Investigation into the Anti-Inflammatory Properties of Metformin as a Potential Therapeutic Agent for Lower Back Pain

by

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Intervertebral disc degeneration (IDD) is closely related to heightened inflammation in the annulus fibrosis (AF) and nucleus pulposus (NP) cells in the intervertebral disc. Gene expression and enzymatic activity of catabolic factors that degrade the extracellular matrix, such as matrix metalloproteinase 13 (MMP-13), IL-1β, PGE$_2$ and COX-2, can be triggered by inflammatory as well as mechanical (tensile/compressive) stresses. An imbalanced matrix homeostasis, i.e., enhanced catabolic and suppressed anabolic activity, has been shown to contribute to disc degeneration and associated discogenic low back pain. There have been multiple efforts to curtail this imbalance through both therapeutic and preventative measures.

Developed in 1922, metformin has recently been the most widely used oral medication for type II diabetes in the United States. Traditionally, metformin has been used to decrease hepatic gluconeogenesis and increase insulin sensitivity by inhibiting mitochondrial pathways in diabetic patients. It has also been noted to exhibit anti-inflammatory properties through upregulation of the AMPK pathway, leading to various pro-autophagy and anti-inflammatory-related responses in hepatocytes. However, it is still unclear how metformin influences disc cellular response to inflammatory stress and the mechanism in which it enacts its effects. Hence, the objective of this study is to elucidate the effects of metformin on expression of key pro-inflammatory, catabolic, and anabolic factors within rat AF cells in response to inflammatory stimulation and mechanical tensile stress.
Five Fischer 344 rats were sacrificed and their spines isolated. AF cells were cultured and plated in flexible silicone membrane-based six-well plates. Wells were split into eight groups and subjected to metformin, IL-1β, mechanical stretch, and combined treatments. Relative gene expressions of MMP-13, COX-2, iNOS, AGC, and Col1 were assessed with qRT-PCR, and downstream PGE$_2$ production was quantified with ELISA.

Metformin in the presence of the combined stress treatments (M+IL/S) significantly decreased COX-2 and iNOS expression, decreased PGE$_2$ production, and increased Col1 expression. The lack of metformin-mediated suppression of inflammatory response in the non-stretch groups indicates that metformin may be enacting its effects through a stretch-dependent manner.

These results suggest a foundation for pursuing further research into metformin’s potential role as an anti-inflammatory agent for curtailing intervertebral disc degeneration.
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1.0 INTRODUCTION

Lower (lumbar) back pain is one of the most common symptoms leading to a clinical visit. Previous literature has indicated an annual healthcare expenditure of $50-$100 billion for low-back pain related conditions (Katz, 2006). The onset of chronic lumbar pain has been shown to interfere with workplace productivity, costing an average of 32 days’ absence per calendar year and $7,800 in lost productivity per employee (Joish & Brixner, 2004). Multiple risk factors have been identified for lumbar back pain in addition to the apparent occupational circumstances and lifestyle choices (e.g. smoking), age, and genetic factors - all of which have been linked to apparent intervertebral disc-related conditions. Particularly in the geriatric population, intervertebral disc degeneration has been documented to play a contributive role in the etiology of chronic lower back pain (Katz, 2006).

Patients that suffer from intervertebral disc degeneration (IDD) present with discogenic pain typically caused by chronic damage to the intervertebral discs between vertebral bodies in the lumbar spine. Damage to intervertebral discs in geriatric patients occur over time, a resultant of the decades of compressive loading, tensile forces, increased local inflammatory profile, and environmental factors. IDD in patients has been linked to radiculopathy, spondylitis, disc herniation, and spinal stenosis in varying degrees of severity (Resorlu et al., 2015). Currently, treatment of IDD is geared towards improving joint mobility in the spine and regaining the ability to perform daily tasks with minimal pain. These treatments can take the form of surgery, medication, physical therapy, injections, and/or lifestyle changes. In addition to surgical procedures involving removal of degenerative disc components, biomedical and pharmaceutical treatments have been gaining much ground recently in the management of IDD.
There is substantial evidence indicating that chronic inflammation and catabolism of the intervertebral disc facilitates extracellular matrix breakdown, accelerating intervertebral disc degeneration (Molinos et al., 2015). Thus, research in biomedical and pharmaceutical treatments for IDD have been geared towards curtailing the inflammatory response and catabolism in the disc.

Metformin, typically used in the treatment of type II diabetes, has been gaining ground as a potential therapeutic agent for its anti-inflammatory properties. Chen et al. have demonstrated that metformin inhibits the expression of pro-inflammatory factors such as the MMPs and cyclooxygenase-2 (COX-2) in nucleus pulposus (NP) cells within the disc in response to tert-Butyl hydroperoxide, an inflammatory response-inducing oxidant (Chen et al., 2016). However, this oxidant very loosely mimics inflammatory progression in the disc and does not consider mechanical loading, which has been shown to contribute to the inflammatory process (Miyamoto et al., 2006). Thus, this dissertation will address the gaps in literature regarding the effect of metformin on the inflammatory profile of the intervertebral disc in response to both inflammatory and mechanical stimulus in a manner that is more representative of causative factors of disc degeneration in vivo.
2.0 BIOLOGICAL AND MECHANOCOLOGICAL CONTRIBUTIONS TO INTERVERTEBRAL DISC DEGENERATION

2.1 Anatomy and Physiology of the Intervertebral Disc

To understand the mechanisms of IDD, we must explore how the structure of the intervertebral discs and the surrounding vertebral constituents relate to the function of the spine as a whole. The intervertebral disc consists of three major types of connective tissue: Nucleus pulposus (NP), annulus fibrosus (AF), and cartilaginous end plate (EP) (Fig. 1). While the NP and AF constitute much of the intervertebral disc volume, the EP is the comparatively thin connective tissue that juxtaposes each adjacent vertebral body to the intervertebral disc.

Figure 1 - Anatomy of the intervertebral disc and associated structures in the lumbar spine. Image courtesy of University of Pecs.
The intervertebral disc region is hypoxic, lacking vascularity and access to active blood supply, aside from the few vessels that innervate the posterior longitudinal ligament and the outermost AF (Yao et al., 2017). Thus, the cells of all three tissues have evolved unique mechanisms to maintain function in the hypoxic environment; albeit, EP provides certain essential nutrients in low concentrations to the AF and NP through simple diffusion. With time, chronic mechanical stressing of the EP tissue can lead to ossification, acting as a catalyst for IDD by reducing nutrient supply to the AF and NP cells of the disc (Moore, 2006). Composing 40-50% of the total disc volume, the NP is the gelatinous interior of the intervertebral disc, circumscribed by the AF. Healthy NP maintains dynamic hydrostatic pressure within the disc due to its high water content, which can be attributed to the localized concentration of hydrophilic proteoglycans. With age, decreased water content within the NP has been linked with the loss of proteoglycans (Newell et al., 2017). A normal range of in vivo pressures in the NP was recorded to be between 460 and 1330 kPa, with the highest pressure recorded during forward flexion with a 20 kg mass at 2300 kPa (Newell et al., 2017). High pressures inside the NP can radially disperse stress onto the AF.

The AF is composed of concentric lamellae surrounding the NP, largely consisting of collagen I and II fibrils. Collagen functions to radially distribute tensile and shear forces within the disc. A rupture of this lamellar collagen matrix from chronic mechanical stress can cause NP to leak out, resulting in disc herniation and various IDD-associated conditions (Fig. 2, Fig. 3).
Figure 2 – Schematic of disc herniation caused by AF rupture. Image courtesy of Mayfield Clinic.

Figure 3 – Lumbar MRI of three swimmers reporting severe lower back pain highlighting the compromising effect of herniated NP on the spinal canal. Image courtesy of Dr. John Mullen, Training COR.
2.2 Chronic Inflammation and its Contributive Role in Intervertebral Disc Degeneration

With age, chronic inflammatory responses localized to the disc have been shown to accelerate degeneration. Current literature supports the hypothesis that immune response is triggered through disc by-products and waste of matrix production. Intermediate compounds of collagen and proteoglycan production, such as fibronectin and hyaluronan, have been shown to increase protease activity and cytokine presence within the disc, such as the upregulation of interleukin-1β (IL-1β) (Molinos et al., 2015). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a protein complex that plays an important role in cytokine production and mechanical signaling, may be activated by genotoxic stresses, cytokines, mechanical stimulation, and/or infection (Fig. 4). Laminin, fibronectin, and other ECM fragments are shown to bind to Toll-like Receptor 2/4 and activate a secondary messenger system that facilitates the nuclear translocation of NF-κB, (Molinos et al., 2015).

![Figure 4](Image Courtesy of Cold Spring Harbor Perspectives in Biology)
Upon activation, NF-κB is responsible for a variety of cellular responses, with a steadfast contribution to immune/inflammatory response via the IL-family, as well as cell proliferation, death, and survival (Lawrence, 2009). The numerous functions of NF-κB have prompted recent research to be directed towards its role in disc degeneration. As noted by Tisherman et al., inhibiting NF-κB dampens the inflammatory response and catabolic factor production in the disc (Tisherman et al., 2016). At the RNA level, NF-κB upregulates the gene expression of pro-inflammatory factors IL-1β, iNOS, COX-2, and catabolic factor MMP-13, facilitating breakdown of collagen and proteoglycan. Therefore, an understanding of the underlying mechanisms of inflammation and its associated factors is crucial to assessing the role of inflammation in disc degeneration.

2.3 Arachidonic Acid Pathway and Role of Prostaglandins in Inflammatory Response

The Arachidonic Acid pathway is a highly researched and established mediator of inflammatory response across various cell and tissue systems in the body. Arachidonic acid is native to the phospholipid membrane, and is released by phospholipase A₂, or less commonly via a two-step process involving phospholipase C and DAG lipase. Downstream, it becomes the substrate of cyclooxygenase-2 (COX-2) and is converted into a variety of prostaglandins. This is a control step for non-steroidal anti-inflammatory drugs (NSAIDs), which target and inhibit COX-2, COX-1, or both to downregulate inflammatory response downstream (Litalien & Beaulieu, 2011). Thus, many prostaglandins are known to be causative agents of inflammation. Prostaglandin E₂ is one of the main downstream products of COX-2, and is of particular interest in the study of disc degeneration. Previous groups have shown that mechanical tensile strain and
inflammatory factors such as IL-1β and TNF-α synergistically increase PGE₂ concentration many-fold within AF cells (Miyamoto et al., 2006). Inflammatory cells have also been shown to co-express both isoforms of COX (1 and 2), and inhibition of either isoforms have been shown to decrease PGE₂ production (Ricciotti & Fitzgerald, 2011). Despite the complexity of inflammation and its underlying mechanisms, there is overwhelming evidence to suggest that suppression of PGE₂ and COX-2 reduces inflammatory response via the arachidonic acid pathway. As inflammation plays a large role in intervertebral disc degeneration, anti-inflammatory agents are currently gaining ground in becoming therapeutic precursors for treating IDD-induced low-back pain.

Figure 5 – Arachidonic Acid pathway highlighting key downstream prostaglandin products. Image courtesy of Emanuela Ricciotti, Arterioscler Thromb Vasc Biol 2011 May; 31(5): 986-1000.
2.4 Biomechanical and Mechanobiological Contributions to Disc Function

Cells of the AF and NP act in conjunction to constitutively produce extracellular matrix (ECM) to maintain disc health and stress distribution properties. While the AF and NP produce collagen type I and proteoglycans respectively in response to deformation, the overall maintenance of the ECM is governed by both biochemical and biomechanical changes. Aging and its associated chronic inflammation in the intervertebral disc bring about biomechanical changes within the AF and NP cells. Annulus fibrosus tissue becomes fibrillated and roughened over time, resulting in a decreased ability to withstand shear force and radially distribute tension (Nguyen, Jacobsen, & Chahine, 2017). The aging nucleus has been shown to exhibit lower hydrostatic pressure and volume. These changes manifest in fibrotic formations in place of the functional nucleus pulposus, ultimately decreasing the ability of the disc to withstand compressive loading. As suggested by Adams, degenerated discs transfer load from the nucleus pulposus to the posterior annulus (Adams, 1996). The increase in load experienced by the annulus weakens it further and heightens the compressive stress peaks in the biomechanical response profile of the functional spine unit up to 160% (Adams, 1996). These areas of high stress within the annulus are hypothesized to be contributors towards lower back pain and disc herniation. While the effect of mechanical stretch on the tissue level is important in the clinic, where stages of disc degeneration are used to characterize age-related diseases, there are notable changes occurring at the cellular level that largely dictate and influence degeneration of the disc tissue. Miyamoto’s group has found mechanical stretch-mediated upregulation of PGE₂ and key pro-inflammatory agents, including COX-2 (Miyamoto et al., 2006). Increased expression of proteases such as MMPs in response to tensile stretch has also been implicated in degenerative discs and noted in elderly patients with advanced disc degeneration (Nemoto, Yamagishi, Yamada, Kikuchi, & Takaishi, 2006). It was
also found that mechanical stretch, in conjunction with IL-1β treatment, synergistically increases PGE₂ synthesis beyond either treatment alone in AF cells (Miyamoto et al., 2006). This combination of stimuli forms a positive feedback loop, where mechanical and cytotoxic (inflammatory) stresses exacerbate the local inflammatory response and accelerate the rate of disc degeneration.

The degree of mechanical strain has also been shown to affect the gene expression profile of the disc cells. Sowa et al. show that at a physiological range of strain (6%) actually exerts a positive and protective effect on AF cells by suppressing pro-inflammatory and catabolic genes such as iNOS, TNF-α, MMP-13 and -3. Conversely, high levels of strain (18%) exhibited the greatest catabolic response, including upregulation of MMPs, iNOS, and COX-2 under inflammatory conditions (Sowa et al., 2011).

2.5 Metformin: Overview and Mechanism of Action

Metformin, synthetically developed in 1922, has been the most widely used oral medication for type II diabetes in the United States since the early 21st century. It is chemically synthesized with organic reactions involving dimethylamine hydrochloride and 2-cyanoguanidine. Traditionally, metformin has been used to decrease hepatic gluconeogenesis and increase insulin sensitivity by inhibiting pathways that promote gluconeogenesis in the liver. Although the exact mechanism in which metformin acts is still largely unknown, Zhou et al. demonstrated that metformin is correlated with increased activation of the AMP-activated protein kinase (AMPK) pathway in primary hepatocytes (Zhou et al., 2001). While the AMPK pathway is largely a homeostatic pathway upregulating glucose and fatty acid uptake when cellular energy is low, it
has also been shown to modulate multiple different aspects of cellular metabolism including autophagy, redox regulation, aging, and inflammatory response (Fig. 6) (Jeon, 2016). In the context of IDD, metformin has been found to increase AMPK activation, which has multiple downstream benefits. Namely, AMPK inhibits NF-κB translocation into the nucleus via SIRT1/FOXO/PGC1α (Fig. 6), downregulating the transcription of pro-inflammatory genes such as COX-2 and iNOS. Metformin’s role in increasing AMPK activation has led to various studies exploring its potential anti-inflammatory and pro-autophagy (cellular recycling) effects on various cell lines (Gu, Ye, Wang, Sun, & Hu, 2014; Isoda et al., 2006). However, there is sparse literature investigating the potential anti-inflammatory properties of metformin in relation to the intervertebral disc to combat disc degeneration.
Figure 6 – Schematic highlighting various discovered pathways associated with AMPK activation. *Image courtesy of Sang-Min Jeon, Experimental & Molecular Medicine 48, e245 (2016).*
3.0 OBJECTIVE

The objective of this dissertation is to investigate the effects of metformin on AF cells under *in vitro* conditions that most closely mimic biological and mechanical contributions to disc degeneration. Cells will be subject to metformin under induced inflammation, traumatic mechanical stretch, and both in conjunction to identify the pathways in which metformin interacts with cellular processes, with a particular focus on changes in the inflammatory response and ECM-associated gene expression of AF cells. Investigation of metformin’s anti-inflammatory properties relating to the intervertebral disc may provide a foundation for further research on metformin’s potential therapeutic uses in treating low-back pain resulting from intervertebral disc degeneration.
4.0 METHODS

4.1 Specimen Procurement

Five Fischer 344 rats (Mean age: 9±2.5 months) were obtained under IACUC protocol 18022330. All rats were visually examined prior to sacrifice to note any abnormalities.

4.2 Spine Isolation

All rats were euthanized with carbon dioxide as stated in the IACUC protocol. A secondary method of euthanasia (cervical dislocation), was used to ascertain cardiac and respiratory arrest. After sacrifice, rats were incised posteriorly from cervical to sacral spine. Rat spines were extracted along with the tail for cell culture. Ligaments, connective tissue, and muscle from the lumbar spine and tail were removed prior to cell isolation to facilitate discectomy.

4.3 AF Cell Isolation and Culture

Annulus fibrosus was collected from as many disc levels as possible and placed in a tube with F-12 media (10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin (PS)). This tissue was then exposed to the proteolytic enzyme pronase, which has been shown to be a rapid and efficient dispersing agent for fibroblastic cell lines (Foley & Aftonomos, 1970). Pronase was diluted in 10% FBS/1% PS to form 0.2% pronase solution. After slow-stir incubation of AF tissue
in 0.2% pronase for 1 hour, cells were washed with Hank’s Balanced Salt Solution (HBSS) twice. AF tissue was then transferred to a .02% collagenase solution (diluted in 10% FBS/1% PS) for further digestion of native collagen fibrils. Tissue was subsequently slow-stir incubated for 3.5 hours with intermittent monitoring. Depending on the amount of tissue, the incubation was halted when little to no macroscopic AF tissue was left visible. AF cells were then washed with HBSS and spun down for 5 minutes at 2000 rpm. Finally, cells were resuspended in F-12 (10% FBS/1% PS) and plated onto T-75 flasks. Cells were stored in a hypoxic chamber (5% CO₂, 5% O₂, 37.5°C) to simulate the intervertebral disc environment. Cell media was changed every few days to facilitate rapid growth. Once cells had grown to ~85% confluence, they were passaged with Trypsin and plated onto six-well FlexCell (FlexCell International Corp., Hillsborough, NC) plates for subsequent treatment (Fig. 7).

4.4 Metformin, IL-1β, and Stretch Treatment

After two days of incubation in FlexCell plates, the cells were checked for sufficient confluence for treatment (~85%). Wells were split into eight groups (Table 1, Fig. 7) for treatment.
Figure 7 - FlexCell plate layout for group treatment conditions.

Table 1 - Names and Abbreviations for Treatment Groups.

<table>
<thead>
<tr>
<th>Group Name</th>
<th>Abbreviation</th>
<th>Treatment Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>C</td>
<td>No treatment</td>
</tr>
<tr>
<td>IL-1β treatment</td>
<td>IL</td>
<td>1 ng/mL IL-1β</td>
</tr>
<tr>
<td>Metformin treatment</td>
<td>M</td>
<td>100µM Metformin</td>
</tr>
<tr>
<td>Metformin + IL-1β treatment</td>
<td>M+IL</td>
<td>100µM Metformin + 1 ng/mL IL-1β</td>
</tr>
<tr>
<td>Control + Stretch</td>
<td>S</td>
<td>18% strain, 24h</td>
</tr>
<tr>
<td>IL-1β/Stretch</td>
<td>IL/S</td>
<td>1 ng/mL IL-1β + 18% strain, 24h</td>
</tr>
<tr>
<td>Metformin + Stretch</td>
<td>M+S</td>
<td>100µM Metformin + 18% strain, 24h</td>
</tr>
<tr>
<td>Metformin + IL-1β + Stretch</td>
<td>M+IL/S</td>
<td>100µM Metformin + 18% strain, 24h + 1 ng/mL IL-1β</td>
</tr>
</tbody>
</table>
Metformin HCl (Sigma-Aldrich; *1,1-Dimethylbiguanide hydrochloride* -- CAS 1115-70-4) was dissolved in F-12 (1% FBS/1% PS) to achieve a 100µM solution. All wells indicating metformin treatment received 2mL of the 100µM solution each after suctioning out old media. In all the other wells, old media was suctioned and replaced with 2mL of F-12 (1% FBS/1% PS) each. All the plates were then pre-incubated in a hypoxic chamber (5% CO₂, 5% O₂, 37.5°C) for four hours prior to inflammatory and stretch treatments. At t=4 hours (Fig. 14, Appendix A), stock IL-1β was diluted to achieve 1ng/mL solution. At the end of the metformin pre-incubation period, 20µL of this solution was added to each well with a group indicating IL-1β treatment. Right after IL-1β treatment, plates with stretch conditions were placed on a FlexCell baseplate (FX-4000; Flexcell International Corp.) with airtight gaskets and subjected to 24 hours of mechanical stretching at 0.5 Hz and 18% strain in the incubator. The FlexCell system consists of compressor and vacuum modules that apply desired strain to a flexible silicone membrane upon which cells are plated (Fig. 8). At t=28 hours, all plates were removed from incubator and treatment media was collected. Cells were then lysed with 10:1 solution of RLT Plus:BME. Cell lysate and treatment media were collected and stored in -80°C freezers for RNA isolation.
Figure 8 – FlexCell plate design emphasizing unit strain mechanism. Amount of vacuum applied corresponds to percentage strain experienced by plated cells on the flexible bottom (*Flex I plate*).

### 4.5 RNA Isolation and qRT-PCR

Within a week of collecting lysate from treated cells, all groups underwent mRNA isolation to establish gene expression profiles. The QIAGEN RNeasy Plus Mini Kit (QIAGEN, catalog no. 74134) was used to isolate mRNA from cell lysate for all groups. Cell lysates were passed through a DNAse step to filter out genomic DNA and increase RNA quality. A nano-spectrophotometer was used to assess RNA concentration and quality. Average RNA concentration was $84.54 \pm 35.37$ ng/µL, with a minimum standard of quality set at a ratio of 1.8 (RNA:protein). qRT-PCR was used to measure gene expression with custom-designed rat primers for MMP-13 (collagenase), COX-2 (cyclooxygenase-2), iNOS (inducible nitric oxide synthase), Col1, and AGC (Table 4, Appendix A). SYBR-green protocol (Applied Biosystems, Carlsbad, CA) detection system was used for thermocycling, with annealing temperature set at 62°C and melting temperature at 95°C.
PCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method for calculating relative gene expression. This particular method of measuring relative gene expression utilizes an internal control/housekeeping gene to normalize the amount of RNA added to the reverse transcriptase reactions. Glyceraldehyde phosphate dehydrogenase (GAPDH) is a constitutively expressed gene shown to be an appropriate housekeeping gene for disc cell mechanobiology testing (Sowa et al., 2011) and was used as such in all samples. Additionally, a calibrator (usually the untreated group) is chosen for direct comparison of gene expression. For example, to observe the effect of IL-1β treatment on nontreated cells, relative gene expression of the control group (C) was set to 1, while the expression of a particular gene in the IL-1β group was presented as a fold-change relative to the control group. Thus, relative gene expression as a fold-change and as a percent-change between groups were compared based on treatment of interest. To explore the effect of induced inflammation under non-mechanical and mechanical conditions, the IL-1β group was compared to control and IL-1β/Stretch was compared to Stretch alone, respectively (i.e. IL vs. C and IL/S vs. S). The effect of mechanical stimulation was investigated by comparing stretch conditions with their analog in the non-stretch group (i.e. IL/S vs. IL). Similarly, the effect of metformin was explored by comparing metformin conditions to their analogs in the non-metformin groups (i.e. M+IL/S vs. IL/S).

4.6 PGE$_2$ ELISA

A Prostoglandin E$_2$ (PGE$_2$) enzyme-linked immunosorbent assay (ELISA) kit was obtained (KGE004B, R&D Systems) for investigating the presence of PGE$_2$ among all treatment groups. Frozen conditioned media (1% FBS/1% PS) from all trials were thawed and diluted 3-fold with calibrator diluent for use. A competitive horseradish-peroxide (HRP) enzyme probe reporter was
used for its high turnover rate and specific enzyme activity. All absorbance values were read using a spectrophotometer (*PerkinElmer*, Waltham, MA) set to 450nm for the main reading, and 570nm for background noise detection. In final analysis of PGE₂ concentration, background absorbance values were subtracted from sample and standard measurements to reflect true PGE₂ concentrations. Standard wells produced a linear absorbance curve with logarithmic equation \( y = 0.622 \ln(x) - 2.2747 \) and a correlation coefficient of 0.9402 (Fig. 9). PGE₂ concentrations of the samples were calculated by inputting absorbance values into the standard curve line of best fit.

![PGE₂ Standard Curve](image)

\( y = 0.622 \ln(x) - 2.2747 \)
\( R^2 = 0.9402 \)

**Figure 9** – Standard curve for PGE₂ ELISA with line of best fit equation and correlation coefficient.
4.7 Statistical Analysis

For statistical analysis of relative gene expression from qRT-PCR, two-way ANOVA was used to compare treatment groups within each gene with Type I error set at $\alpha=0.05$. Main column effects were calculated with Dunnett’s multiple comparisons test.

Statistical analysis of PGE$_2$ concentrations among groups was conducted with one-way ANOVA, with Type I error set at $\alpha=0.05$. Inter-group averages were compared using Tukey’s multiple comparisons test.

All statistical analyses were performed in the statistical software Prism 8.0 (GraphPad Software, San Diego, CA).
5.0 RESULTS

5.1 Gene Expression – Inflammatory Conditions

Treatment groups were split into inflammatory (IL-1β treatment) and non-inflammatory (without IL-1β treatment) conditions for analysis. In the inflammatory profile, all relative gene expression was normalized to the IL-1β group. Figure 10 presents the inflammatory gene expression profile for AGC, COX-2, MMP-13, Col1, and iNOS. All bars depict relative percent change in gene expression compared to the IL-1β group. For example, the addition of mechanical stretch (IL/S) increased COX-2 expression 64.5% compared to IL-1β treatment alone. In all trials, the addition of IL-1β to untreated cells substantially increased gene expression of pro-inflammatory genes MMP-13, COX-2, and iNOS, indicating successful inflammatory stimulation.

During combined inflammatory and mechanical stretch treatments (IL/S), AGC gene expression was upregulated by 155.8%. Col1 production was downregulated -26%. COX-2 was the only pro-inflammatory gene showing substantial upregulation under combined inflammatory/stretch conditions (64.5%). MMP-13 and iNOS were downregulated by -70.3% and -66.3%, respectively (Fig. 10).

Metformin added to IL-1β stimulated cells (M+IL) increased AGC and Col1 expression by 135.5% and 26.5% respectively compared to IL-1β alone. Additionally, only COX-2 was upregulated with the addition of metformin to IL-1β treatment (10%), while both MMP-13 and iNOS were downregulated (-28.8% and -30.5% respectively).

The addition of metformin to combined treatment (M+IL/S) upregulated AGC by 163.5%, a 7.7% increase from combined treatment alone (IL/S, 155.8%). Metformin also significantly
upregulated Col1 expression under combined treatment (99%, p < .0001) compared to the IL/S group (-26%). COX-2 and MMP-13 showed upregulation in M+IL/S conditions (35.8% and 20% respectively). However, metformin decreased COX-2 expression by 28.7% in the presence of combined treatment (M+IL/S) compared to IL/S alone (Fig. 10).

Table 2 provides an executive summary of gene expression modulation under each inflammatory condition.

![Inflammatory Gene Expression Profile](image)

**Figure 10 - Inflammatory gene expression profile, with all groups normalized to IL group. For example, in the M+IL group, gene expression of AGC was 135.5% greater than IL alone.**
Table 2 - Inflammatory conditions highlighting modulation of gene expression with various treatment groups.

<table>
<thead>
<tr>
<th>Inflammatory Conditions (IL-1β)</th>
<th>AGC</th>
<th>Col1</th>
<th>COX-2</th>
<th>MMP-13</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Mechanical stretch</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Metformin + Stretch</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

5.2 Gene Expression – Non-inflammatory Conditions

In the non-inflammatory conditions, all relative gene expression was normalized to the control group. Figure 11 presents the non-inflammatory gene expression results for AGC, COX-2, MMP-13, Col1, and iNOS. All bars depict relative percent change in gene expression compared to the control group. For example, the introduction of metformin to untreated cells (M) increased AGC expression by 14.5% relative to the control group (C).

The addition of mechanical stretch to untreated cells (S) increased AGC expression by 15.8% and downregulated Col1 expression by -52.8%. Mechanical stretch (S) also increased baseline levels of COX-2 by 40.3%. MMP-13 expression was suppressed by -28.3% (Fig. 11).

Metformin added to untreated cells (M) upregulated AGC (14.5%) and pro-inflammatory genes COX-2 and MMP-13 (7.4% and 25.3%, respectively). Col1 expression decreased 31.3% with the addition of metformin to untreated cells (M), in contrast to the analogous inflammatory group (M+IL) which exhibited Col1 upregulation.

Metformin in the presence of stretch stimulus (M+S) increased AGC expression significantly (27.5%, p < .05) compared to metformin alone (M). A large degree of Col1 expression was observed in the M+S group compared to metformin alone (87.8%, p < .0001). Metformin and
stretch stimulus in conjunction upregulated COX-2 expression (63.3%) further than either condition alone. While metformin alone (M) increased MMP-13 expression by 25.3%, the contribution of stretch stimulus (M+S) decreased expression to -6.7% relative to control.

iNOS expression was infinitely small or undetectable in all non-inflammatory conditions, as expected. Percent changes in relative gene expression of iNOS are not representative of true gene expression, as PCR threshold counts (Ct) were very high or nonexistent, and thus small changes in Ct registered as large percentages.

Table 3 provides an executive summary of gene expression modulation under each non-inflammatory condition.

![Non-Inflammatory Gene Expression Profile](image)

* Figure 11 - Non-inflammatory gene expression profile, with all groups normalized to C group. For example, in the S group, gene expression of AGC was 15.8% greater than C alone.
Table 3 - Non-inflammatory conditions highlighting modulation of gene expression with various treatment groups.

<table>
<thead>
<tr>
<th>Non-Inflammatory Conditions</th>
<th>AGC</th>
<th>Col1</th>
<th>COX-2</th>
<th>MMP-13</th>
<th>iNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Mechanical Stretch</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Metformin + Stretch</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

5.3 Prostaglandin E₂ Detection and Quantification

PGE₂ ELISA was used to identify and quantify the amount of PGE₂ synthesized in each treatment group. PGE₂ concentration was lowest in the control group (1941 pg/mL) and peaked during IL/S treatment group (4481 pg/mL). All stretch conditions, with the exception of the M+IL/S group, exhibited overall greater concentrations of PGE₂ compared to non-stretch groups. Combined stress treatments (IL/S) significantly increased PGE₂ expression (p < .0001). The presence of metformin under combined stress treatments (M+IL/S), however, significantly decreased PGE₂ expression to 2714 pg/mL from the 4481 pg/mL observed in the IL/S group (Fig. 12). This trend was not observed under non-inflammatory conditions, where the addition of metformin to mechanical stretch (M+S) increased PGE₂ expression from 3898 pg/mL to 4341 pg/mL.
Figure 12 - PGE\(_2\) concentration of all groups in pg/mL.
6.0 DISCUSSION

6.1 Metformin acts through Mechanical Stretch-Mediated Inhibitory Action

The difference in gene expression profiles between non-inflammatory and inflammatory conditions highlights key differences that may provide insight into metformin’s mechanism of action. Metformin in the combined presence of mechanical stretch and IL-1β (M+IL/S) suppressed COX-2 (Fig. 10), but substantially increased COX-2 mRNA transcripts in the non-inflammatory (M+S) group (Fig. 11). Thus, metformin may not be acting to enzymatically inhibit COX-2, unlike traditional NSAIDs.

As previously described, NF-κB is a prominent mediator in mechanical and inflammatory signaling pathways. Mechanical stimulation upregulates nuclear translocation of NF-κB, resulting in transcription of a range of inflammatory cytokines, including the production of IL-1β (Tisherman et al., 2016). AMPK is a well-known inhibitor of NF-κB and downregulates its translocation into the nucleus. Since AMPK shows increased activity in the presence of metformin, it is possible that metformin is acting to downregulate NF-κB translocation via AMPK. However, this phenomenon only seems to occur during stretch conditions, as the M+IL group does not show any significant downregulation of pro-inflammatory genes explored in this study, while the M+IL/S group does. This may be in part due to the greater hypothesized NF-κB translocation associated with the stretch conditions compared to the non-stretch groups, although an immunofluorescence assay is necessary to confirm this hypothesis.

Although the inflammatory response seems synergistic and positively reinforced between stretch and IL-1β as previously described by Miyamoto (Miyamoto et al., 2006), the stretch
condition seems to be a greater contributor to inflammation compared to IL-1β alone, as evidenced by the PGE\(_2\) concentrations (Fig. 12). The significant increase in PGE\(_2\) concentration from IL to IL/S treatments suggests that IL-1β alone produces a moderate inflammatory response, which may be further upregulated by the NF-κB pathway through mechanical signaling in the IL/S group. When metformin is added to the IL/S group however, significant inhibition of PGE\(_2\) is seen. Strikingly lower PGE\(_2\) levels in the M+IL/S group compared to the IL/S group suggests that metformin may be enacting its anti-inflammatory effects in a stretch-dependent manner. As metformin is known to upregulate AMPK, which acts to downregulate NF-κB downstream, it is hypothesized that metformin blunts the stretch-mediated inflammatory response through NF-κB inhibition or another stretch-mediated inflammatory pathway (Fig. 13). Synergistic stretch and IL-1β-mediated exacerbation of inflammation through upregulation of PGE\(_2\) has been shown to accelerate degeneration by emphasizing production of pro-inflammatory factors (Miyamoto et al., 2006). Metformin’s significant inhibitory effect on PGE\(_2\) synthesis suggests that it may play a mediatary role in PGE\(_2\)-related inflammation in the disc.

The homeostasis of disc cells is closely governed by the balance of anabolic and catabolic factors, and thus anabolic gene profiles were also explored. In both inflammatory and non-inflammatory conditions, Col1 production was greatly increased through the synergistic effects of metformin and stretch (Fig. 10, 11). The lack of a similar effect in the metformin and stretch groups separately further suggests that metformin’s mechanism of action is dependent on mechanical signaling pathways. The immense upregulation of Col1 in combined stress and metformin treatments implies improved ECM production, which is crucial for balancing catabolic factors that simultaneously degrade the disc. Additionally, as AF cells are primarily composed of Collagen I
and II fibers, increased Col1 expression may be beneficial in protecting against disc herniation by strengthening the AF in the posterolateral plane.

6.2 Implications

Our findings provide evidence to suggest that metformin may be a viable anti-inflammatory alternative to NSAIDs. From gene expression analysis and downstream PGE$_2$ quantification, it is possible that metformin acts at the gene transcription level through a separate pathway to curb inflammation compared to the currently prescribed COX-1 and -2 inhibitors, which act as enzymatic inhibitors. Traditional NSAIDs, and particularly COX-1 inhibitors, have documented side effects such as the thinning of the gastric lining. Further biochemical assays are necessary to identify the exact pathway in which metformin enacts its anti-inflammatory effects; however, the evidence suggesting its stretch-mediated inhibition of NF-$\kappa$B via AMPK provides a basis for further studies comparing its effectiveness as an anti-inflammatory agent over traditional NSAIDs. Metformin’s blanket anti-inflammatory effects may prove not only useful in the intervertebral disc as a retardant of disc degeneration, but also as a system-wide anti-inflammatory agent with potential for ameliorating a plethora of inflammation-related conditions.
Figure 13 - Simplified schematic highlighting hypothesized mechanism of metformin and its effect on intervertebral disc degeneration.
Appendix A Miscellaneous

![Figure 14 – High-level timeline overview of methods.](image)

Table 4 – Rat primer sequences used in qRT-PCR assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Fwd: 5’-ATGACTCTACCCACGGCAAG-3’</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-GATCTCGCTCCTGGAAGATG-3’</td>
</tr>
<tr>
<td>AGC</td>
<td>Fwd: 5’-AGACACCCCTTACCCTTGTT-3’</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-AAAGATGTCCCAAGGCATCCAC-3’</td>
</tr>
<tr>
<td>Col-1</td>
<td>Fwd: 5’-TTCTGAAACCCTCCCCTTT-3’</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-CCACCCCGAGGATAAAAAACT-3’</td>
</tr>
<tr>
<td>iNOS</td>
<td>Fwd: 5’-CCTGTGTGCCACCGAGGAGAT-3’</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-CGCTTTCCACCAAGACTGTGA-3’</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Fwd: 5’-GCAGCTCACAAGGGCTACAAC-3’</td>
</tr>
<tr>
<td></td>
<td>Rev: 5’-GAAATGGCTTTTGGCCAGTG-3’</td>
</tr>
</tbody>
</table>


