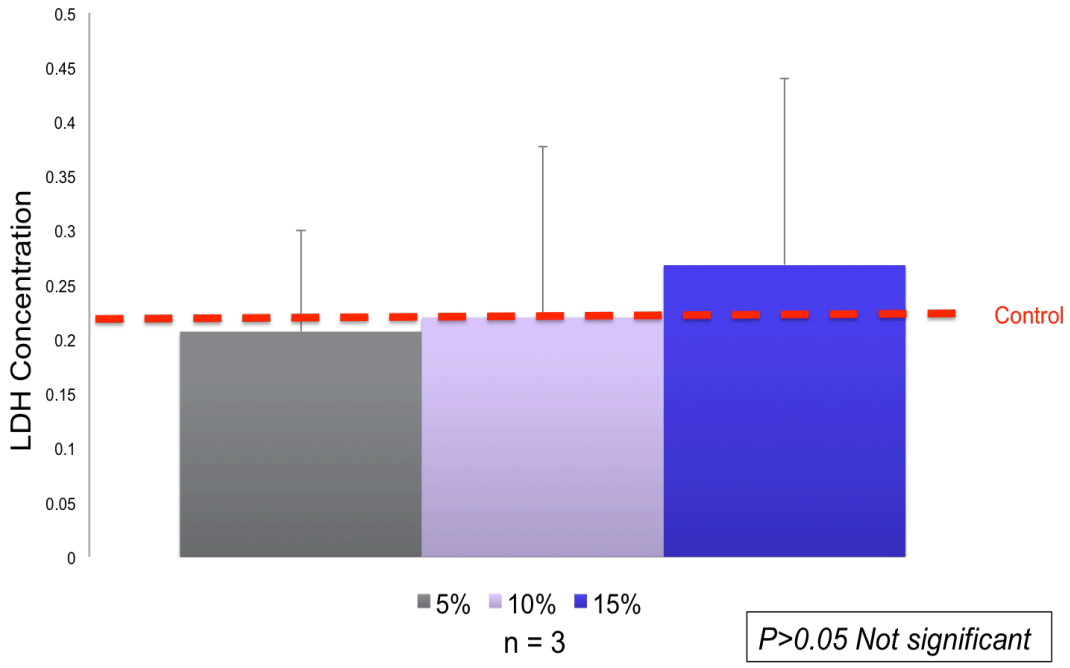


Figure 2. LDH concentration in HTMCs after dose-response stretch vs. control conditions.

Dose-response increase in LDH levels after cells are stretched at 5%, 10%, 15% stretch for 48 hours.

Lactate Dehydrogenase Release Time-Response:

LDH concentration of samples was measured at various time points from 24hr, 48hr, and 72hr at the same degree of stretch of 15% and frequency of 1Hz. Results in Figure 3 show that there is a trend of time-dependent increase of cell death with stretch. As cells are subjected to 15% stretch for longer time, there appears to be more cell death as measured by LDH concentration. There is no significant difference between stretched cells and control (n=3, P>0.05). The mean of the control samples for all three conditions is represented by the red dotted line.

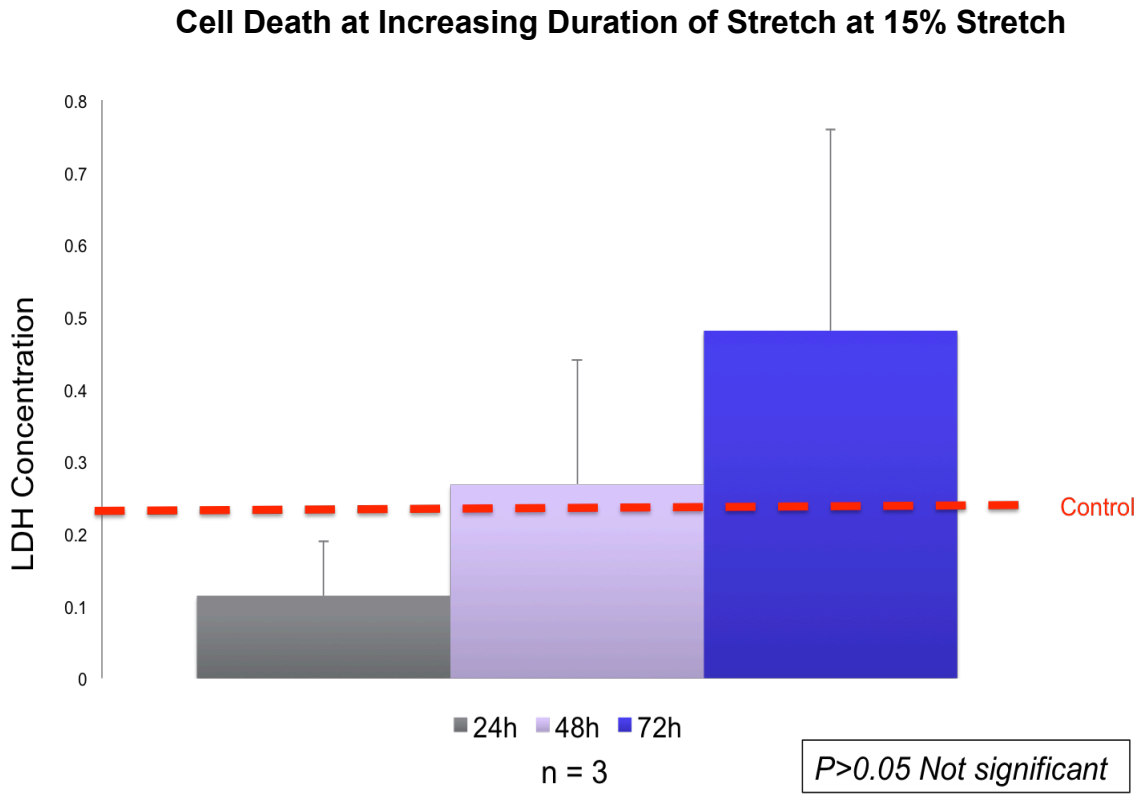


Figure 3. LDH concentration in HTMCs after time-response stretch vs. control conditions. Time-response increase in LDH levels after cells are stretched at 5%, 10%, 15% stretch for 48 hours. No significant difference was observed.

ELISA Apoptosis Analysis:

ELISA Apoptosis was performed in cells after being stretched of 10% and 15% at 48hr, and 1Hz. The amount of apoptosis was determined by measuring the nucleosome concentration in stretched cells compared to control. Greater nucleosome concentration is believed to indicate greater degrees of apoptosis. Results in Figure 4 demonstrate that in both 10% and 15% stretch, there appears to be a decrease in apoptosis compared to control. At 15% stretch, there is a significant decrease in apoptosis (n=6, P=0.04). Results were normalized with protein concentration to ensure results are normalized to cell number.

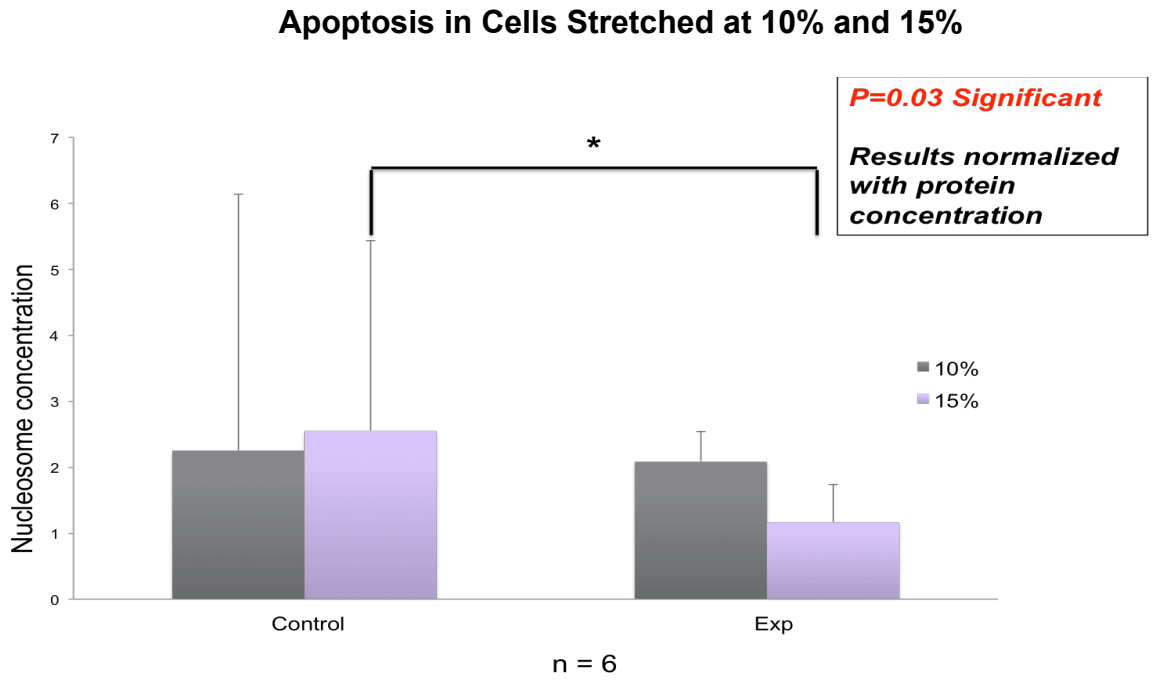


Figure 4. Level of apoptosis in HTMCs after 10% and 15% stretch compared to control. ELISA apoptosis assay was performed using Roche apoptosis kit. There is a significant decrease in apoptosis after cells were subjected to 15% stretch. Statistical analysis was performed using p-test in Microsoft Excel.

Western Blot Analysis of Connexin43:

After cells were stretched at 10% and 15%, western blot was preformed to determine gap junction connexin43 level. Results from Figure 5 show that there is an increase in connexin43 after cells were subjected to stretch compared to control in both 10% and 15% conditions. Although there is a trend of up-regulation of protein, there is no significant difference in levels of Connexin43 in stretched cells compared to control (n=3, P>0.05).

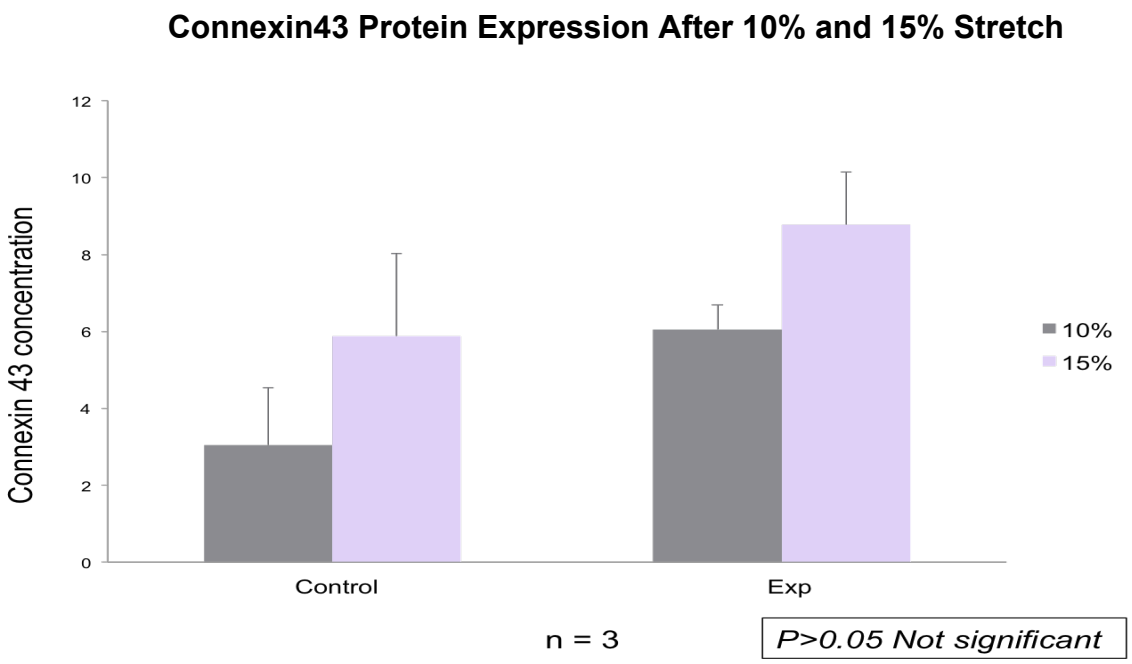


Figure 5. Connexin43 protein expression from Western Blot.
Western blot was used to investigate connexin 43 expression. There appears to be an increase in protein expression after both 10% and 15% stretch. There is no significant difference.

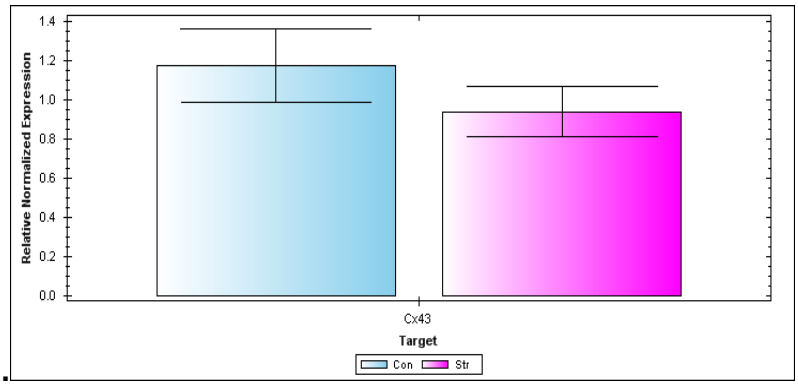
Real Time qPCR Results of Connexin43:

Real time qPCR was preformed to determine levels of mRNA for connexin43 in stretched cells compared to control. Results from Figure 6a and 6b show that there is a

slight decrease in connexin43 mRNA in both 10% and 15% stretch compared to control; the difference is not significant (n=3, P>0.05).

RNA Levels of Connexin43 After 10% Stretch

6a.



RNA Levels of Connexin43 After 15% Stretch

6b.

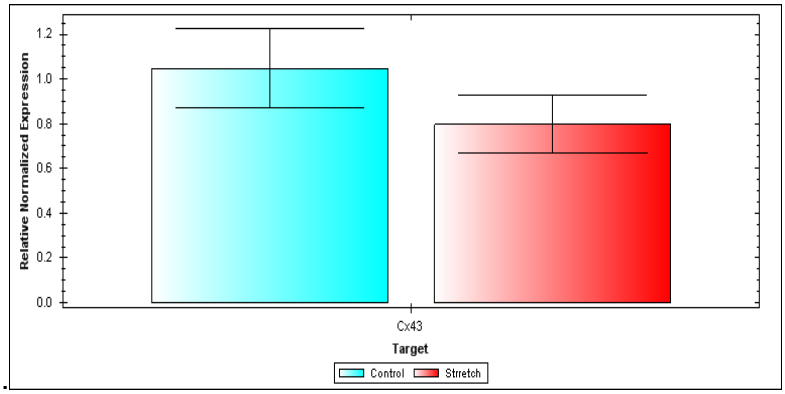


Figure 6. Real time qPCR results of Connexin 43 mRNA.
6a. There is a slight decrease in RNA levels after 10% stretch. **6b.**There is a slight decrease in RNA levels after 15% stretch. Both qPCR was performed with n=3, statistical analysis was performed and the result is insignificant (P>0.05).

Discussion:

The trabecular meshwork has been an increasing target for treatment of glaucoma due to its role as the major outflow pathway for draining the aqueous humor. The most prevalent glaucoma is primary open-angle glaucoma (POAG).[12] POAG is the leading cause of irreversible and preventable blindness worldwide, with its main risk factor

being high intraocular pressure (IOP). Current medication aims to manage glaucoma by reducing the IOP, but none of the current classes of drugs can reduce IOP by more than 25-30%.[13] There is an increasing interest in targeting the trabecular meshwork for development of a new class of drugs however, there is little known about the response of trabecular meshwork to high IOP in glaucoma. High IOP can act as a form of mechanical stress for the trabecular meshwork, and there is no study to date that quantitates the response of HTMC to stretch. By using the FLEXCELL stretch machine, we were able to deliver a form of mechanical stress to cultured primary HTMC, which was measured by percent stretch; that is the percent change in surface area when stretched. The primary goal of this study was to investigate various degrees of stretch and duration of stretch on the health of HTMC. The present study reports three major findings: (1) There is an increase of HTMC death under stretch conditions at a dose- and time-response manner, (2) the mechanism of cell death does not seem to be apoptosis and (3) there is an up-regulation in the expression of the gap junction protein connexin43. Taken together, these results suggest that there is a dose- and time-dependent increase in necrotic cell death in trabecular meshwork after being subjected to mechanical stress.

Our results show that primary HTMC cultured *in vitro* demonstrate an increase in cell death after stretch compared to control as measured by LDH concentration. This increase in cell death due to stretch is seen in a dose- and time-dependent manner. This research has provided evidence that, *in* vitro, 5% stretch for 24 hours may represent a model of physiological stretchas there was no discernible difference

between LDH release in stretched compared to control cells. Pathological stretch could be induced 15% for 72 hours.

Although there was a distinct trend, there was no statistically significant difference between LDH release in stretched cells as opposed to control. This result, could indicate that a larger sample size is needed or that variability in donor responses to mechanical stretch may be variable. It may be useful to examine, in replicates, how individual donors behave to stretch rather than combining the results of several donors in a single experiment.

As apoptosis is common pathway leading to cell death, it was anticipated that stretch would cause increased apoptosis. In contrast, the results demonstrated a significant decrease in apoptosis after cells were stretched at 15% for 72 hours. The result was normalized with protein concentration to ensure the decrease was not due to large differences in cell numbers between stretched and control cells. The decrease in apoptosis could be attributed to the increasing necrotic cell death as measured by LDH release discussed earlier. Necrosis is morphologically defined by cytoplasmic swelling, dilation of organelles which causes cellular vacuolation and rupture of the plasma membrane, and thus can be measured by the level of LDH released in the supernatant.[8] Apoptosis however, is controlled, programmed cell death characterized by chromatin condensation and fragmentation and thus can be measured by the level of cytosolic nucleosome content by the ELISA apoptosis assay.[8] These results suggest that when mechanical stress is applied, HTMC tend to die through the necrotic pathway instead of apoptosis. Differentiating the two in understanding our results is important as it can have clinical implications when determining potential therapeutic targets for glaucoma.

Confirmation of this finding may be obtained in the future by other assays of apoptosis such as caspase-3. While the ELISA assay focuses on measuring cytoplasmic nucleosomes, the caspase-3 assay measures a key enzyme involved in induction of apoptosis.[8]

An up-regulation of connexin43 protein after HTMC were subjected to 15% stretch for 72 hours was observed by Western blot. The up-regulation of connexin43 may be a stress response to mechanical stretch. Connexin43 has been implicated in a number of cell types in the stress response. However, its regulation may go up or down, depending on the cell type. Evidence that suggests connexin43 can modulate apoptosis includes a study done by Giardina et al. that found connexin 43 can confer resistance to hydrogen peroxide mediated apoptosis and Plotkin et al. have shown that connexin 43 transduces pro-survival signals in osteocytes.[14,15] However, Sun et al and Ramachandran et al demonstrates the opposite is happening; connexin43 increase apoptosis in pancreatic cancer cells and in tumor cells respectively.[16,17] Thus it is worthwhile to investigate the link between connexin43 expression and apoptosis in HTMC under stretch conditions.

Despite the increase in protein expression of connexin43 that was observed with western blot, there was a slight decrease in connexin43 RNA after 10% and 15% stretch detected by real time qPCR. The discrepancy between protein and RNA level could be attributed to the fact that the cells were being stretched for 72 hours in serum-free medium. Essentially the cells were being starved and slowly declining in health. The increase in protein level could be attributed to the fact that the cells had successfully translated the available RNA in the cell to protein, but were losing ongoing

transcription due to prolonged stretch. Future experiments will stretch cells at 48 hours and of 72 hours as previous work 48 hours may be less harsh, yet significant as determined by previous work that did demonstrate connexin43 up regulation under these milder conditions. Conditions of 15% and 72 hours stretch may be ideal for a cell death endpoint whereas 15% at 48 hours may be more of an injury model. Future experiments will be needed to determine this.

Currently there is no medication available which directly targets the trabecular meshwork despite it being the site of dysfunction, and hence, pathogenesis of glaucoma. Studies such as those presented in this research have the potential to provide insight into the contribution of mechanical stretch to impaired trabecular meshwork, thus potentially providing novel targets for drug development.

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